Eedor Measurements. We showed above that a number of odmr lines may be associated with a given trap emission, in general. There is thus ambiguity in the assignment of D and E values to a particular triplet site, since it is not known which line of the multiplet appearing in the D − E region originates from the same triplet as a particular line in the 2E region. This ambiguity may be resolved in this case by use of the eedor optical method.[4] Microwave power was applied at the fixed frequency of the center of an odmr line in either the D − E or 2E region as in the hole-burning experiment, while a second microwave source was swept slowly through the D + E frequency region. In each case, a signal was observed in the D + E region in the presence of the fixed frequency power, whereas nothing was observed in its absence. An accurate measurement of the frequency of the eedor response in the D + E region allows the assignment of D and E values to the triplet being pumped by the continuous wave microwave power. In this manner it was found, for example, that the odmr responses observed for trap 1 emission of indole-h_1 in indan originate from three distinguishable triplets with ($|D|$, $|E|$) cm^{-1} values of (0.10379, 0.04570), (0.10383, 0.04596), and (0.10394, 0.04624).

Conclusions

Odmr, eedor, and hole-burning measurements have been made on the triplet state of indole in various environments. It is concluded that the triplet energy (origin of the phosphorescence spectrum) as well as the zero-field splitting is very sensitive to the environment. The emission from pure crystalline indole and indole dissolved in EG–H_2O glasses is broad and originates from an inhomogeneous distribution of traps. Indole dissolved in crystalline indan exhibits a Shpol'skii effect and at least four optically resolvable origins are present. The optical origins themselves originate from distinguishable sites as evidenced by multiplet structure in the odmr lines and optical eedor measurements which show that the multiplicity is due to a system of triplets with a distribution of zero-field splittings. It is probable that the width of the 0–0 bands of indole in indan ($\sim$12 cm^{-1}) contains a contribution due to the discrete trapping sites which we are able to resolve by odmr. The behavior of the odmr lines of indole in the pure crystal as well as dissolved in indan upon hole burning point to an apparent "homogeneous" line width of about 10 MHz. It is important to note that *in zero field* the major contribution to the odmr line width of ^{14}N-containing triplet states is due to "forbidden" satellite transitions involving simultaneous ^{14}N-electron spin flips.[8] These are split out from the "allowed" transitions by the ^{14}N quadrupole splitting frequencies, or typically from ± 1 to ± 4 MHz in azaaromatics. Thus, the line is not inhomogeneously broadened over its entire width but only over a width represented by *second-order hyperfine splittings*, typically $\sim$0.5 MHz. Consequently, spin diffusion need only be effective over the latter frequency band to allow saturation of the entire 10-MHz width by monochromatic microwave power. It is also possible that frequency instability of our signal source could result in saturation over a 0.5-MHz bandwidth. The situation in zero field is thus fundamentally different from that encountered in a strong magnetic field in which first-order hyperfine interactions are responsible for the major portion of the line width and monochromatic microwave power burns a hole in a portion of the nuclear spin population.

Comparison of the results for indole-h_1 with those for indole-d_1 and *N*-methylindole reveal a great similarity. Consequently, we conclude that the sensitivity of the indole triplet state optical and magnetic properties to the environment does not involve direct interactions of the N–H bond.

Acknowledgment. This work was supported by a U. S. Public Health Service grant (GM 12327). J. U. v. S. received support from the Deutsche Forschungsgemeinschaft (Schu 317).

Resolution of Tryptophan Phosphorescence from Multiple Sites in Proteins Using Optical Detection of Magnetic Resonance

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Abstract: A new method is presented which makes it possible to detect heterogeneity in the phosphorescence emission of tryptophan in different protein sites. By observing optically detected magnetic resonance (odmr) signals through a monochromator with a narrow optical window, multiple sites are revealed by discontinuities in a plot of zero-field splitting parameters *vs.* monitored wavelength. It is shown that the phosphorescence emission of lysozyme and of the lysozyme–tri-*N*-acetylglucosamine complex at 1.2°K originates from two distinct types of tryptophan sites, although the 0–0 origins of the sites are not resolved as separate optical bands. Odmr hole-burning experiments and optical eedor experiments are presented which show that the homogeneous magnetic resonance line width in the horse liver alcohol dehydrogenase triplet state is $\sim$10 MHz in zero field (in accord with measurements on the isolated tryptophan triplet), whereas the homogeneous magnetic resonance line width of the principal lysozyme triplet is $\sim$100 MHz under the same conditions. The latter result is probably due to a motional averaging process present at 1.2°K, the nature of which is not presently understood.

The aromatic amino acids, tryptophan and tyrosine, which are components of many proteins, can be optically excited to give reasonably strong fluorescence and phosphorescence emission. These spectra have been studied extensively in the past with a view toward obtaining information about the interactions of the

aromatic amino acids with their environment and with each other.[1] The optical emission bands of excited tryptophan in proteins are generally broad even at pumped-helium temperatures due to a quasi-continuous distribution of environments at each site, and it is usually impossible to resolve the emission bands from tryptophans in quite different regions of the protein molecule. Since it is expected that other properties of the excited states (such as the zero-field splittings of the triplet) might also be sensitive to the environment, we have used the new method of optically detected magnetic resonance (odmr)[2] in zero field in an attempt to further characterize the triplet states of native proteins and to resolve the magnetic resonance signals of tryptophans which are located in different positions on the protein chain.[3] In the previous studies,[3] tryptophan phosphorescence was monitored through a monochromator which emitted light only near the maximum of the 0–0 band, and fast-passage methods were used to detect the odmr signals. In this paper, we report the results of odmr experiments carried out over a range of monitored wavelengths (λ) throughout the 0–0 emission bands of several tryptophan-containing samples. We expected to find correlations between the optical wavelength and the zero-field splitting parameters. Furthermore, if the correlation should vary with the tryptophan site in the protein, discontinuities in the plot of zero-field parameters *vs.* monitored wavelength would indicate emission from distinct tryptophan sites. The measurements are made using slow-microwave-passage methods in order that accurate odmr frequencies and line widths are obtained. In addition, hole-burning and eedor measurements have been made in order to determine whether or not the odmr lines are inhomogeneously broadened and to obtain an estimate of the homogeneous line width of the magnetic resonance signal. Measurements are reported for indole, tryptophan, horse liver alcohol dehydrogenase (HLAD), lysozyme (hen), lysozyme–tri-*N*-acetylglucosamine (tri-NAG) complex in ethylene glycol–water (EG–H_2O) glasses, and crystalline hen lysozyme.

Sample Preparation. L-Tryptophan (Cal Biochem), HLAD (Worthington Biochemical Corp.), and egg white lysozyme (Schwarz/Mann, Inc.) were used as purchased, while indole was carefully zone refined (200 passes). The substrate tri-NAG was kindly donated by Professor John A. Rupley and the lysozyme crystals by Dr. Wolfgang Kabsch. These crystals were grown from a buffered acetate solution with pH 4.8.

All samples, except the lysozyme crystals, were dissolved to concentrations of 10^{-3} mol/l. in EG–H_2O. Detailed information about the optical and odmr apparatus can be obtained from the preceding paper.[4]

Results and Discussion

Phosphorescence Spectra. Phosphorescence spectra of the systems studied here have been published in earlier reports.[3,5] The phosphorescence emissions of lysozyme and lysozyme substrate in EG–H_2O are similar to those of the crystalline samples[3] but with somewhat narrower bandwidths (Table I). The 0–0 phosphorescence bands of each of the samples are plotted in Figure 1. Interference from tyrosine emission in HLAD is minimized by proper selection of exciting wavelength. The emissive properties of tryptophan residues are quite sensitive to the immediate environment as seen from the shifts between the 0–0 wavelengths of the various samples (Figure 1).

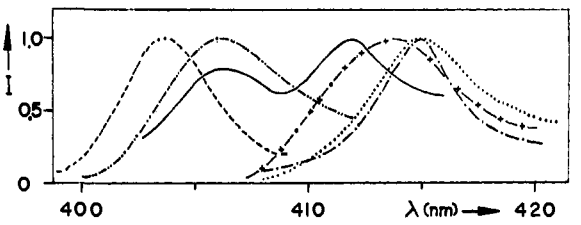

Figure 1. The 0–0 phosphorescence bands of indole (EG–H_2O) (---), tryptophan (EG–H_2O) (—·—), HLAD (EG–H_2O) (—), crystalline lysozyme (×—×), lysozyme (EG–H_2O) (····), and lysozyme–tri-NAG (EG–H_2O) (—·—).

Table I. Optical Data for Investigated Samples

	Excitation wavelength, nm	Phosphorescence peak, nm	Phosphorescence line width, cm^{-1}
Indole (EG–H_2O)	288.0	403.8	340
HLAD-1 (EG–H_2O)	285.0	406.0	180
Tryptophan (EG–H_2O)	290.0	407.5	370
HLAD-2 (EG–H_2O)	300.0	412.5	180
Crystalline lysozyme	290.0	414.0	430
Lysozyme (EG–H_2O)	300.0	415.1	220
Lysozyme–tri-NAG (EG–H_2O)	300.0	415.5	170

Phosphorescence Decay. At 77°K, the tryptophan phosphorescence decay in most proteins appears to be a single exponental with a lifetime of ~6 sec.[5] We have found this to be true also at 4.2°K for all proteins which we have studied. At pumped helium temperatures (1.2°K) the spin–lattice relaxation rates become slow enough to lead to nonexponential decays with components from each of the now partially decoupled triplet sublevels.[6]

An anomalous phosphorescence decay has been reported for egg white lysozyme at 77°K.[7] We have observed a similar result at 77°K; the decay is composed of two exponentials with lifetimes of 1.36 and 4.07 sec and approximately equal preexponential coefficients.[8] At 4.2°K, however, we find a single exponential decay with a 6.3-sec lifetime with no evidence of a short component. There is no apparent change in the wavelength or general shape of the phosphorescence spectrum at the two temperatures, the emission seem-

(1) I. Weinryb and R. F. Steiner in "Excited States of Proteins and Nucleic Acids," Steiner and Weinryb, Ed., Plenum Press, New York, N. Y., 1971.
(2) M. Sharnoff, *J. Chem. Phys.*, **46**, 3263 (1967); A. Kwiram, *Chem. Phys. Lett.*, 1, 272 (1967); J. Schmidt, I. A. M. Hesselman, M. S. deGroot, and J. H. van der Waals, *ibid.*, 1, 434 (1967).
(3) J. Zuclich, D. Schweitzer, and A. H. Maki, *Photochem. Photobiol.*, **18**, 161 (1973).
(4) J. Zuclich, J. U. von Schütz, and A. H. Maki, *J. Amer. Chem. Soc.*, **96**, 710 (1974).
(5) J. W. Longworth in ref 1.
(6) J. Zuclich, J. U. von Schütz, and A. H. Maki, *Mol. Phys.*, in press.
(7) J. E. Churchich, *Biochem. Biophys. Acta*, **92**, 194 (1964); J. W. Longworth, *Biopolymers*, **4**, 1131 (1966).
(8) The ratio of the preexponential coefficients changes from sample to sample and can be affected by the variation of the exciting wavelength.

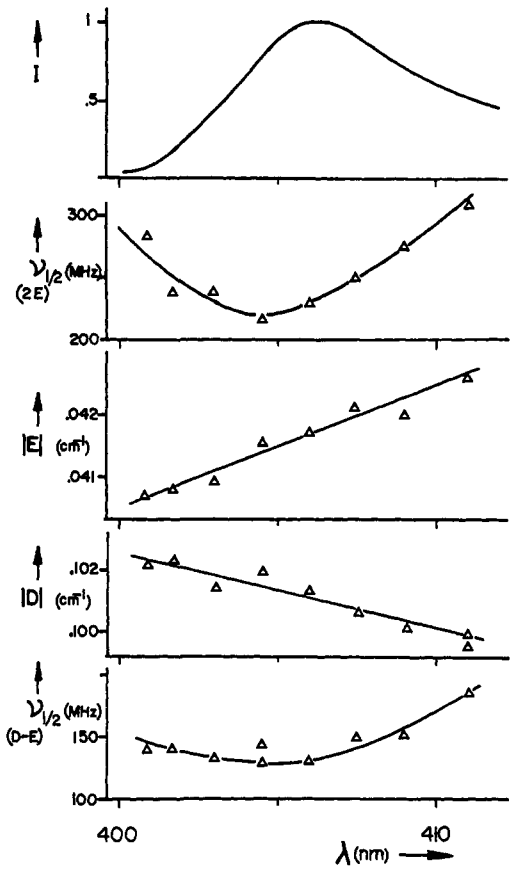

Figure 2. The zero-field splitting parameters |D| and |E|, the odmr line widths $\nu_{1/2}$, and the phosphorescence intensity I of tryptophan (EG–H_2O) as functions of the monitored wavelength.

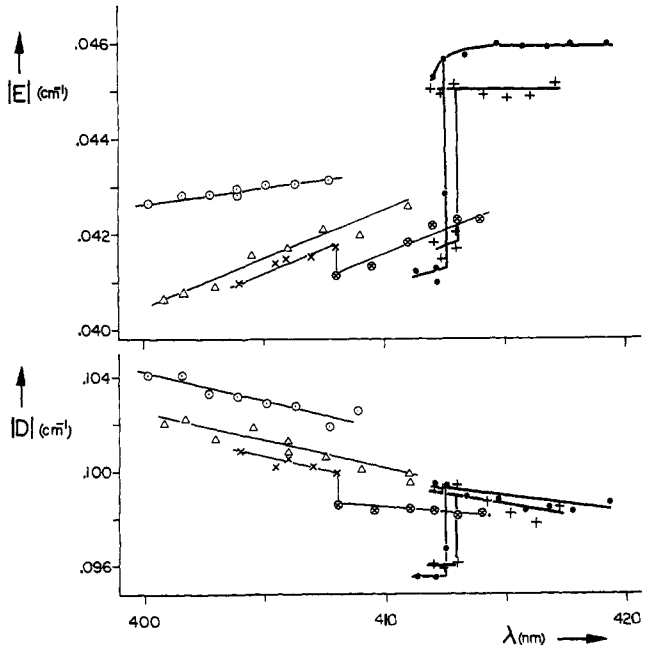

Figure 3. The zero-field splitting parameters |D| and |E| as functions of the monitored wavelength in (EG–H_2O) glasses of indole (⊙), tryptophan (Δ), HLAD-1 (×), HLAD-2 (⊗), lysozyme (●), and lysozyme-tri-NAG (+).

ingly being due solely to tryptophan residues in each case. Further investigations as to the cause of the anomalous temperature dependence of phosphorescence lifetime are in progress.

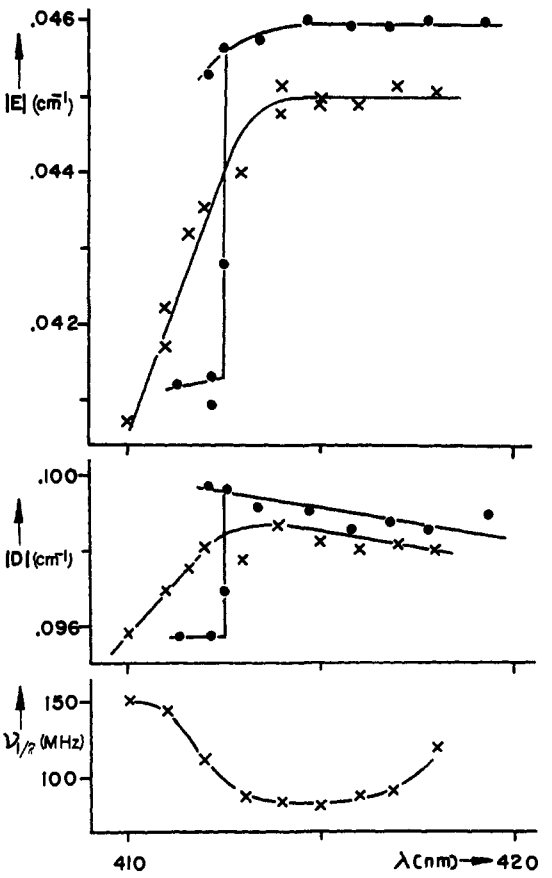

Figure 4. The zero-field splitting parameters and the odmr line width (D — E transition) of crystalline lysozyme (×) as functions of the monitored wavelength. For comparison, the wavelength behavior of the zero-field splitting parameters of lysozyme (EG–H_2O) (●) are plotted also.

Odmr. The results reported here were obtained by slow-passage microwave sweeps[4] through the |D| — |E| and 2|E| transitions of the tryptophan triplet state. The sweep rate was 5–8 MHz/sec and the monochromator slits were set for 1-Å resolution. The pumped helium temperature varied over the range of 1.25–1.34°K from day to day but the results were reproducible within this range.

Tryptophan (EG–H_2O). Figure 2 illustrates the results for tryptophan in EG–H_2O. The zero-field splitting parameters |D| and |E|, the odmr line widths, and the phosphorescence intensity are plotted as functions of the monitored wavelength. It is seen that |E| increases and |D| decreases linearly with increasing detected wavelength. Similar behavior of the |D| and |E| parameters is found in the other samples studied here (Figures 3 and 4), but the curves have discontinuities indicating contributions from more than one tryptophan site. The odmr line widths are significantly broader when monitored at the periphery of the optical bands than when monitored near the center as shown in Figure 2. The more red shifted the 0–0 phosphorescence peak the narrower is the odmr line width of the EG–H_2O samples when monitored at the peak (Figure 5).

The fact that the |D| and |E| values of the tryptophan emission show a dependence on the emission wavelengths is proof that there is a contribution to the width of the optical emission lines from a distribution of

environments. Although the optical window which is monitored is only 1 Å in width, the odmr line width is the order of 100 HMz. As will be shown below, this is at least an order of magnitude larger than the line width expected for a homogeneous distribution of tryptophan triplet sites. It may be concluded, then, that there is only a weak correlation between the zero-field splitting parameters and the triplet energy, *i.e.*, that there is a distribution of |D| and |E| values among the triplets emitting at a given wavelength. Furthermore, the width of this distribution increases as the monitored wavelength approaches the edges of the 0–0 band. Comparison of Table I with Figure 5 shows that narrower optical lines yield narrower odmr signals, in general.

Because of the low concentration of 10^{-3} mol/l., direct tryptophan–tryptophan interactions may be neglected in the frozen glass. Consequently, the width of the optical emission lines and the microwave frequency shifts (Figures 1 and 2) may be ascribed to a distribution of interactions between the triplet molecules and the polar solvent. The change in |E| is about +5% between 401.0 and 411.0 nm, while |D| decreases by about 2% in the same wavelength range.

Indole (EG–H_2O). The behavior of indole, the aromatic portion of tryptophan, in EG–H_2O is qualitatively similar to tryptophan as seen in Figure 3. Although there is a displacement of the |D| and |E| values and the phosphorescence due to the absence of the amino acid segment, the slope of the zero-field parameter |D| is similar on the wavelength scale. On the other hand, |E| varies much less with λ in indole than in tryptophan. This result suggests that |D| is principally affected by solvent interactions directly with the aromatic part of the molecule, whereas |E| is influenced mainly by solvent interactions with the amino acid side chain.

HLAD (EG–H_2O). HLAD is a rare example of an enzyme which exhibits resolvable phosphorescence origins from distinct tryptophan sites.[9] The peak at 406.0 nm has been assigned to a solvent-exposed tryptophan (HLAD-1), while the peak at 412.5 nm was assigned to a tryptophan effectively buried in a hydrophobic region of the enzyme (HLAD-2).[9] Zuclich, *et al.*,[3] found significant differences in the |D| value but only a small difference in the |E| value, obtained by monitoring the two peaks. The wavelength dependence is given in Figure 3 and shows the discontinuity in both the |D| and |E| values which occurs in the region of 408 nm where the emissions of HLAD-1 and HLAD-2 overlap. Since the change in microwave frequency at this wavelength is the same order as the line width, a clearer resolution of the two signals can be obtained by shifting the exciting wavelength from 285 to 300 nm, whereupon the contribution of HLAD-1 to the odmr signal and the phosphorescence is absent.

It is seen from Figure 3 that the |D| and |E| values of HLAD-1 fall very nearly on the same line as those of tryptophan in EG–H_2O confirming the assignment of HLAD-1 as a solvent-exposed tryptophan by Purkey and Galley.[9] HLAD-2 deviates significantly from the tryptophan curves, indicating a different type of environment. The slopes of the HLAD-1 and HLAD-2 curves are similar, however, indicating a similar correlation of the zero-field splitting parameters with optical shift for the two sites.

(9) R. M. Purkey and W. C. Galley, *Biochemistry*, 9, 3569 (1970).

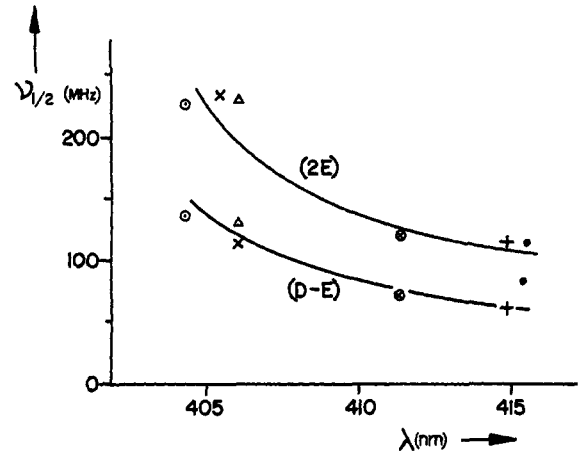

Figure 5. Odmr line widths, monitored at the peak of the 0–0 phosphorescence band, are plotted *vs.* the peak wavelength: indole (⊙); HLAD-1 (×); tryptophan (Δ); HLAD-2 (⊗); lysozyme–tri-NAG (+); and lysozyme (●). All measurements were made in EG–H_2O glass.

Lysozyme (EG–H_2O). Lysozyme (hen) has a molecular weight of 14,300 and contains six tryptophan residues at positions 28, 62, 63, 108, 111, and 123. From the work of Imoto, *et al.*,[10] it is known that about 80% of the room-temperature fluorescence originates from tryptophans 62 and 108 which are situated near the active site of the enzyme. It is probable, considering the average intersystem crossing efficiency of 10%,[5] that the phosphorescence originates from these sites as well. Our results presented in Figure 3 indicate that the phosphorescence originates from at least two distinct types of tryptophan, although the phosphorescence spectrum (Figure 1) shows no evidence of resolved origins. One site which is responsible for about 30% of the phosphorescence intensity has a 0–0 band peak at about 412 nm and an average |E| value of 0.041 cm^{-1}, whereas the major emitting site (~70% of the phosphorescence) has its 0–0 band peak red shifted to 416 nm and a 10% larger |E| value. As in HLAD a sudden discontinuity in |E| occurs in the overlapping wavelength region around 412.5 nm. The odmr signal in this wavelength region is a clearly resolved doublet. There appears to be little, if any, change in the |E| value with wavelength for the red-shifted site. This is interesting in light of the hole-burning experiments to be discussed below.

Lysozyme–Tri-NAG (EG–H_2O). Binding of the trisaccharide involves hydrogen bonding with the active site tryptophans 62, 63, and 108 and is accompanied by an important structural change involving mainly a change in the position of tryptophan 63.[11,12] Our results for the enzyme–inhibitor complex are, in fact, quite similar to those for the enzyme itself and are given in Figure 3. The binding of the inhibitor shifts the |E| values of the two sites slightly, but differently. The red-shifted site |E| value is shifted downwards, whereas the blue-shifted |E| value is shifted upwards.

(10) T. Imoto, L. S. Forster, J. A. Rupley, and F. Tanaka, *Proc. Nat. Acad. Sci. U. S.*, 69, 1151 (1972).
(11) C. B. Blake, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sharma, *Proc. Roy. Soc., Ser. B*, 167, 365 (1967).
(12) C. B. Blake, L. N. Johnson, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sharma, *Proc. Roy. Soc., Ser. B*, 167, 378 (1967).

Again, there is no observable dependence of the |E| value of the red-shifted site on the observing wavelength.

The similarity of the results for the enzyme and the enzyme–inhibitor complex is surprising in light of the site of inhibitor binding[11,12] and the supposed origin of the phosphorescence.[10] It is possible that the triplet-state properties are not greatly changed on displacement of solvent molecules in the active site by the trisaccharide. On the other hand, the luminescent tryptophans at low temperature might differ from those at room temperature.

Crystalline Lysozyme. Our results for crystalline lysozyme are similar to those obtained for lysozyme in EG–H,O, except that the line width is greater. Apparently, there exists a larger distribution of tryptophan environments in the crystals than in the glass. It may be seen in Figure 4 that the sharp discontinuity in |D| and |E| *vs.* wavelength is not observed, but rather, that we observe a relatively sharp monotonic increase in |D| and |E| in the wavelength region where the discontinuity occurs in the EG–H_2O sample, as well as an increase in the overall line width in the region of overlap of the emissions of the two sites. This behavior is consistent with the presence of the same sites as in the glass but with line widths too large for resolution of the two individual odmr signals.

Hole Burning and Eedor. Hole-burning experiments[13] on indole and tryptophan in various solvents are reported in the previous paper.[4] These were continued in the present work in order to investigate the nature of the odmr line broadening and to determine the homogeneous line width in the protein samples. In all measurements, we monitored the phosphorescence at the center of the 0–0 band with narrow slits.

Whereas in HLAD (EG–H_2O) we were able to burn a hole of 10 MHz width in the odmr line with the application of continuous wave (CW) microwave power, no hole could be burned in the odmr lines of lysozyme or of the lysozyme–inhibitor complex in EG–H_2O. Instead, the entire odmr line decreased in intensity upon application of CW microwave power at the center frequency and could be nearly totally saturated with the application of approximately 0.5 W of power. Hole-burning experiments were attempted on the blue-shifted site of lysozyme, but these were inconclusive due to the poor signal-to-noise ratio in this region.

Eedor experiments[14] were done as described in the previous paper.[4] No eedor signal could be detected at the |D| + |E| frequency when CW microwave power was applied at the |D| − |E| or 2|E| frequencies in HLAD, but the eedor signal was observed in the case of lysozyme and the lysozyme–tri-NAG complex. In the cases where the eedor signal was observed, sufficient CW microwave power was applied to significantly saturate the odmr line, and the width of the eedor response at |D| + |E| was comparable to that of the directly observable 2|E| and |D| − |E| signals. These results indicate that the odmr signals of HLAD are inhomogeneously broadened with a "homogeneous" line width of about 10 MHz, whereas the odmr line of lysozyme and the lysozyme–inhibitor complex appear to have "homogeneous" widths the order of 100 MHz. This surprising result suggests that some sort of motional averaging process is present in the lysozyme systems at 1.2°K which makes it possible to saturate all triplets with magnetic resonance frequencies over a 100-MHz range by application of monochromatic microwave power. Spin diffusion is not expected to be effective over such a large frequency range; the averaging may be related to quantum mechanical exchange between neighboring tryptophans in the enzyme. The lack of the wavelength dependence of the |E| value in the lysozyme systems which is characteristic of the other systems studied also is consistent with motional averaging of the zero-field parameters. Further experiments are in progress on related enzymes and model systems in an attempt to clarify the nature of the line broadening in lysozyme.

(13) M. Leung and M. A. El-Sayed, *Chem. Phys. Lett.*, **16**, 454 (1972).

(14) T. S. Kuan, D. S. Tinti, and M. A. El-Sayed, *Chem. Phys. Lett.*, **4**, 507 (1970).

Conclusion

We have shown that it is possible to detect heterogeneity in the phosphorescence emission from proteins even if the heterogeneity is not optically resolvable. By observing the odmr with a narrow optical window as a function of wavelength through a broad emission envelope, the presence of multiple emitting sites is revealed as a discontinuity in a plot of zero-field splitting parameters *vs.* observing wavelength. Two emitting tryptophan sites are shown to be present in lysozyme and in lysozyme–tri-NAG complex although separate 0–0 bands are not resolved optically.

A "homogeneous" line width of ~10 MHz in zero field is found for the tryptophans in HLAD, a value which is typical of tryptophan in EG–H_2O, as shown in the previous paper.[4] The major emitting sites of lysozyme and the lysozyme inhibitor complex are shown to have "homogeneous" line widths of about 100 MHz by hole-burning experiments. This result is indicative of some sort of motional averaging process taking place at 1.2°K, the nature of which is not now clear.

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