Phosphorescence and Optically Detected Magnetic Resonance Studies of a Class of Anomalous Tryptophan Residues in Globular Proteins†

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ABSTRACT: A class of tryptophans in globular proteins which display unusual emission properties and are typified by the lone tryptophan residue in RNase T_1 has been investigated. Phosphorescence and optically detected magnetic resonance (ODMR) data for RNase T_1 and phospholipase A_2 in 1:1 ethylene glycol–buffer at ~1.1 K reveal that the zero-field splitting (zfs) frequencies along with the phosphorescence wavelength maxima are characteristic of tryptophan residues exposed to the polar solvent. The narrow emission and ODMR line widths on the other hand indicate a more homogeneous local environment. The gross correlation found to exist between emission wavelength maxima and the zfs parameters for a number of proteins in addition to the ones studied here suggests that both the optical and magnetic transitions are influenced in a similar way by local polarity and polarizability. These considerations are consistent with the presence of a stabilizing polar interaction between tryptophan and other functional groups in the protein as a source of the anomalous triplet properties of RNase T_1 and phospholipase A_2. Despite the evidence for a relatively homogeneous environment for the lone tryptophan in RNase T_1, minor shifts in the phosphorescence spectrum as a function of exciting wavelength and inhomogeneously broadened lines revealed by ODMR hole-burning experiments indicate the presence of a spectrum of local perturbations experienced by the chromophore as a consequence of microheterogeneity in the protein structure.

Early observations of protein emission revealed that both the tryptophan fluorescence (Teale & Weber, 1959) and low-temperature phosphorescence (Konev, 1967) maxima varied from protein to protein. This variation arises from the fact that the excited-state properties of the aromatic amino acids in proteins depend on the interactions between the chromophores and the local environment. The triplet-state emission has proved to be informative in this regard for several reasons. (1) The resolution inherent in the low-temperature phosphorescence spectra permits not only the facile separation of tyrosine and tryptophan emissions (Longworth, 1971) but also in addition the detection of spectral heterogeneity arising from tryptophan residues in differing local environments (Purkey & Galley, 1970; Beyer et al., 1974; Galley, 1976). (2) The zero-field splittings (zfs),[1] obtained from the frequencies of the magnetic transitions detected optically in zero field, ODMR, provide an additional sensitive probe to the nature of and the heterogeneity in the perturbing local surroundings (Zuclich et al., 1973; von Schütz et al., 1974; Ugurbil et al., 1977; Ross et al., 1977; Deranleau et al., 1978; Rousslang et al., 1978). (3) For proteins in solutions, marked variation in the sensitivity of tryptophan residues to phosphorescence quenching (Saviotti & Galley, 1974; Imakubo & Kai, 1977; Kai & Imakubo, 1979) provides a measure of protein flexibility.

The differences between the interactions experienced by a tryptophan residue exposed to the polar solvent and one buried in the more polarizable interior of a protein give rise to distinct low-temperature phosphorescence maxima and zfs frequencies. Solvent-exposed tryptophans show 0,0 emission maxima in the region of 405–409 nm whereas buried residues normally emit to the red (410–415 nm). These two classes of tryptophan residues also tend to show characteristic ranges for the D–E and $2E$ transitions observed in ODMR studies.

Several examples of buried tryptophan residues in proteins exist which are anomalous in that their phosphorescence maxima based on the above classification would suggest that the chromophores are solvent exposed. The resolution and blue wavelength maxima observed for the room-temperature fluorescence from the lone tryptophan in RNase T_1 indicate, on the other hand, that the residue is not exposed to the polar solvent (Longworth, 1968). This latter conclusion is supported by (a) the invariance of the fluorescence efficiency to exchange of the solvent from H_2O to D_2O (Eisinger & Navon, 1969), (b) the relative resistance of the fluorescence to acrylamide quenching (Eftink & Ghiron, 1975), (c) the observation of tryptophan phosphorescence at room temperature with a 3.5-ms lifetime (Imakubo & Kai, 1977), and (d) the absence of a solvent relaxation induced red shift in the phosphorescence (J. Kossowski and W. C. Galley, unpublished data) at a temperature characteristic of solvent relaxation around tryptophan in these solvents (Galley & Purkey, 1970; Milton et al., 1978). The blue component in the phosphorescence of BSA at low ionic strength (Larkindale, 1971; Galley, 1976) and the phosphorescence of phospholipase A_2 (W. C. Galley and S. Strambini, unpublished observations) would appear to represent other anomalous tryptophans of this type.

We have examined the phosphorescence and ODMR parameters of representatives of this class of tryptophans at ~1.1 K with a view to developing insights into the nature of the perturbations responsible for these anomalies and at the same time probing the basis of the relationship between phosphorescence wavelength maxima and the zfs. The data suggest that polar interactions in the protein which stabilize the ground electronic state of tryptophan are responsible for the emission properties of RNase T_1 and phospholipase A_2. Hole-burning experiments on the ODMR transitions and shifts in the emission spectrum as a function of excitation wavelength in-

† From the Department of Chemistry, University of California, Davis, Davis, California 95616. *Received July 18, 1979.* This work was partially supported by grants from the National Science Foundation (NSF CHE76-19826), the National Institutes of Health (ES-01268), and the National Sciences and Engineering Council of Canada.

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[1] Abbreviations used: zfs, zero-field splitting; ODMR, optically detected magnetic resonance; RNase, ribonuclease; $\lambda_{0,0}$, wave maximum of the 0,0 band of the phosphorescence; $\nu_{1/2}$, full width of an ODMR transition at half-maximum intensity; EG, ethylene glycol; LADH, liver alcohol dehydrogenase; HCAB, human carbonic anhydrase B; ACTH, adrenocorticotrophic hormone; PTH, human parathyroid hormone; NAG, *N*-acetylglucosamine.

0006-2960/80/0419-2204$01.00/0

Table I: A Comparison of Triplet-State Parameters for a Variety of Buried and Exposed Residues

	λ_{0-0} (nm)	D (cm^{-1})	E (cm^{-1})	D-E (cm^{-1})	$\nu_{1/2}(D-E)$ (MHz)	$\nu_{1/2}(2E)$ (MHz)	ref
RNase T_1	404.5	0.0997	0.0413	0.0584	50	125	this work[a]
phospholipase A_2	406.5	0.0990	0.0416	0.0574	72	190	this work[a]
LADH	406	0.1013	0.0415	0.0597	125	175	von Schütz et al. (1974)[a]
Trp	407	0.0988	0.0407	0.0580	165	345	Zuclich et al. (1973)[a]
HCAB	407	0.1010	0.0425	0.0585			Co (1976)[a]
Lys-Trp-Lys	407	0.0988	0.0415	0.0573			Co (1976)[a]
ACTH	408	0.0993	0.0413	0.0581	152	296	Deranleau et al. (1978)[b]
glucagon peptide	409	0.0992	0.0418	0.0574	140	263	Deranleau et al. (1978)[b]
PTH peptide	409	0.0989	0.0419	0.0570	134	291	Deranleau et al. (1978)[b]
somatostatin	409	0.0993	0.0415	0.0578	147	296	Deranleau et al. (1978)[b]
glucagon	411	0.0994	0.0432	0.0562	102	206	Deranleau et al. (1978)[b]
azurin B	411	0.1000	0.0465	0.0535	40	60	Ugurbil et al. (1977)[a]
LADH	412	0.0977	0.0421	0.0556	75	125	von Schütz et al. (1974)[a]
HCAB	413	0.1005	0.0452	0.0553			Co (1976)[a]
chymotrypsin	413	0.0985	0.0450	0.0535	42	110	Maki & Co (1976)[a]
lysozyme	415	0.0968	0.0453	0.0515	80	89	this work[a]
lysozyme-$(NAG)_3$	415	0.0965	0.0445	0.0520	62	115	von Schütz et al. (1974)[a]

[a] The ODMR transitions were measured at the maximum of the 0,0 transition. [b] The transitions were measured at the λ_{max} of the spectrum: ~432 nm.

dicate a distribution of local perturbations and therefore protein conformations.

Materials and Methods

Ribonuclease T_1 (EC 3.1.4.8) from *Aspergillus oryzae* and phospholipase A_2 (EC 3.1.1.4) from bee venom were purchased from Sigma Chemical Co. Lysozyme (EC 3.2.1.17) from egg white was purchased from Schwarz/Mann, while azurin B from *Pseudomonas aeruginosa* was a generous gift from Professor R. Bersohn (Columbia University, New York).

The ammonium sulfate suspension of RNase T_1 was dialyzed against 0.1 M phosphate buffer (pH 7.5), concentrated, and mixed 1:1 v/v with ethylene glycol (Matheson Coleman and Bell; chromatoquality) to yield a final concentration of 5×10^{-4} M. Phospholipase A_2, lysozyme, and azurin were lyophilized powders and were dissolved in ethylene glycol–buffer mixtures (1:1 v/v) of 0.05 M phosphate (pH 8.0), 0.05 M phosphate (pH 7.5), and ammonium acetate (pH 6.0), respectively. The final protein concentration in each case was $\sim 5 \times 10^{-4}$ M. Phosphorescence spectra were recorded with a resolution of 1 nm or better by using a previously described instrument (Maki & Co, 1976). The spectra were not corrected for the wavelength-dependent sensitivity variations of the MacPherson monochromator or the EMI 6256S photomultiplier tube. Excitation (~10-nm band-pass) was at 295 nm unless otherwise stated.

Slow-passage ODMR spectra were recorded by sweeping in both directions at sweep rates of 4 MHz/s or less by using previously described equipment (Zuclich et al., 1974). Hole-burning experiments on the D–E transitions of the different proteins were performed by using two Hewlett-Packard 8690B sweep oscillators with one set at a fixed frequency and 10 mW of power after passing through a 2-GHz low-pass filter and a 6-dB attenuator. The second sweep oscillator is swept and isolated from the first by a 1–2-GHz circulator terminated into a 50-Ω load and is also equipped with a 2-GHz low-pass filter. The microwaves from the two sources were coupled through a Narda 4312-2 power divider to the slow-wave helix. A similar arrangement was used for hole burning in the $2E$ transition except that the fixed-frequency microwave source was a Hewlett-Packard 8616B signal source.

Results and Discussion

The phosphorescence spectra of RNase T_1, phospholipase A_2, and tryptophan at ~4.2 K are shown in Figure 1. While the wavelength maxima (Table I) are similar, the resolution apparent in the protein spectra is clearly greater and at this temperature is higher for RNase T_1 than that observed at 77 K (Longworth, 1968; Imakubo & Kai, 1977). The spectrum of phospholipase A_2, which possesses two tryptophans, is somewhat less resolved than that of RNase T_1. These spectra suggest that while the perturbations experienced by the tryptophans in these proteins are similar to those for the indole chromophore in a polar solvent, the spectrum of perturbations experienced by the chromophore is distinctly narrower.

The zfs frequencies observed for tryptophan in RNase T_1 and phospholipase A_2 appear in Table I along with values for typical solvent-exposed and buried residues. The frequencies found for these two proteins are more nearly characteristic of those found for solvent-exposed rather than buried residues and appear to fit in with the variations of zfs frequencies with phosphorescence wavelength maxima apparent from observations on a sizable number of peptides and proteins. These trends have been alluded to previously (Ugurbil et al., 1977; Ross et al., 1977; Deranleau et al., 1978). An analogous variation of zfs as a function of wavelength is found to exist *across the 0,0 vibronic emission band* for solvent-exposed tryptophans (von Schütz et al., 1974; Kwiram et al., 1978). A general increase in E and concomitant decrease in D with increasing wavelength of the 0,0 phosphorescence maxima for tryptophan in a wide variety of protein environments are apparent from the data plotted in Figure 2. We have included a plot of D–E in Figure 2, as well, since it was found to yield the most favorable correlation with $\lambda_{0,0}$ when a multiple linear regression analysis is done. It can be noted particularly in the plots of E and D–E that the frequencies for the proteins studied here are far removed from the values found for more typical buried tryptophan residues which emit farther to the red but rather correlate along with other protein residues with their blue emission.[2]

The D and E values of indole have been measured in ethylene glycol–H_2O (von Schütz et al., 1974). The absence of the side chain at the 3-carbon position is expected to in-

[2] The magnitude of the slopes of D,E and D–E as a function of $\lambda_{0,0}$ in Figure 2 was larger than those observed for D,E and D–E vs. λ measured across the 0,0 transition of tryptophan in polar solvents (von Schütz et al., 1974). This is likely a consequence of both the overlapping of the intrinsic components within the envelope of the 0,0 band for tryptophan in solution and the finite emission slits employed.

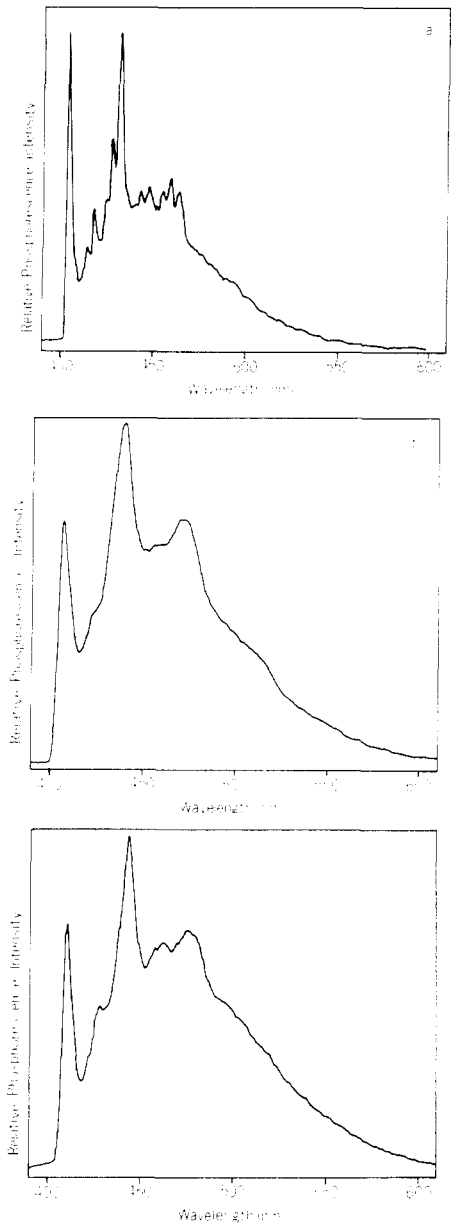

FIGURE 1: Phosphorescence spectra at 4.2 K of (a) RNase T_1 in 1:1 EG–buffer (0.05 M phosphate, pH 7.5), (b) tryptophan in the same solvent, and (c) phospholipase A_2 in 1:1 EG–buffer (0.05 M phosphate, pH 8.0).

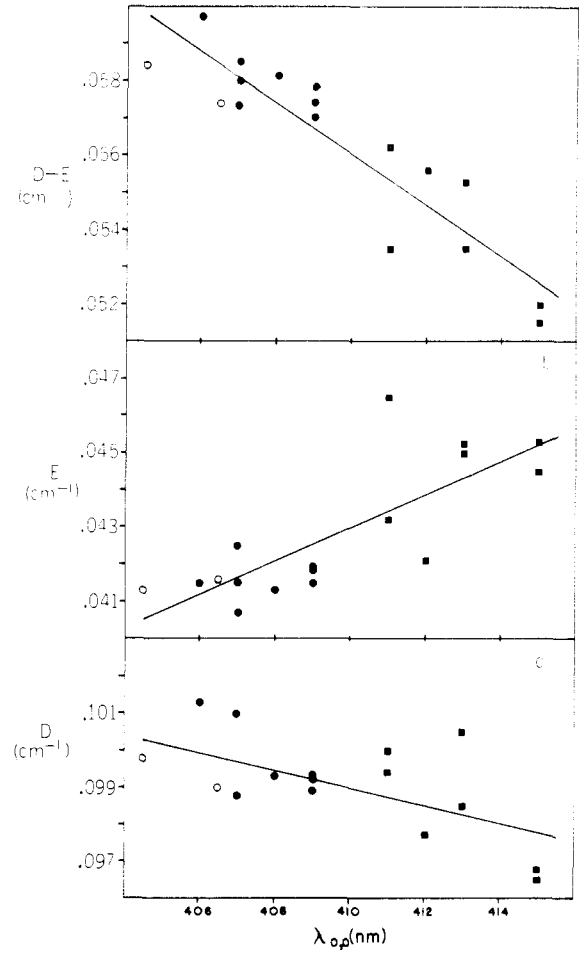

FIGURE 2: zfs parameters for tryptophan in a variety of protein and peptide environments as a function of the phosphorescence wavelength maxima ($\lambda_{0,0}$). Tryptophan residues exposed to solvent (●); tryptophan residues which are buried (■) (glucagon which may be partially exposed has been included in this latter category); anomalous buried tryptophans (○). Solid lines are the least-squares linear regressions. For D–E, E, and D vs. $\lambda_{0,0}$, the correlation coefficient is $r = -0.91$, 0.79, and −0.61, respectively.

fluence the zfs parameters, as well as the 0,0 phosphorescence maximum. Indeed, the latter is shifted to 404 nm, 3 nm to the blue of tryptophan in the same solvent. Although D and E of indole do not correlate well individually with the tryptophan data plotted in Figure 2, D–E = 0.0602 cm^{-1} correlates very well.

Model for the Relationship between Phosphorescence Red Shift and zfs Parameters. The changes in D and E as a function of emission wavelength maxima can be considered to arise ultimately from the local polarity and polarizability of the chromophore environment. The anticipated influences of these parameters on (a) the ground- and triplet-state energies and (b) the electronic distribution of the chromophore itself can account not only for the trends observed for the distinct tryptophans in a wide variety of proteins but also for the variations that are observed across the 0,0 vibronic band of tryptophan in polar solvents as well.

(*a*) *Effect of Environment on Phosphorescence Wavelength.* Solvent polarity and protein polarizability have been proposed to be the cause of differences in phosphorescence maxima of solvent-exposed and buried tryptophans (Purkey & Galley, 1970). Red-shifted phosphorescence was viewed as arising from the greater stabilization of the excited state of the chromophore with its larger excited-state dipole moment through dipole-induced dipole interactions with the polarizable protein interior. The more blue phosphorescence typical of free tryptophan in a polar solvent, on the other hand, was proposed to occur not only by virtue of the lower polarizability of the environment but also as a consequence of the poor stabilization of the triplet state by a rigid solvation geometry that was organized to effectively stabilize the ground state by dipole–dipole alignment. It is significant for the considerations in the present study that in transferring from a rigid polar solvent (stabilized ground state → less stabilized triplet state) to a more polarizable protein interior (stabilized ground state → more stabilized triplet state) the accompanying red-shifted emission displayed by tryptophan is associated with an *increase in stabilization of the triplet state.*

Within a polar solvent itself this relation between emission wavelength and triplet-state stabilization is again encountered. Heterogeneity in the solvation-site geometry of indole and other polar chromophores leads to a distribution of transition energies in absorption and emission (Galley & Purkey, 1970; Milton et al., 1978). The emission components which are encountered

in proceeding from the blue to the red side of the vibronic transition correspond to molecules which are progressively less effectively solvated in the ground state but possess solvation geometries which happen to accommodate the excited state. This is evidenced by the observations that (a) the more red-emitting components in the emission which can be selectively excited at the red edge of the absorption band make a progressively smaller contribution to the intensity as the temperature is lowered, indicating that they arise from molecules with higher ground-state energies (Purkey, 1972), and (b) when the solvent becomes sufficiently mobile at temperatures > 170 K, the tryptophan phosphorescence spectrum in general red shifts in response to solvent reorganization about the molecule in its triplet state (Galley & Purkey, 1970). The general relation between phosphorescence emission wavelength and triplet-state stabilization is contained implicitly in this earlier work, but attention is drawn to it here in that it is the influence of this increasing triplet-state stabilization on the electronic distribution of the chromophore itself which is considered to give rise to the trends observed in the zfs parameters.

(*b*) *Influence of Excited-State Stabilization (Red Shift) on the zfs Parameters.* Effective stabilization of the electronic energy of a chromophore in its triplet state by the dielectric of the local environment can be expected to perturb the π-electronic distribution of the molecule itself and therefore the zfs parameters. The zfs parameters D and E are determined by the symmetry and magnitude of the magnetic–dipolar interactions between the unpaired electrons in the triplet state and can be expressed as (McGlynn et al., 1969)

$$D = \frac{3}{4}g^2\beta^2\frac{r_{12}^2 - 3z_{12}^2}{r_{12}^5} \qquad E = \frac{3}{4}g^2\beta^2\frac{y_{12}^2 - x_{12}^2}{r_{12}^5}$$

in which x, y, and z are the electron–electron separations along the principal axes and the coordinate functions are averaged over the triplet spacial wave function. The z direction is perpendicular to the plane of the indole ring, and the x and y principal axes lie in the plane of the indole chromophore (Zuclich, 1970). Either (a) an increase in the local polarizability or (b) a favorable alignment between the solvent dipoles and the dipole of the chromophore *in its triplet state* has the effect of reducing the Coulombic attraction of the π electrons for the nuclei with a resulting expansion of the molecular wave function and concomitant increase in the average separation r_{12}. The decrease observed in the value of D with increasing red shift for tryptophan in proteins has been attributed to this effect (Ugurbil et al., 1977). The large value of E results from significant localization in the ^3L_a state of the electron pair in the "ethylenic" 2–3 bond of indole (Lami, 1977; Gouterman & Moffitt, 1959). From the magnetophotoselection measurements of Zuclich (1970), the principal y and x axes are close to being parallel and perpendicular, respectively, to the 2–3 bond. If the triplet electron distribution were to expand isotropically, we would expect both D and E to become smaller in a more polarizable environment. Since we actually observe E to increase with increasing triplet-state stabilization, we can conclude that the expansion of the electron distribution does not occur isotropically. The changes of D and E with increasing $\lambda_{0,0}$ seen in Figure 2 can be reproduced by allowing y_{12} to selectively expand in comparison with x_{12} and z_{12}.

In view of the general nature of these considerations, it follows that very blue tryptophans which correspond to residues stabilized in the ground, but not the excited, state by polar interactions are associated with zfs parameters which reflect the poorly stabilized triplet state. While these are the type of interactions experienced by the indole chromophore in a polar solvent, the independent evidence cited earlier, in addition to the resolution apparent in the phosphorescence spectrum in Figure 1, makes it clear that the tryptophan in RNase T_1, in particular, is buried in the protein interior. The ODMR of RNase T_1 and phospholipase A_2 were examined to test the validity of the general considerations of the model. The fact that their zfs frequencies do indeed correlate with emission wavelength maxima as predicted reveals that while the zfs frequencies and low-temperature emission maxima per se provide some measure of the nature of the excited- and ground-state perturbations suffered by a tryptophan residue, they cannot be used to unambiguously distinguish between buried and exposed locations in proteins. Additional ODMR and phosphorescence data were sought to distinguish the type of local environment present in these proteins from that surrounding a chromophore in a polar solvent.

Measurements of both the D–E and $2E$ transition frequencies across the narrow 0,0 vibronic band in RNase T_1 gave values which within experimental error did not differ from the frequency measured at the wavelength maximum. In phospholipase A_2, variation of zfs with λ across the 0,0 band is observed, but this could be due to unresolved emission from both sites. Constancy of the zfs frequencies across the 0,0 vibronic band has been previously shown to be characteristic of the buried tryptophan residues of lysozyme (von Schütz et al., 1974). This implies that either some buried tryptophans such as those of lysozyme and that of RNase T_1 lie in a homogeneous microenvironment in the protein interior or the correlation between electronic-transition energies across the 0,0 band and zfs parameters does not hold for some buried tryptophans. The results of hole-burning measurements (see below) indicate that the latter is the case. Hence, while the model presented above accounts for the variation of zfs with the phosphorescence 0,0-peak wavelength, it does not account for the behavior of the zfs parameters within the 0,0 band of certain buried residues.

It is most accurate to think of the relationship between D–E and the wavelength of the 0,0-band maximum as a trend, or a tendency, rather than as a rigorous correlation. The scatter of the individual points in Figure 2a about the least-squares regression line is well beyond the range of experimental error in many cases. This implies, not unsurprisingly, that these parameters are influenced independently by certain specific details of the local interactions. As phosphorescence and ODMR data of additional tryptophan sites in proteins become available, it is likely that examples of large deviations from the observed "correlation" will be found.

Heterogeneity in the Local Environment of Buried Tryptophan Residues in Proteins. While the phosphorescence spectrum of the lone tryptophan in RNase T_1 is sharper than that observed in other proteins to date and the ODMR frequencies are constant across the 0,0 vibronic band, the chromophore does not lie within a homogeneous environment in the protein. Heterogeneity in the perturbations influencing the ground and triplet state of the aromatic group can be detected optically in terms of a shift in the emission spectrum with changes in the excitation wavelength. For these measurements, the excitation monochromator slits were set at 5-nm band-pass, and those of the emission monochromator were narrowed to 0.25 nm. When the excitation wavelength was changed from 295 to 280 nm, a 0.4-nm blue shift of the 0,0 peak in the phosphorescence spectrum (Figure 1a) was observed. The shift is outside of the estimated precision of this measurement (±0.1 nm). The observed shift of the phos-

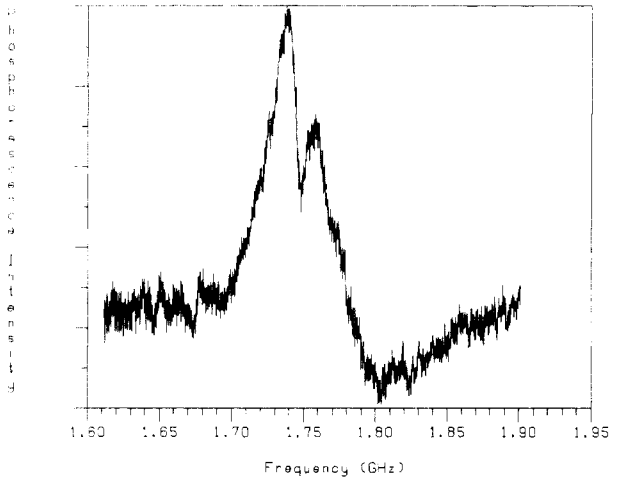

FIGURE 3: Evidence for heterogeneity in the environment of the lone tryptophan in RNase T_1. Hole burning in the D–E transition of RNase T_1. 10 mW of microwave power was employed at 1.746 GHz for the hole burning. The microwave sweep rate is 2 MHz/s.

phorescence spectrum is significantly smaller than that observed for tryptophan exposed to the spectrum of interactions in a rigid polar solvent (Galley & Purkey, 1970; Milton et al., 1978) but reveals a heterogeneity of local interactions nonetheless.

The extent of the heterogeneity in the protein conformation of RNase T_1 resulting in a distribution of chromophore–protein interactions can be more readily detected and compared with the heterogeneity observed with polar solvents by using ODMR. As a consequence of solvation-site heterogeneity, the ODMR transitions for tryptophan in polar solvents and for solvent-exposed residues in proteins are very broad (von Schütz et al., 1974; Ugurbil et al., 1977; Ross et al., 1977; Deranleau et al., 1978; Rousslang et al., 1978). The width values, $\nu_{1/2}$, of the D–E and $2E$ transitions shown in Table I for the tryptophans in RNase T_1 and phospholipase A_2 are considerably narrower than for tryptophan in polar solvents and more characteristic of those observed for buried residues in proteins. The line widths of RNase T_1 [$\nu_{1/2}(D$–$E)$ = 50 MHz; $\nu_{1/2}(2E)$ = 125 MHz], while comparable to those observed for the lone tryptophan in azurin B (Ugurbil et al., 1977), remain considerably broader than those observed for indole in the Shpol'skii matrix provided by indan [$\nu_{1/2}(D$–$E)$ and $\nu_{1/2}(2E)$ $\sim$ 10 MHz] (Zuclich et al., 1974). Attempts at hole burning in the ODMR lines of indole in indan were not successful (Zuclich et al., 1974). It is unlikely that this will be easily accomplished in light of the experience that the minimum hole width thus far obtained in inhomogeneously broadened indole and tryptophan ODMR lines also is on the order of 10 MHz (Zuclich et al., 1974; Rousslang et al., 1978). Thus, 10 MHz appears to be about the effective range of spin diffusion in the indole chromophore. It follows that one should not expect to observe heterogeneity by hole burning if the ODMR line width of a tryptophan or indole site is $\lesssim$10 MHz.

Variability in the "native" conformation of RNase T_1 leads to a spectrum of local environments for the lone tryptophan in the interior of the protein, resulting in inhomogeneously broadened ODMR lines. This inhomogeneity is revealed in hole-burning experiments of the type depicted in Figure 3. The 7-MHz width of the hole burnt in the ODMR lines of both RNase T_1 and phospholipase A_2 indicates that despite the relatively narrow ODMR line widths in comparison with those associated with solvent-exposed residues, site heterogeneity within the protein interior is responsible for broadening the lines beyond the spin diffusion limit. Apparently this is true for all proteins thus far studied by ODMR as we have recently been able to burn holes in lysozyme and azurin which are previously reported to be homogeneously broadened (Zuclich et al., 1974; von Schütz et al., 1974; Ugurbil et al., 1977).

The structural variability in the vicinity of the tryptophan residue in RNase T_1 must be rather subtle. The emission and ODMR parameters reflect a stabilizing polar interaction in the ground state involving the tryptophan residue and the protein interior. The structural heterogeneity cannot involve significant disruption of this interaction or else much broader emission vibronic and ODMR transitions would be anticipated Studies of the present type on proteins crystallized in cryogenic solvents would be of interest since they may make it possible to obtain a sensitive measure of the restrictions of conformational variability imposed by crystal-packing interactions.

Summary and Conclusions

Phosphorescence and ODMR data provide sensitive probes of the local environment of aromatic amino acids in proteins and can be used to complement information provided by fluorescence experiments. The wavelength maxima and sharpness of the room-temperature fluorescence spectra of both RNase T_1 and azurin B clearly establish that the lone tryptophan residues in these proteins are not exposed to the polar solvent. This has been taken as evidence for burial of the emitting residues in a hydrophobic environment in the protein. While this appears to be true in the case of azurin B (Adman et al., 1978), the low-temperature phosphorescence wavelength maxima and zfs frequencies indicate that the tryptophan residue in RNase T_1 must be stabilized in the ground state by a polar interaction. Similar interactions apparently occur for the emitting tryptophan(s) of phospholipase A_2. The resolution of the phosphorescence spectrum and the relatively narrow line width of the ODMR transitions are triplet-state parameters which reveal that these polar interactions occur with other groups in the protein rather than with the polar solvent. Minor shifts in the phosphorescence spectrum as a function of exciting wavelength and the occurrence of hole burning in the ODMR transitions are used to reveal heterogeneity in the local perturbations and therefore in the conformations of RNase T_1 and of phospholipase A_2.

Acknowledgments

We thank Professor R. Bersohn for a generous sample of azurin B.

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Proton Nuclear Magnetic Resonance Study on the Roles of Histidine Residues in the Binding of Polypeptide Chain Elongation Factor Tu from *Thermus thermophilus* with Aminoacyl Transfer Ribonucleic Acid and Guanine Nucleotides†

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ABSTRACT: Proton nuclear magnetic resonance (^{1}H NMR) spectra were measured of the polypeptide chain elongation factor Tu (EF-Tu) from an extreme thermophile, *Thermus thermophilus* HB8 [Nakano, A., Miyazawa, T., Nakamura, S., & Kaziro, Y. (1979) *Arch. Biochem. Biophys. 196*, 233–238], in order to elucidate the environment around functionally important histidine residues. In the present study, the behavior of five histidine C_2 proton signals was studied in more detail. A hydrogen–deuterium exchange experiment was carried out on the histidine C_2 protons of free EF-Tu, and the previous assignments of C_2 proton signals were revised in part. An analysis of the ^{1}H NMR spectra of EF-Tu photooxidized under various conditions indicates that a histidine residue is located in the aminoacyl-tRNA binding site and is probably essential for the binding with aminoacyl-tRNA. A solvent-accessible histidine residue is found to lie near the aminoacyl-tRNA binding site. Furthermore, the effect of paramagnetic hexacyanochromate(III) ion on the ^{1}H NMR spectra of free EF-Tu suggests that another histidine residue lies near the guanine nucleotide binding site.

The polypeptide chain elongation factor Tu (EF-Tu)[1] promotes the GTP-dependent binding of aminoacyl-tRNA (aa-tRNA) to the A site of ribosomes [for reviews, see Miller & Weissbach (1977) and Kaziro (1978)]. EF-Tu has at least two active sites, one for binding with GDP or GTP and the other for interaction with aa-tRNA. The conformation around the aa-tRNA binding site is altered upon ligand substitution from GDP to GTP, and only EF-Tu·GTP, but not EF-Tu·GDP, can bind aa-tRNA to form a ternary aa-tRNA·EF-Tu·GTP complex. Upon binding of the ternary complex to the A site of ribosomes, GTP is hydrolyzed and then EF-Tu·GDP is released. For EF-Tu from *Escherichia coli*, various spectroscopic studies (Arai et al., 1974a, 1975, 1976; Crane & Miller, 1974; Wilson et al., 1978) have shown that the conformational transitions induced by the ligand change in fact occur near the cysteine residue in the aa-tRNA binding site. Recently, the complete amino acid sequence of *E. coli* EF-Tu has been determined (Arai et al., 1980) and the tertiary structure of the modified form of *E. coli* EF-Tu·GDP has been reported by X-ray analyses at 6.0-Å (Kabsch et al., 1977) and 2.6-Å (Morikawa et al., 1978) resolution. According to Morikawa et al. (1978), the molecule of *E. coli* EF-Tu consists of two or three domains and tracing of the main chain is difficult in the loose domain(s) which corresponds to ~60% of the whole molecule, probably because the local conformation in the loose domain(s) is more disordered than in the tight domain.

Nuclear magnetic resonance (NMR) spectroscopy provides important information about dynamic structure–function re-

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[1] Abbreviations used: EF-Tu, polypeptide chain elongation factor Tu; aa-tRNA, aminoacyl transfer ribonucleic acid.

0006-2960/80/0419-2209$01.00/0