Molecular Luminescence Studies of Flavins, II

Interactions Involving the Excited States*

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The effects of experimental geometry on the theoretical polarizations of the $S_1 \leftarrow S_0$ and $S_2 \leftarrow S_0$ bands of $\pi \rightarrow \pi^*$ type in riboflavin have been examined. Polarizations of these two bands are characterized by an angle between them in the range of $20-28^\circ$ and are relatively insensitive to the input geometry. Thus the predicted polarizations are generally in agreement with fluorescence polarization spectrum of riboflavin at 77 K. Alloxazine forms a strong complex with KI, and the fluorescence and phosphorescence from the charge transfer states have been characterized by means of luminescence and photoselection measurements. Riboflavin did not form a strong complex with KI, but it forms aggregates (dimer) more readily than alloxazine. The excited states of flavins can be populated by the weak dipole-dipole coupling mechanism of energy transfer from $1_{\Lambda}$ states of indoles to the $S_2$ state of flavins. The measured critical distances estimated from the fluorescence depolarization experiments range from 31 Å for indole to 40 Å for indole-2-carboxylic acid in glycerol-methanol mixture (9:1) at 263 °K.

Abbreviations: AL, alloxazine; CT, charge transfer; LE, locally excited; RF, riboflavin; RFTB, riboflavin tetra-butylate; I, indole; CA, carboxylic acid; IAA, indole acetic acid; P, polarization in degrees.

The present report describes interactions of flavin-flavin, flavin-iodide, and indole-flavin systems involving the electronically excited states. In the previous paper (Part 1, referred to as 1 hereafter), excited states of individual flavins have been examined 1. We begin our discussion by examining the polarization direction of the two absorption bands of RF. Fig. 1 shows the absorption spectrum of RF along with its polarized excitation spectrum with respect to the fluorescence emission. After the polarizations of the 450 and 360 nm bands of RF were calculated 1, the complete experimental geometries of the flavin nucleus were published 2, 3.

New results of the polarization calculations using the observed geometries 2, 3 (3 and 4) are shown in Fig. 1, along with the data in 1 obtained using the crystal structure of RF hydrobromide monohydrate 4 and in ref. 5 using an assumed geometry.

It can be seen that the results of the four different geometries are similar *, and are consistent

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Fig. 1. Absorption spectrum ( ), polarized fluorescence excitation spectrum ( — o — o — ) in ethanol at 77 °K, and calculated polarizations of two absorption bands at 350 and 445 nm. Calculation 1 based on assumed geometry 5, and 2, 3, 4 based on observed geometries of ref. 4, 2, and 3, respectively. Calculated transition energies (vertical lines) and oscillator strengths (f) were obtained with the geometry 3.

* If we accept the existence of the weak $\pi \rightarrow \pi^*$ band in the region of 300 nm 1, as deduced from the linear dichroic spectra of RF in a stretched film 6 and from log-normal analysis of the absorption spectrum 7, results of the MO calculations using the geometry of ref. 4 are not satisfactory because of the high oscillator strength predicted for the weak transition.
with the polarized fluorescence excitation spectrum shown in Fig. 1 and the spectra recorded in 1. The longest wavelength absorption transition is predicted to be polarized along the axis containing N3 and C8, with the absolute direction of the transition moment pointing toward C8. Having described the polarization characteristic of the typical flavin, RF, and using data on the triplet states of flavins in 1, some examples of interactions in the excited states of flavins will be discussed below.

1. Dimer Luminescence of Flavins

Fig. 2 shows the phosphorescence excitation and emission spectra of 5 × 10⁻⁵ M RF or RFTB in ethanol at 77 °K. The phosphorescence excitation spectrum shows some intensity beyond 500 nm. Further, the intensity of the excitation at wavelength longer than 500 nm increases with concentration, with the decrease in the excitation intensity below 500 nm due to the front surface absorption. It was found that the phosphorescence emission maximum shows a red shift of about 35 — 40 nm as the excitation wavelength is changed from 465 nm to 510 nm. The lifetime of the emission maximum at 605 nm with excitation wavelength of 465 nm was found to be 0.17 ± 0.01 sec, while the emission maximum at 640 nm has a lifetime of ~ 0.1 sec, indicating that two maxima represent different emitting species. Since the short lifetime phosphorescence predominates at higher concentrations, partly because of the front surface absorption, it can be assigned to a RF dimer emission. The relative yield of the dimer phosphorescence is about 3 times higher than that of the monomer (based on rough estimates of the O.D. at 510 nm and the phosphorescence intensity at the maxima).

In order to demonstrate clearly the concentration dependence of the dimer phosphorescence, RFTB was used since it is considerably more soluble in many organic solvents than RF itself. The gradual and parallel increases in the 510 nm excitation and 650 nm dimer emission was observed, with eventual alteration of the excitation spectrum at the highest concentration. Typical spectra at highest concentration used are shown in Fig. 2. The dimer emission is even more predominant in isopentane than in polar matrices. When RFTB (3 × 10⁻⁵ M) is dissolved in isopentane, the longer wavelength emission completely obscures the shorter wavelength monomer phosphorescence.

The polarized dimer phosphorescence is substantially less negative than the monomer phosphorescence, as can be seen from Fig. 2, implying that it has more mixed components than the monomer. Because of the front surface absorption and monomer phosphorescence of concentrated solutions of RFTB, it was not possible to obtain the polarized excitation spectrum for the dimer phosphorescence at wavelengths shorter than 510 nm. However, approximate depolarization of the dimer phosphorescence is suggestive of configurational and spin-orbit mixing of various excited states having different polarization. Similar depolarization has been observed in the case of excimer emission from anthracene sandwich dimers. A crude MO calculation (PPP SCF MO CI, closed shell) also predicts mixed polarizations of S₁ → S₀ and T₁ → S₀ for a dimer in which the two flavin planes incompletely stack onto each other. Such an incompletely stacked dimer has been confirmed in solution by NMR spectroscopy.

Attempts to observe dimer fluorescence of RF and RFTB at 77 °K were not successful owing mainly to a strong scatter and weak dimer emission, compared to the dominant monomer emission. However, an extremely weak dimer fluorescence emission (λₑ ~ 540 nm) can be demonstrated for solutions at room temperature, after correcting for the inner filter effect, provided sufficiently concentrated solutions are prepared.
The monomer phosphorescence and its polarization spectrum of AL have been described previously. The dimer phosphorescence spectrum of AL cannot be readily obtained due to difficulty in preparing sufficiently high concentrations in organic solvents and in water. To obtain more concentrated solutions, the ethanol solution containing excess AL was heated at 80 °C in the dark for several hours, and the concentration of AL reached \(2 \times 10^{-4}\) M. This solution was immediately frozen to 77 °K without apparent precipitation during freezing. A red shift of 9 — 10 nm in the phosphorescence maximum was observed for this solution with the excitation at the red edge of the absorption spectrum (430 nm), as compared to the monomer excitation at 378 nm. This behavior is essentially the same as the one demonstrated by RF and RFTB, except that the red shift and extent of the alteration of the excitation spectrum of AL appear to be less distinctive than that of the two flavin compounds.

2. AL-Iodide Complex: CT Luminescence

Spectrophotometric measurements of AL — KI indicated that a strong complex (1:1) formed with its charge transfer absorption maximum at about 430 — 440 nm. All of the AL is complexed with iodide at concentrations of KI greater than 0.2 M in ethanol.

With 0.2 M KI we observe no original AL fluorescence as it is completely quenched by KI, but its position is indicated in Fig. 3 as a reference. If the frozen solution is excited at 445 nm, the new absorption band observable only in the presence of KI, a new fluorescence band is observed at 480 nm and phosphorescence at 585 nm. This new absorption band and its corresponding luminescence is attributed to a strong charge transfer complex formed between I⁻ and AL. The phosphorescence of the complex has a lifetime of \(\leq 0.014\) sec and a reasonably exponential decay curve. There is a deviation from the exponential decay at the later stage. AL itself in ethanol in the absence of KI has a lifetime of 0.23 sec with exponential decay. Non-exponential decay is observed for the uncomplexed AL in the presence of KI, as was the case with RF.

The polarization of the O — O phosphorescence of AL is slightly negative or close to zero degree. With 0.2 M KI added, the polarization is clearly positive when the solution is excited at 400 nm, as shown in Fig. 3. This implies that in-plane contributions to the phosphorescence are enhanced by KI. The phosphorescence polarization of the AL — KI complex is slightly positive with respect to the 445 nm excitation. However, polarization across the original AL band is significantly positive with respect to the CT excitation at 445 nm, suggesting that the phosphorescent triplet state is probably perturbed by the CT state. A satisfactory characterization of the CT phosphorescence requires the polarized CT phosphorescence excitation spectrum across the entire absorption spectrum, and this is not feasible experimentally because of the heavy atom enhanced emission from the localized \(\pi,\pi^*\) state of AL with \(\lambda_{ex}\) shorter than 410 nm. However, the CT fluorescence shows a high polarization with respect to the CT absorption and about zero value with respect to \(S_1 \leftarrow S_0\) and \(S_2 \leftarrow S_0\) excitations (\(\pi \rightarrow \pi^*\) type). Fig. 4 illustrates these CT luminescence bands diagramatically. The dotted arrow represents the transition which is not observed in AL — KI complex.

A non-exponential decay of RF phosphorescence in the presence of KI and ethyl iodide has been observed. The simplest interpretation of the non-exponential decay is that the system is inhomogeneous due to uneven distributions of heavy atoms in the frozen lattice sites. An alternative interpretation is that the system consists of complexed and uncomplexed flavins with iodide. Although no quanti-
tative estimates of the equilibrium constants have been made, RF complexes weakly with iodide, as shown by the difference spectrum. However, neither of these interpretations are sufficient to explain the slow component of the phosphorescence having a lifetime longer than the RF phosphorescence in the absence of KI and ethyl iodide. We ascribe the existence of the slow component of phosphorescence to the uncomplexed RF in which the $3(n,\pi^*)$ states, in particular, $3(n,\pi^*)$ states, are raised in energy due to the polar environment provided by iodide. This type of effect then results in lengthening of the lifetime, since the vibronic coupling between $3(\pi,\pi^*)$ and upper $3(n,\pi^*)$ states affects the lifetime. If this interpretation is correct, such slow components will not be observed in purely aromatic systems in the presence of iodide. To support the above interpretation, we observed the exponential decay of the RF phosphorescence in the presence of 0.011 M KCl. The exponential decay, low concentration of KCl, and its subsequent little effect on the phosphorescence intensity, all point to the situation that RF does not complex with chloride significantly under these conditions. An aqueous solution of a higher concentration of KCl (about 0.1 M) was also added to the alcoholic solution of RF and was frozen to form a snowy matrix. Again, the exponential decay was observed. The lifetime observed is 0.24 sec, a considerably larger value than the lifetime of the unperturbed RF phosphorescence! This seems to demonstrate clearly the increase in $3(n,\pi^*)$ state energy due to polarity effect of iodide and chloride in the local lattice sites. The lack of a detectable red shift of the phosphorescent $3(\pi,\pi^*)$ state of RF in the presence of halides can be explained by presuming that the halide is located in the plane of the molecule nearest to the heterocyclic centers (perhaps $N_5$), thus affecting the $n,\pi^*$ states but with negligible effects on the lowest triplet state. AL does not show such an effect presumably because of a strong complexing and vibronic coupling between $3(n,\pi^*)$ and $3(\pi,\pi^*)$ states which is sufficiently strong so that the halide polarity effect is not revealed.

3. Energy Transfer

There are two major obstacles in determining the mechanism of energy transfer from indole to flavin as a model for flavoproteins containing tryptophan residues. These are: a) absorption of the exciting and emitted light intensities by the acceptor flavin, resulting in trivial transfer, and b) intrinsically low polarization values ($P$) and difficult $0-0$ excitation for the indole fluorescence, making it difficult to estimate the transfer radius. To avoid these difficulties which can be eliminated in theory but are extremely difficult to eliminate in practice, we have adopted the stepwise transfer model developed by Weber and shown in the following equation which relates the polarization of the donor fluorescence to the acceptor concentrations using a polarized excitation source:

$$\frac{1}{P} - \frac{1}{3} \simeq \left[ 1 + \frac{4 \pi N(RF)}{15} \left( \frac{R_0}{2r} \right)^3 \cdot 10^{-3} \right]$$

where $r$ is the molecular radius in cm and $R_0$ is the critical transfer distance in Å. Results after least-

![Fig. 4. Energy level diagram for AL-KI complex.](image)

![Fig. 5. A typical plot of the RF concentration dependence of the reciprocal polarization of the donor (IAA) fluorescence. Other indole donors have yielded similar straight lines, and critical distances from the slopes of such plots are also included.](image)
square analysis are shown in Fig. 5 along with the theoretically calculated values for selected indole derivatives which have been obtained using the spectral overlap between the $^1L_a \rightarrow \Lambda$ and $S_2 \leftarrow S_0$ bands and observed fluorescence lifetimes of indole$^{14,15}$. Previously, KARREMAN et al.$^{16}$ calculated $R_0$ of 26 Å for the indole $\rightarrow$ RF transfer.

Energy transfer appears to be via the weak dipole-dipole coupling mechanism. Thus, quenching of fluorescence of tryptophanyl residues in flavoproteins (e.g. flavodoxin)$^{17}$ can be attributed to the long-range energy transfer, as suggested in the present model system. The trivial mechanism was found to be predominant in the case of energy transfer from the triplet state of indoles to flavins. Finally, it should be pointed out that critical distances evaluated from depolarization in viscous solutions (e.g. Fig. 5) are subject to errors, particularly in view of the non-identity of donor and acceptor molecules and low depolarization range. A critical analysis of the stepwise transfer model has been made recently$^{18}$.

8. S. K. CHAKRABARTI, Molecular Physics 18, 275 [1970].