line of a reciprocal Stern-Volmer plot ($F_0/\Delta F$ vs. $1/[X]$) provides an effective quenching constant, $K_e = \Sigma f_i K_i^2 / \Sigma f_i K_i$, and an effective fractional accessible fluorescence, $f_e = (\Sigma f_i K_i)^2 / \Sigma f_i K_i^2$. These parameters provide a better index of the properties of the most accessible fluorophors than the corresponding parameters obtained from the limiting slopes of simple Stern-Volmer plots, $K_e = \Sigma f_i K_i$; $f_e = (\Sigma f_i K_i^{-1})^2 / \Sigma f_i K_i^{-2}$ (5). With the use of sensitive differential fluorometers these parameters may reveal subtle changes of accessibility accompanying a variety of protein interactions.

## REFERENCES


1. LEHRER, S. S., and G. D. FASMAN. 1967. Fluorescence of lysozyme and lysozyme substrate complexes. Separation of tryptophan contributions by fluorescence difference methods. *J. Biol. Chem.* **242**:4644.
2. LEHRER, S. S. 1967. The selective quenching of tryptophan fluorescence in proteins by iodide ion: lysozyme in the presence and absence of substrate. *Biochem. Biophys. Res. Commun.* **29**:767.
3. LEHRER, S. S. 1971. Solute perturbation of protein fluorescence. The quenching of the tryptophyl fluorescence of model compounds and of lysozume by iodide ion. *Biochemistry.* **10**:3254.
4. ELKANA, Y. 1968. Differentiation between tryptophan residues in proteins. *J. Phys. Chem.* **72**:3654.
5. LEHRER, S. S., and P. C. LEAVIS. 1977. Solute quenching of protein fluorescence. *Methods Enzymol.*, Part F, Enzyme Structure. In press.


# IS OPTICAL DETECTION OF MAGNETIC RESONANCE USEFUL IN DETECTING HETEROGENEITY IN PROTEIN PHOSPHORESCENCE?


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Optical methods have been used for some time in detecting heterogeneity in the tryptophan sites of proteins. Some of these methods rely on the sensitivity of the Stokes shift of the tryptophan phosphorescence origin to the nature of the environment, particularly its electrical polarizability.

A good example of the sensitivity of the Stokes shift of the indole chromophore phosphorescence to its environment is found in indole itself dissolved in the crystalline Shpol'skii matrix, indane. Four well-resolved O—O bands with 12 $cm^{-1}$ widths are observed at 1.25 K spread over a range of about 300 $cm^{-1}$. These discrete origins probably result from distinct guest sites in the matrix which have differing host polarizabilities along the direction of dipole moment change in the $S_0 - T_1$ process.

Possible heterogeneity within these optically resolved bands was investigated by utilizing another property of the excited state that also is expected to vary with the environment–the zero field splittings (ZFS). The splitting pattern of the magnetic sublevels is determined principally by the magnetic dipole-dipole coupling of the spin-parallel electrons and is characterized by the ZF parameters $D$ and $E$. In zero magnetic field, magnetic dipole-allowed transitions can be induced between the sublevels at the frequencies $(|D| + |E|)c$, $(|D| - |E|)c$, and $2|E|c$, where $c$ is the speed of

light and $D$ and $E$ are in reciprocal centimeters. The assignment of any two of these transitions allows determination of $|D|$ and $|E|$. When spin alignment can be achieved during optical pumping by quenching of spin-lattice relaxation at sufficiently low $T$, these transitions can be observed as changes in the phosphorescence intensity (optical detection of magnetic resonance, ODMR) by conventional slow passage methods if the radiative quantum yields of the coupled levels differ. This situation occurs in indole and in tryptophan, where $Q_x \sim 1$, $Q_y, Q_z \sim 0$; thus the transitions $T_x \leftrightarrow T_y$ ($2|E|$), and $T_x \leftrightarrow T_z$ ($|D| - |E|$) can be observed by monitoring the phosphorescence intensity.

Each of the phosphorescence origins of indole/indane was isolated in turn with a monochromator utilizing 20-$cm^{-1}$ slits, and slow passage ODMR signals were detected at 1.25 K in the $2|E|c$ and $(|D| - |E|)c$ regions of the zero field spectrum. A total of eight (possibly nine) distinct, apparently homogeneous, sets of ZF transitions were obtained utilizing the combination of optical and radio-frequency resolution. The highest energy O—O band, for example, was found to originate from three distinct triplet states with $(|D|, |E|) = (0.10379, 0.04570)$, $(0.10383, 0.04596)$, and $(0.10394, 0.04624)$ $cm^{-1}$. $|D|$ is larger while $|E|$ is smaller for the triplets emitting in the three red-shifted origins, but there is otherwise no correlation apparent between the ZFS and the Stokes shift.

It is convenient to classify the heterogeneity of tryptophan sites in proteins into two types: Structural heterogeneity: The location of tryptophan at different positions in the polypeptide chain, thus with different neighboring groups in the tertiary and quaternary structure; and Statistical heterogeneity: A basically continuous distribution of microenvironments at each structurally distinct site within the ensemble of protein molecules.

The indole/indane system exhibits mainly structural heterogeneity, since the variation in microenvironments is severely limited by the crystalline regularity of the host. The statistical heterogeneity of sites in frozen glassy (and also crystalline) proteins, on the other hand, leads to a spread in the Stokes shifts usually comparable to or greater than the shifts between the structurally heterogeneous sites. The resulting inhomogeneous broadening of the spectra rarely makes it possible to resolve the emission from structurally distinct sites of proteins optically.

ODMR would be useful in the detection and isolation of structural heterogeneity if the ZFS parameters, $|D|$, and $|E|$, of structurally distinct sites correlate differently with the Stokes shift. The idea is to select, using narrow band optical detection, narrow wavelength regions of the inhomogeneously broadened O—O phosphorescence band of tryptophan-containing protein, measure the ZFS of the contributing triplets by ODMR, and plot $|D|$, and $|E|$ vs. the Stokes shift. A specific response of these parameters to the onset of the emission from a structurally distinct site would result in a discontinuity in the plot. The ability to resolve multiple triplets by ODMR within bands of extremely narrow Stokes shift in the indole/indane system already suggests that the ZFS correlate with site heterogeneity differently from the Stokes shift.

As a model of a system characterized by statistical heterogeneity only, we have in-

vestigated tryptophan in EG:$H_2O$ glass using the method described above. There is only a weak correlation of the ZF parameters with Stokes' shift that is monotonic and continuous.

A similar set of measurements was made on horse liver alcohol dehydrogenase, an enzyme characterized by two structurally distinct tryptophans, one solvent-exposed pair, and one pair inside the contact region of the dimer. The inhomogeneous broadening resulting from statistical heterogeneity is less than the Stokes shift between the structurally distinct sites; the phosphorescence is a partially resolved doublet. Plots of $|D|$ and $|E|$ vs. Stokes shift reveal a clear discontinuity at the wavelength where the O—O bands of the structurally distinct sites merge.

Hen lysozyme, its complex with tri(*N*-acetylglucosamine), human carbonic anhydrase B, and its complex with *m*-acetylbenzenesulfonamide show similar discontinuities in the ZFS parameters plotted against Stokes shift over the O—O bands, even though in lysozyme and its complex no distinct site emissions can be resolved optically.

We conclude that ODMR with narrow band wavelength selection over an inhomogeneously broadened phosphorescence band can reveal, at least in some cases, emission from structurally heterogeneous sites even if these emissions are not optically separable into distinct bands. The success of this method rests upon sufficient differences in the relationship between the ZFS and the Stokes shift between structurally distinct sites.

## HETEROGENEITY IN THE CIRCULAR POLARIZATION OF PROTEIN FLUORESCENCE

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Optical activity has been extensively used in the investigation of the conformation of biopolymers. This property is usually measured by the optical rotatory power or the circular dichroism (CD) of the studied biopolymers. We have shown that the optical activity of chiral molecules, including proteins, can be manifested by an additional spectroscopic property, its circular polarization of the luminescence (CPL) (1, 2). CPL is the emission analogue of CD and is related to the molecular conformation of the first electronic excited state in the same way that CD is related to the molecular conformation of the ground state (1, 2). The CPL spectrum is expressed by the emission anisotropy factor defined as $g_{em} = 2\Delta f/f$, where $f$ is the total fluorescence intensity and $\Delta f$ is the intensity of the circularly polarized component in the emitted light.

If the conformation and the environment of the chromophore are the same in the ground state and excited state, $g_{em}$ should be equal to the absorption anisotropy factor $g_{ab}$ (1, 2) defined as $2(\epsilon_l - \epsilon_r)/(\epsilon_l + \epsilon_r)$ where $\epsilon_l$ and $\epsilon_r$ are the molar extinction coefficients for left-handed and right-handed circularly polarized light. Since only lumines-