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A Stark Spectroscopic Study of *N*(3)-Methyl, *N*(10)-Isobutyl-7,8-Dimethylisoalloxazine in Nonpolar Low-Temperature Glasses: Experiment and Comparison with Calculations

Robert J. Stanley* and M. Salim Siddiqui

Department of Chemistry, Temple University, Philadelphia, Pennsylvania 19122

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Flavins have unique electronic properties that have led to their utilization in biological redox chemistry. Despite this there is relatively little experimental information about such basic electronic properties as dipole moments and polarizabilities for these molecules. We have explored the electronic structure of the ground and first two excited electronic states of an oxidized flavin in nonpolar organic glasses using Stark spectroscopy. The dipole moment change for the $S_0 \rightarrow S_1$ transition is about 3 times smaller than the dipole moment change for the $S_0 \rightarrow S_2$ transition. Dipole moment directions for these excited states have been calculated using the experimental results along with predictions from calculations. The experimental results are consistent with computational predictions of intramolecular charge transfer for the $S_0 \rightarrow S_2$ transition. In addition, we have verified that there is a large change in polarizability for the S_2 state vs the S_0 and S_1 states. Both excited-state polarizability changes show a slight dependence on the polarity of the solvent. The physical origin behind this trend is explored.

Introduction

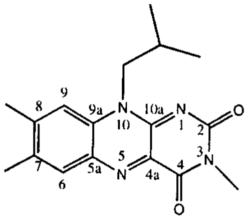
Flavins are distributed widely in nature as protein cofactors that are useful in one- and two-electron-transfer reactions.[1,2] These molecules are highly asymmetric structurally and therefore electronically. This asymmetry leads to a large ground-state dipole moment (about 7 D from gas-phase calculations[3]). Studies have shown the importance of the protein binding site in setting the redox potentials of flavins.[4−6] For example, the redox potential of the electron transport protein flavodoxin can be altered by the mutagenesis of a methionine residue near the polarizable xylene ring of the flavin molecule.[7] In other studies, disruption of flavin−aromatic residue stacking interactions and changes in the electrostatic environment of the flavin binding site modulates the redox potential of the flavin.[8] In addition, certain flavoproteins are light-driven, highlighting the importance of the excited state in photoreception, DNA repair, and signal transduction.[9−13] However, the electronic structure of the excited states of these systems are relatively unexplored.

We have applied Stark spectroscopy to the study of the electronic properties of flavins in polar solvents.[14] Stark spectroscopy measures the difference in the electronic structure of molecules between ground and excited states.[15] The technique employs an externally applied electric field to perturb the energies of these electronic states and thus the absorption spectrum. Encoded within the field-dependent line shape is information on the electronic structure of the ground and excited states of the chromophore. We now extend these results to include a wider range of solvent polarity.

The object of this study is to provide reference data that will more closely simulate the nonpolar environment the flavin cofactor experiences in flavoproteins. As will be shown, the effect of the solvent (reaction) field on the Stark spectra is modest. However, much larger electric field effects on flavins are expected in proteins. Many flavoproteins have charged residues in proximity to the flavin cofactor. The electric field due to these exposed charges is likely to be at least an order of magnitude larger than those produced by simple (isotropic) solvents. Other workers have used this idea to interpret anomalously large dipole moment changes in carotenoid molecules in the bacterial photosynthetic reaction center.[16] A similar argument has been made for the Stark effect on the bacteriochlorophyll special pair in the reaction center.[17] Thus an important goal of this report is to provide reference data that elucidate the influence of simple nonpolar solvents on the electronic structure of the flavin.

In our previous work, several flavins were studied using solvents of which the polarity change was relatively modest (i.e., water to butanol).[14] Additionally, quite high concentrations were used (typically 4 mM) which led to the formation of dimers and higher aggregates. In this work, we present Stark spectra of a single flavin (*N*(3)-methyl, *N*(10)-isobutyl-7,8-dimethylisoalloxazine, hereafter referred to as the "*N*(3)-flavin", see Scheme 1), taken at lower concentrations. This flavin has a methyl group at the N(3) nitrogen, effectively blocking hydrogen bonding at this point. Many studies have shown that hydrogen bonding by solvent to N(3)−H perturbs the electronic structure and spectrum of the flavin.[18−21] Methylation of the flavin at this site removes some of this perturbation and focuses the interpretation of the Stark spectra on effect due to the polarity of the solvent alone. In addition, the *N*(3)-flavin is soluble in a

SCHEME 1



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* To whom correspondence should be addressed. E-mail: rstanley@nimbus.temple.edu.

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wide variety of less polar solvents in which dimer formation is minimized relative to aqueous solutions.[22]

Materials and Methods

N(3)-Methyl, *N*(10)-isobutyl-7,8-dimethylisoalloxazine was a generous gift of Prof. Vincent Rotello (University of Massachusetts, Amherst). Ethanol and 1-butanol were dried over molecular sieves (4 Å). 2-Methyl tetrahydrofuran (2-MTHF) was passed through an alumina column (Brockman Activity I) to remove the stabilizer and water. *N*(3)-flavin was dissolved in dried ethanol, 1-butanol, and 2-MTHF to 800 μM, 860 μM, and 900 μM, respectively. Thereafter, all solutions were stored under argon at −20 °C and handled under yellow lamps to avoid undesirable photoreactions. Coumarin 540A (Exciton) was dissolved in toluene (to 700 μM) that had been dried over 4 Å molecular sieves for one week.

Low-temperature absorption spectra were obtained using a liquid nitrogen Dewar flask (H. S. Martin). A fused silica demountable cuvette (0.5 mm path length) was attached to a copper coldfinger using spring clips. The coldfinger was screwed into a $^1/_2$ in. fiberglass rod and plunged into liquid nitrogen for the flavin-containing solution. Helium gas was sprayed over the surface of the liquid N_2 to prevent bubbling. A 150 W Xe arc lamp was filtered through a $^1/_8$ m monochromator (1−2 nm band-pass) and recollimated using a 10 cm planoconvex fused silica lens. A calcite polarizer was placed after the collimating lens to obtain linearly polarized light. The polarizer was calibrated using a linearly polarized laser and a power meter. Transmission spectra were recorded using a chopper, a UV-enhanced silicon photodiode, and a digital lock-in amplifier. The transmission spectra for the solvent alone were taken at room temperature in the same manner.

The monochromator was stepped by equal wavenumber intervals rather than in wavelength. This was done to provide equal coverage of the lower and higher energy absorption bands. Typically, the spectra were taken using 58 cm^{-1} intervals between 310 and 560 nm. Because the monochromator can only step in minimum wavelength increments of 1 Å, there is the potential for a small loss of accuracy in setting the monochromator for the low-wavelength part of the scan. However, this procedure results in an acceptable error of ± 0.05 nm at 310 nm, and the error is much less at higher wavelengths. Wavelength calibration of the spectra was achieved by directly correcting the transmission spectra using the peak of the Xe Arc lamp (467.5 nm).

The Stark spectrometer is similar to that used previously[14] except that an immersion Dewar flask was used and the monochromator was stepped in equal wavenumber increments as described above. Use of the immersion Dewar flask resulted in higher applied fields. In addition, it was found that higher fields were obtained when the high-voltage power supply frequency was set below about 300 Hz; 217 Hz was used.

The angle between the probe beam and the applied electric field in the cuvette could be varied by rotation of the sample, the polarizer, or both. The polarizer was set to deliver light polarized perpendicular to the applied field when the sample was rotated normal to the direction of the probe beam ($\chi = 90°$). Phase-sensitive detection of the change in the extinction coefficient, $\Delta\epsilon$, was obtained by using an AC electric field and measuring the field-modulated transmitted light ($I(F)$) using a UV-enhanced Si photodiode (photovoltaic mode) and a digital lock-in amplifier. Because the modulation due to the field was very small ($<10^{-4}$), the transmission at zero field, $I(F{=}0) \approx I_0$, could be obtained directly by measuring the photodiode output with a 16-bit analog-to-digital converter. Usually, two to three scans were taken for a given Stark spectrum. The change in the extinction was obtained using

$$\Delta\epsilon = \frac{2\sqrt{2}I(F)}{2.303 I_0 c l'} \tag{1}$$

where c is the concentration of the solution and l' is the path length (corrected for χ and the refractive indexes of the solvent and liquid nitrogen, see below). The factor of $2\sqrt{2}$ corrects for the use of an AC electric field and detection at the 2nd harmonic of the field frequency.

Cuvettes for the Stark experiments were constructed of two 1 in. × 1 in. × 1.1 mm aluminosilicate glass slides coated on one side only with indium tin oxide (resistivity = 100 Ω/cm^2). The two slides were offset to allow connection of the cell to the AC power supply using alligator spring clips and small pieces of indium foil to ensure good electrical contact. A 29 μm Kapton film was used as a spacer. The actual path length was 32 ± 3 μm, as determined interferometrically at room temperature. These cuvettes could sustain applied fields of $(3-6) \times 10^5$ V/cm before electrical breakdown.

Values for the refractive index (n) and the dielectric constant (ϵ_s) for the solvents were obtained from standard sources.[23] Values of these constants for 2-MTHF are somewhat controversial. We use the low-temperature value determined by Eweg et al.[24] for the dielectric constant, $\epsilon_s^{\text{2-MTHF}} = 7.58$ at 77 K.

Data Analysis

The data were fitted using the Liptay formalism of electroabsorption. A particularly lucid account of electroabsorption and its relationship to solvatochromism is available.[25] Liptay modeled the changes in line shape for a chromophore in an applied electric field using 0th, 1st, and 2nd derivatives of the absorption spectrum.[26] The scaling of these derivatives to fit the Stark spectrum ($\Delta\epsilon/\nu$) reflects changes in the transition dipole moment, polarizability, and dipole moment, respectively, between the ground and excited state of the chromophore:

$$\frac{\Delta\epsilon}{\nu} = (f\vec{F})^2\left\{A_\chi \frac{\epsilon(\nu)}{\nu} + \frac{B_\chi}{15ch}\frac{\mathrm{d}[\epsilon(\nu)/\nu]}{\mathrm{d}\nu} + \frac{C_\chi}{30c^2h^2}\frac{\mathrm{d}^2[\epsilon(\nu)/\nu]}{\mathrm{d}\nu^2}\right\} \tag{2}$$

where c is the speed of light and h is Planck's constant. The external field is modified by the local field factor, $f = 3\epsilon_s/(2\epsilon_s + \epsilon_c)$, where ϵ_s is the dielectric constant of the solvent and ϵ_c is the dielectric constant of the chromophore.[27] This factor, usually in the range of 1.0−1.5, takes into account the effect of the solvent cavity on the electric field strength at the flavin chromophore. This is correct strictly for a spherical solvent cavity (flavins are highly ellipsoidal, see below).

The scaling factors, A_χ, B_χ, and C_χ, are related to the intrinsic electronic properties of the chromophore. The A_χ term reflects poling of the molecule possessing a ground-state dipole moment in the applied field. We work in frozen media so that the molecules are not free to rotate, therefore $A_\chi \approx 0$. We also make the assumption that any field-induced changes in the transition dipole moment are small for strongly allowed transitions.[28] The B_χ and C_χ factors are related to the difference polarizability and difference dipole moments, respectively:

$$B_\chi \approx \frac{5}{2}\,\mathrm{tr}\Delta\overleftrightarrow{\alpha} + (3\cos^2\chi - 1)\left(\frac{3}{2}\,\hat{\mathbf{m}}\cdot\Delta\overleftrightarrow{\alpha}\cdot\hat{\mathbf{m}} - \frac{1}{2}\,\mathrm{tr}\Delta\overleftrightarrow{\alpha}\right) \tag{3}$$

where $\hat{\mathbf{m}}$ is the transition dipole moment, $\mathrm{tr}\Delta\vec{\vec{\alpha}} = \mathrm{tr}(\vec{\vec{\alpha}}_e - \vec{\vec{\alpha}}_g)$ is the mean difference polarizability where $\vec{\vec{\alpha}}_e$ is the polarizability tensor of the excited state and $\vec{\vec{\alpha}}_g$ is the polarizability of the ground state, and χ is the angle between the polarization vector of the probe light and the applied electric field ($\chi = 90°$ when the Poynting vector of the probe beam is parallel to the applied electric field). The projection of $\mathrm{tr}\Delta\vec{\vec{\alpha}}$ along the transition dipole moment is $\hat{\mathbf{m}}\cdot\Delta\vec{\vec{\alpha}}\cdot\hat{\mathbf{m}}$.

The change in the dipole moment between the ground and excited states is related to

$$C_\chi = |\Delta\vec{\mu}|^2\{5 + (3\cos^2\chi - 1)(3\cos^2\zeta_A - 1)\} \qquad (4)$$

where $\Delta\vec{\mu} = \vec{\mu}_e - \vec{\mu}_g$ is the difference dipole moment and ζ_A is the angle between $\Delta\vec{\mu}$ and the transition dipole moment, $\hat{\mathbf{m}}$.

Initially, the absorption spectrum is fitted to a set of Gaussian functions. This provides a smoothing of the absorption spectrum so that derivatives of the spectrum are relatively noise-free. However, the absorption spectrum (and therefore the Stark spectrum) of oxidized flavin consists of (at least) two overlapping transitions, the $S_0 \rightarrow S_1$ band (band I, centered at approximately 450 nm) and the $S_0 \rightarrow S_2$ band (band II, centered roughly at 370 nm). The two excited states naturally have different electronic structures. This necessitates partitioning the absorption spectrum into two sections and fitting each section with its own set of A_χ, B_χ, and C_χ terms.[14]

The parameters from the fit of the absorption spectrum were then used to fit the Stark spectrum. We took the approach of fitting both the absorption and Stark spectra simultaneously because it is often the case that the absorption spectrum is more difficult to measure accurately. In this case, errors in the absorption spectrum are amplified when fitting the Stark spectrum.[17] Typically, the fit was weighted equally between the two spectra. It was interesting to note that if the fit to the absorption spectrum was weighted less than the Stark fit the B_χ factor tended to be somewhat lower than if equal weighting was used. Stark spectra were obtained at several fields for at least two different angles of χ, and all of these data were fitted separately. The A_χ, B_χ, and C_χ parameters represent averages from these fits. The errors in the parameters were obtained by fitting at least six data sets as a function of χ and using these A_χ, B_χ, and C_χ parameters to derive the values for the difference dipole and polarizability constants. Estimates of the error in these constants were obtained by simulating random A_χ, B_χ, and C_χ parameters sets about the mean values from the data analysis to obtain the sensitivity of the fitted parameters to the randomly generated parameter sets.

The data acquisition and analysis were checked by taking the absorption and Stark spectrum of coumarin 540A (data not shown). The Stark spectrum of this molecule is dominated by a single electronic transition that has been well-studied by several methods. We obtained a difference dipole moment of about 8 D with an average polarizability change of about 10 Å^3 ($f = 1.3$), in good agreement with previous studies.[29]

Results

The energy-weighted absorption spectra (ϵ/ν) for the *N*(3)-flavin in the different solvents are shown in Figure 1a−c. The solvent polarity, E_N^T, is given on the figures and is derived from the solvatochromic behavior of a betaine dye.[23] Virtually no solvatochromic shift is observed for band I in EtOH and BuOH solvents, while the position of band I shifts to the blue by about 270 cm^{-1} in 2-MTHF. The shift for band II follows a similar trend though the magnitude of the shift is about three times larger, about 800 cm^{-1}. This is indicative of an increase in the dipole moment of the S_2 state relative to the S_1 state.

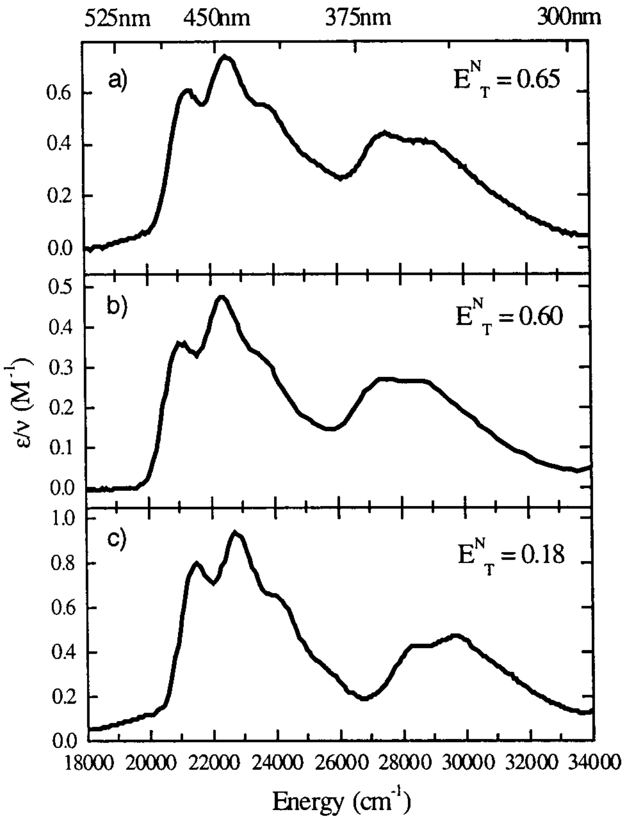

Figure 1. The energy-weighted absorption spectra of *N*(3)-flavin in ethanol (a), 1-butanol (b), and 2-MTHF (c) at 77 K and at 800 μM, 860 μM, and 900 μM, respectively. The step size was 58 cm^{-1}, and the monochromator band-pass was 2 nm. The fused silica cuvette had a path length of 500 μm. The polarities (E_N^T) of the different solvents are given for reference.

The energy-weighted Stark spectra ($\Delta\epsilon/\nu$) of *N*(3)-flavin in the different solvents are shown in Figure 2a−c. These spectra have been normalized to 10^6 V/cm for comparison, although they were obtained over a range of $(3-6.2) \times 10^5$ V/cm. Each spectrum was constructed from an average of 2−4 data sets at each angle for each solvent. The change in extinction, $\Delta\epsilon/\epsilon$, for the maximum applied field (about 6×10^5 V/cm) was about 4×10^{-4} for the largest feature at ~ 27 000 cm^{-1} (cf. Figure 1). Note that because of the derivative nature of Stark spectroscopy it is easier to discern the solvatochromic shifts for the two transitions.

Spectra were obtained at two angles by rotating the sample to approximately the magic angle ($\chi = 55°$) relative to the Poynting vector of the probe light. χ was then varied by rotating the polarizer so that the polarization of the probe light was either $\chi = 90°$ or $\chi = 50-55°$ to the applied field direction. It is immediately apparent that the response of the Stark spectrum to changes in χ is different for the two electronic transitions. The $S_0 \rightarrow S_1$ transition shows very little dependence on χ, while the intensity of the $S_0 \rightarrow S_2$ transition depends much more strongly on χ.

A representative fit to these data is shown in Figure 3a−d for *N*(3)-flavin in ethanol. Figure 3a shows the Stark spectrum and the fit. The residuals of the fit are shown in Figure 3b. Residuals for the fit of the absorption spectrum are similar in the lack of structure (fit not shown). Figure 3c shows the 1st derivative contributions for bands I and II obtained from the fit plotted against the experimental data. These contributions were

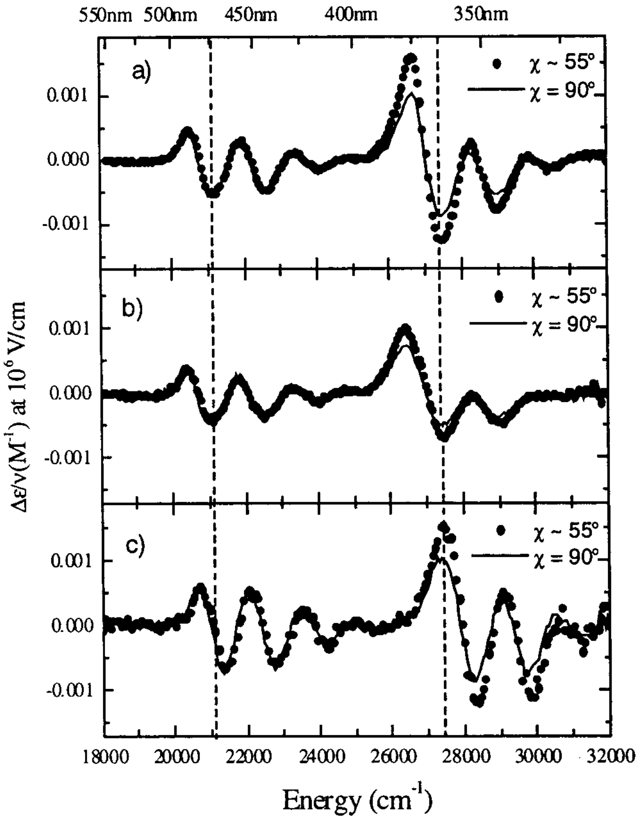

Figure 2. Representative energy-weighted Stark spectra of *N*(3)-flavin in ethanol (a), 1-butanol (b), and 2-MTHF (c) at 77 K and at 800 μM, 860 μM, and 900 μM, respectively. The step size was 58 cm^{-1}, and the monochromator band-pass was 2 nm. The spectra have been normalized to 10^6 V/cm for comparison. The probe light was polarized at both normal incidence (χ=90°, —) and at the magic angle (~55°, •••) to the applied electric field. These data have been normalized to $\Delta\epsilon$ using the concentration and the path length, adjusted for χ. The path length of the cuvette was 32 μm as determined interferometrically.

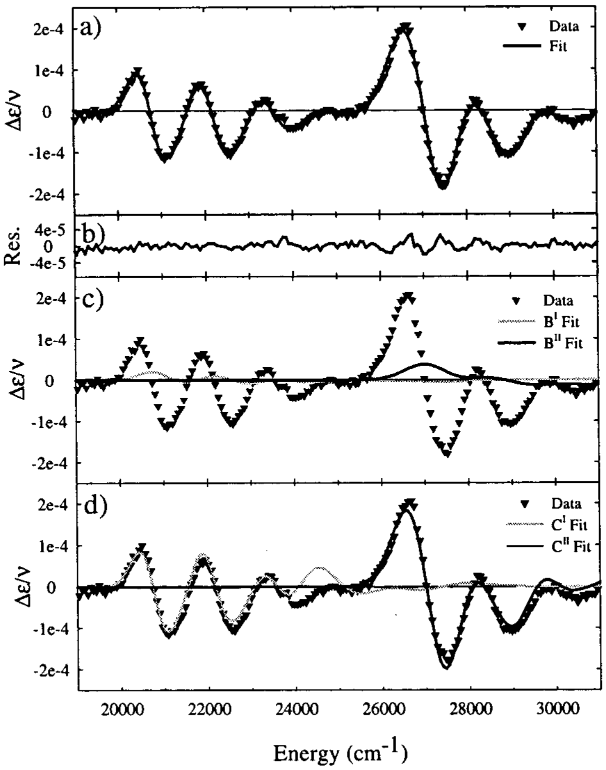

Figure 3. (a) A representative fit (—) to the Stark-effect data for *N*(3)-flavin in EtOH (▼) using 1st and 2nd derivatives obtained from the absorption spectrum fit (not shown). (b) The residuals (data−fit) have been scaled for direct comparison with panel a. Panel c shows the 1st derivative components: band I (gray line, B^{I}) and band II (black line, B^{II}) from the two-band analysis plotted against the Stark data (▼). Panel d shows the 2nd derivative components: band I (gray line, C^{I}) and band II (black line, C^{II}) from the two-band analysis plotted against the Stark data (▼).

obtained by summing the 1st derivatives of the Gaussian components for indicated bands. It is evident that band I (B^{I}) requires a smaller 1st derivative component than band II (B^{II}). The 2nd derivatives needed to produce the fit are shown in Figure 3d. Again, the spectrum requires larger 2nd derivative components for the $S_0 \rightarrow S_2$ transition than for the $S_0 \rightarrow S_1$ transition.

Dipole Moment Changes. These spectra have been fitted using the Liptay analysis and the results for the dipolar parameters are tabulated in Table 1. The difference dipole moment for band I is about the same in each solvent within experimental error, $|\Delta\vec{\mu}_{I}| \approx 2.5f$ D. The only other measurements of this quantity come from transient microwave conductivity and solvatochromism,[30] which gave a value of about 1.1 D. To compare the Stark result with other methods, it is necessary to find an appropriate value for the local field correction factor, *f*. Flavins are ellipsoidal so that the Lorentz cavity field correction factor is not terribly useful. We have calculated the local field factors for an ellipsoidal molecule of minor axes $a_x = 6$ Å, $a_y = 12$ Å, and $a_z = 2.4$ Å (twice the hydrogen atom van der Waals radius) using the prescription in Premvardhan and Peteanu[31] for obtaining the local field factors for each axis of the ellipsoid (see Table 2):

$$f_i = \frac{\epsilon_0}{\epsilon_0 - A_i(\epsilon_0 - 1)} \tag{5}$$

where

$$A_i = \frac{a_x a_y a_z}{2} \int_0^\infty \frac{ds}{(s + a_i^2)\sqrt{(s + a_x^2)(s + a_y^2)(s + a_z^2)}} \tag{6}$$

where *s* is a dummy variable. Averaging the *f* factor over all directions gives $f \approx 1.7$ for the alcohols and $f \approx 1.6$ for 2-MTHF. Applying these factors to the values in Table 1 leads to $|\Delta\vec{\mu}_{I}| \approx 1.5$ D, in reasonable agreement with the other methods.

TABLE 1: Dipolar Parameters for *N*(3)-Flavin in Various Solvents

solvent	solvent polarity (E_N^T)	$\lvert\Delta\vec{\mu}_{I}\rvert$ (D·*f*)	ζ_A^{I} (deg)	$\lvert\Delta\vec{\mu}_{II}\rvert$ (D·*f*)	ζ_A^{II} (deg)
ethanol	0.65	2.4 ± 0.2	55 ± 8	7.6 ± 0.3	27 ± 7
butanol	0.60	2.5 ± 0.2	63 ± 6	7.3 ± 0.3	15 ± 9
2-MTHF	0.18	2.5 ± 0.2	58 ± 11	7.8 ± 0.5	30 ± 9

TABLE 2: Local Field Correction Factors for an Ellipsoidal Molecule

solvent (ϵ_s)	f_x	f_y	f_z	f_{avg}
EtOH (24.6)	1.31	1.10	2.72	1.71
BuOH (17.5)	1.30	1.10	2.64	1.68
2-MTHF (7.6)	1.27	1.09	2.33	1.56

The angle, ζ_A^{I}, between $\Delta\vec{\mu}_{I}$ and the transition dipole moment $\vec{m}_{I}$ is 59° ± 3°. Note that this vectorial information is not available from other methods. To further specify the S_1 dipole moment, $\vec{\mu}_1$, it is necessary to have some knowledge of

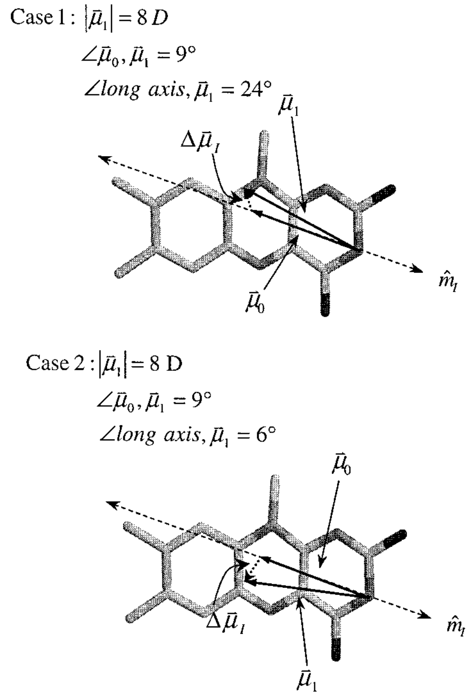

Figure 4. Dipole vectors for the S_0 and S_1 states plotted against the framework of the flavin molecule (the isobutyl group at N(10) was simplified to a methyl group and the N(3) methyl was omitted for clarity). $\vec{\mu}_0$ was obtained from MINDO/3 calculations and is a gas-phase value. $\Delta\vec{\mu}_{\mathrm{I}}$ and $\zeta_{\mathrm{A}}^{\mathrm{I}}$ are from this work and $\vec{\mu}_1$ was calculated as described in the text. The magnitudes of the experimentally determined dipole moments are adjusted using a local field factor $f = 1.6$ for comparison with $\vec{\mu}_0$.

the transition dipole moment direction as well as some estimate of the ground-state dipole moment direction and magnitude. The transition dipole moments for both band I and band II are known from linear dichroism studies on flavodoxin single crystals[32] and from measurements made using flavins oriented in liquid crystals.[33] The $\vec{m}_{\mathrm{I}}$ direction is about $-15°$ from the long axis of the molecule, lying roughly along the N(3)$-$CH$_3$ axis (refer to Figure 4). Gas-phase calculations for *N*(3)-methyllumiflavin give the ground-state dipole moment as 7.1 D, lying parallel to the transition dipole moment.[3] Because of the nature of Stark spectroscopy, the direction of $\Delta\vec{\mu}$ is only constrained to lie on a cone about the transition dipole moment with the angle specified by ζ_{A}. Physical intuition can be used to eliminate certain directions, and we have applied this intuition in specifying the directions of both $\vec{\mu}_1$ and $\vec{\mu}_2$ (i.e., we assume that $\Delta\vec{\mu}$ in both cases lies in the plane of the isoalloxazine moiety).

With this caveat and information about the direction of $\vec{m}_{\mathrm{I}}$ and $\vec{\mu}_0$, both the direction and magnitude of $\vec{\mu}_1$ can be estimated using the law of sines and cosines. Four orientations of $\vec{m}_{\mathrm{I}}$ and $\Delta\vec{\mu}_{\mathrm{I}}$ are possible. Two of these orientations lead to $|\vec{\mu}_1| < |\vec{\mu}_0|$, so we reject these outright because solvatochromism predicts a blue shift if this were true.[25] In the remaining two cases, the vector $\vec{\mu}_1$ lies about 9° from $\vec{\mu}_0$ with a length of about 8.0 D, a 13% increase (see Figure 4). Case 1 illustrates $\vec{\mu}_1$ pointing in the direction of N(10) while case 2 shows $\vec{\mu}_1$ pointing more toward N(5).

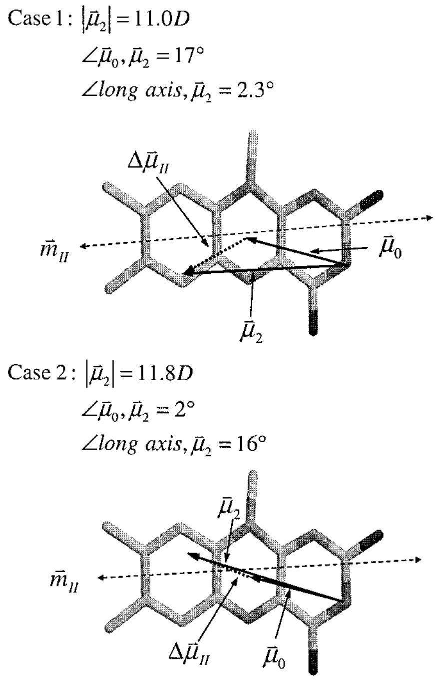

Figure 5. Dipole vectors for the S_0 and S_2 states plotted against the framework of the flavin molecule (the isobutyl group at N(10) was simplified to a methyl group and the N(3) methyl group was omitted for clarity). $\vec{\mu}_0$ was obtained from MINDO/3 calculations.[3] $\Delta\vec{\mu}_{\mathrm{II}} = 4.7$ D and $\zeta_{\mathrm{A}}^{\mathrm{II}} = 24°$ are from this work. $\vec{\mu}_2$ was calculated as described in the text. Because $\vec{m}_{\mathrm{II}}$ and $\vec{\mu}_0$ are no longer collinear (at least) two possibilities for the direction of $\vec{\mu}_2$ are considered. Case 1 represents the resultant excited-state dipole moment, which forms a 17° angle between $\vec{\mu}_0$ and $\vec{\mu}_2$. In this case, $|\vec{\mu}_2| = 11.0$ D ($f = 1.6$) and lies nearly parallel to $\vec{m}_{\mathrm{II}}$. Case 2 shows the situation when the angle between $\vec{\mu}_0$ and $\vec{\mu}_2$ is about 2°. In this case, $|\vec{\mu}_2| = 11.8$ D.

To distinguish between these two cases, we look to calculations that predict the molecular orbital structure of the lowest unoccupied molecular orbital, because there is general agreement from different calculations that the lowest energy transition is a HOMO → LUMO jump.[34−38] Semiempirical[36] and ab initio calculations[38] show that the HOMO → LUMO transition for oxidized lumiflavin involves a reduction of electron density at N(10) and N(1) with an increase in electron density near N(5) and C(4a). On this basis, we tentatively place the direction of $\vec{\mu}_1$ in the plane of the isoalloxazine molecule pointing toward N(10) (i.e., case 1).

The difference dipole moment for band II is significantly larger than that for band I and has never been reported for EtOH and 2-MTHF solvents.[39] All three solvents give the same value within experimental error, about 7.5D·f, which is a factor of 3 larger than that obtained for band I. Again, if we choose $f = 1.6$, then $|\Delta\vec{\mu}_{\mathrm{II}}| \approx 4.7$ D. The angle that $\Delta\vec{\mu}_{\mathrm{II}}$ makes with the transition dipole moment $\vec{m}_{\mathrm{II}}$ is $\zeta_{\mathrm{A}}^{\mathrm{II}} = 24° \pm 7°$. However, the band II transition dipole moment is offset from $\vec{m}_{\mathrm{I}}$ (and therefore $\vec{\mu}_0$) by about 20° so that $\vec{m}_{\mathrm{II}}$ would be about 5° above the long axis (see Figure 5). The direction of $\vec{\mu}_2$ therefore is complicated by the fact that the transition dipole moment $\vec{m}_{\mathrm{II}}$ is not collinear with $\vec{\mu}_2$, as was the case for the $S_0 \rightarrow S_1$ transition. This means that $\vec{\mu}_{\mathrm{II}}$ might vary from about 11 (case 2, Figure 5) to 12 D (case 1, Figure 5), assuming that $f = 1.6$ and that the excited-state dipole moment must lie in the plane of the molecule. Note that case 1 places $\vec{\mu}_2$ along $\vec{m}_{\mathrm{II}}$ while case 2 places $\vec{\mu}_2$ roughly parallel to $\vec{\mu}_0$. It may be possible to use quantum-mechanical calculations to decide between these two alternatives (see below). In either case, the increase in dipole moment upon excitation is large, at least 55%, and about four times larger than the same increase for the $S_0 \rightarrow S_1$ transition.

Polarizability Changes. The polarizability of the S_1 state shows a small change compared to the S_0 state as evidenced by the small $\mathrm{tr}\Delta\vec{\vec{\alpha}}_{\mathrm{I}} \approx 17$ (Å^3·f^2) for the $S_0 \rightarrow S_1$ transition (see Table 3). This value is similar in magnitude to coumarin 540A, which also contains an aromatic ring. For comparison, anthracene, which is completely aromatic, has a difference

TABLE 3: Polarizability Parameters for *N*(3)-Flavin in Various Solvents

solvent	solvent polarity (E_N^T)	$\mathrm{tr}\Delta\vec{\vec{\alpha}}_I$ ($Å^3\cdot f^2$)	$\hat{\mathbf{m}}\cdot\Delta\vec{\vec{\alpha}}_I\cdot\hat{\mathbf{m}}$ ($Å^3\cdot f^2$)	$\mathrm{tr}\Delta\vec{\vec{\alpha}}_{II}$ ($Å^3\cdot f^2$)	$\hat{\mathbf{m}}\cdot\Delta\vec{\vec{\alpha}}_{II}\cdot\hat{\mathbf{m}}$ ($Å^3\cdot f^2$)
ethanol	0.65	14 ± 2	7 ± 3	79 ± 10	59 ± 27
butanol	0.60	17 ± 2	7 ± 2	149 ± 8	106 ± 24
2-MTHF	0.18	22 ± 5	32 ± 15	162 ± 34	183 ± 100

polarizability that is a factor of about three larger than *N*(3)-flavin. This is not the case for the S_2 state in which the $\mathrm{tr}\Delta\vec{\vec{\alpha}}_{II}$ is from 5 to 9 times larger than that for the $S_0 \rightarrow S_1$ transition. Both $\mathrm{tr}\Delta\vec{\vec{\alpha}}_{II}$ and its projection on $\vec{m}_{II}$ grow as the solvent polarity decreases though the effect of solvent polarity seems to saturate below $E_N^T \approx 0.5$. A similar trend was observed for the $S_0 \rightarrow S_1$ transition. A measurement of the ground-state polarizability of oxidized flavins was made using refractometry.[40] These measurements suggest that this polarizability is on the order of 50 $Å^3$. Using our value for $\mathrm{tr}\Delta\vec{\vec{\alpha}}_{II} \approx 50-100$ $Å^3$ ($f = 1.3$) leads to an S_2 polarizability of about 100−150 $Å^3$. This value is somewhat less than that for a highly conjugated system such as anthracene[26] but significantly larger than that obtained for dye molecules such as coumarin ($\mathrm{tr}\Delta\vec{\vec{\alpha}} \approx 12$ $Å^3$).[29] We anticipate that the difference will be traced to the xylene ring of the isoalloxazine heterocycle.

The term ($\hat{\mathbf{m}}\cdot\Delta\vec{\vec{\alpha}}\cdot\hat{\mathbf{m}}$) is useful because it gives the magnitude of one component of the 5-component polarizability tensor, namely, the component that lies along the transition dipole moment direction. If this component is comparable to $\mathrm{tr}\Delta\vec{\vec{\alpha}}$, then the polarizability change would be fairly well localized along the transition dipole moment. For measurements in the alcohols, the ratio of ($\hat{\mathbf{m}}\cdot\Delta\vec{\vec{\alpha}}_{II}\cdot\hat{\mathbf{m}}$)/$\mathrm{tr}\Delta\vec{\vec{\alpha}}_{II}$ is larger than that obtained for the $S_0 \rightarrow S_1$ transition. This suggests that the polarizability for the S_2 state is much more anisotropic than that for the S_1 and S_0 states. This in turn suggests that the molecular orbitals occupied by the transition from $S_0 \rightarrow S_2$ are very different structurally from those accessed during the $S_0 \rightarrow S_1$ transition. In the case of 2-MTHF, both ($\mathbf{m}\cdot\Delta\vec{\vec{\alpha}}_I\cdot\hat{\mathbf{m}}$)/$\mathrm{tr}\Delta\vec{\vec{\alpha}}_I$ *and* ($\mathbf{m}\cdot\Delta\vec{\vec{\alpha}}_{II}\cdot\hat{\mathbf{m}}$)/$\mathrm{tr}\Delta\vec{\vec{\alpha}}_{II}$ are greater than unity. This is consistent with the transition dipole moment and $\mathrm{tr}\Delta\vec{\vec{\alpha}}_{II}$ being parallel but not coincident with any of the principal axes of the molecule.

One explanation for the observed trend in $\mathrm{tr}\Delta\vec{\vec{\alpha}}$ is dimer formation in 2-MTHF vs the alcohols. Eweg et al have reported delayed fluorescence of flavins in low-temperature alkane glasses, which they attributed to aggregate formation.[24] This is in contrast to the observations by Weber and others that base stacking is maximized in aqueous solution and thwarted in nonpolar solvents.[22,41,42] We have also seen that band II is somewhat sensitive to flavin concentration in 2-MTHF at low temperature (data not shown). Dimerization should result in a larger available charge density, increasing the apparent polarizability of the sample. On the other hand, the bulky isobutyl side chain on N(10) should sterically hinder any complexation. It should be pointed out that the values in Table 3 are given in terms of the local field correction, *f*, which will be different for each solvent. Because the precise value of *f* depends on the molecular details of solvation, it is difficult to determine whether the observed trend is due solely to changes in *f* or whether aggregation is in fact responsible.

It is instructive to ask whether values of $\mathrm{tr}\Delta\vec{\vec{\alpha}}$ in this range would be observable as an induced dipole moment in the value of $\Delta\vec{\mu}$ as a function of solvent polarity. The induced dipole moment should be proportional to the difference polarizability and the reaction field of the solvent: $\Delta\vec{\mu}_{\mathrm{ind}} \propto \mathrm{tr}\Delta\vec{\vec{\alpha}}\cdot\vec{F}_R$ where in its simplest form the reaction field $\vec{F}_R = [2(\epsilon_{DC} - 1)/(a^3(2\epsilon_{DC} + 1))]\vec{\mu}_g'$. Here, ϵ_{DC} is the static dielectric constant of the solvent, a is the solvent spherical cavity radius, and $\vec{\mu}_g'$ is the total ground-state dipole moment of the dissolved molecule (we ignore dispersion interactions that will be proportional to the refractive index).[25] For $\epsilon_{DC} = 40$, $a = 10^{-9}$ m, and $|\vec{\mu}_g'| = 7$ D, the reaction field is $|\vec{F}_R| = 2 \times 10^8$ V/m. For $\epsilon_{DC} = 18$, $|\vec{F}_R| = 1.9 \times 10^8$ V/m, a difference of 3%. Thus for the range of solvent dielectric used in this study, we would not be able to see the effect of a solvent-induced dipole moment within the experimental error for $\mathrm{tr}\Delta\vec{\vec{\alpha}} = 160$ $Å^3$.

Discussion

There have been only a few measurements of the electronic properties of the excited states of flavins, though a number of calculations are available. Solvatochromic and transient microwave conductivity[30] measurements of the electronic structure of oxidized flavins have focused on the first excited singlet state. Neither technique has been able to retrieve vectorial information or contribute to our knowledge of the polarizability of these excited states. Our measurements agree with and extend these previous determinations.

While Stark spectroscopy yields valuable information about the magnitudes of the difference dipole moments in several electronic states, the associated directions of these vectors in the molecular frame are less satisfactorily defined. This in turn leads to ambiguities with regard to the direction of the excited-state dipole moments even with a perfect knowledge of the ground-state dipole moment direction. It may be useful to utilize quantum-mechanical calculations for guidance in obtaining more definitive information at the molecular level. It should be pointed out that the electronic structure of flavins has attracted considerable computational effort since the 1960s, though not all such calculations are relevant to this study.

Several theoretical studies are available that predict the electronic structure of oxidized flavins. Gas-phase calculations by Hall et al at the MINDO/3 level give ground-state dipole moments and directions for a large number of derivatized flavins; we have used these values in this work ($|\vec{\mu}_0| = 7.1$ D).[3] However, there is no accounting for solvation on the magnitude or direction of the calculated dipole moment. A larger value for the ground-state dipole moment (7.8 D for 10-methylisoalloxazine) was obtained by Platenkamp and co-workers using ab initio methods. Again, solvation was not taken into account.

Semiempirical methods have been employed to calculate the properties and spectra of excited states in flavins while taking into account solvation effects. An interesting set of calculations was performed by Hanson at the INDO/s level using doubles excitations in which charges were placed at various points in a plane containing the isoalloxazine molecule.[35] These charges were used to mimic the internal electrostatic environment of a flavoprotein. A negative charge placed near the benzenoid end of the molecule increased the π-LUMO energy, while a positive charge placed near the pyrimidine end of the flavin lowered the π-HOMO, producing a red shift in the calculated absorption spectrum.

Song performed Pariser−Parr−Pople SCF MO calculations in 1969 on methyl flavins.[34] He predicted that the ground-state permanent dipole moment should be parallel to the transition dipole moment for the lowest $\pi \rightarrow \pi^*$ transition, which further strengthens the basis for our assignment of excited-state dipole moment directions. Remarkably, the calculations predicted that the $S_0 \rightarrow S_2$ state will have charge-transfer character. This could be taken as support of either case 1 or case 2 for the assignment of the direction of $\vec{\mu}_2$, but not enough information was given to decide between these two possibilities. The work by Song also highlights the possible existence of an $n \rightarrow \pi^*$ transition around 370 nm. This has been something of a controversial issue.[43,44] It would be expected that an $n \rightarrow \pi^*$ transition would show a significant change in charge distribution. Our data require only two sets of Stark parameters for a reasonable fit, suggesting that only two transitions span this wavelength range. There is no evidence for a third transition in the near-UV that has an appreciable change in dipole moment.

Perhaps most relevant to the Stark experiments is the study of Zuccarello et al. who used the INDO/s semiempirical method and a Kirkwood solvation model modified to include changes in the electronic polarization of the solvent to predict the optical spectra of isoalloxazine and 10-methylisoalloxazine.[37] They employed a spherical solvation cavity ($r = 3.4$ Å) to take into account both low-frequency (dielectric constant) and high-frequency (index of refraction) contributions of the solvent to the energy of the dipolar isoalloxazine. While the use of a spherical solvent cavity is clearly incorrect in detail, the calculations predict that the $S_0 \rightarrow S_2$ transition involves charge transfer from the xylene/N(5)−N(10) part of the isoalloxazine to the entire molecule. This is consistent with our result that $\vec{\mu}_2 > \vec{\mu}_1 \approx \vec{\mu}_0$. Unfortunately, specific information about excited-state dipole moment directions was omitted. On the other hand, the authors expect that the $S_0 \rightarrow S_2$ transition is a HOMO-1 → LUMO orbital change. As pointed out by Hall et al.[3] (see above), the LUMO involves electron density around N(5) and C(4a). If we take this at face value, it implies that the $\vec{\mu}_2$ dipole moment points away from N(5) rather than toward it, lending support for case 2 (Figure 5).

Despite this extensive computational effort, none of these calculations (all reported in the mid-1980s) accurately reproduce the absorption spectrum of flavin or its solvatochromic behavior. However, more accurate predictions about excited-state electronic structure will be useful in deciding between the various directions offered by these Stark experiments. Conversely, our experimental results can be used to test the accuracy of these methods. It is worth mentioning that density functional theory has been applied to the calculation of energies of the three redox states of ground-state isoalloxazine using the self-consistent isodensity method (SCI−PCM).[45] This technique provides a solvation cavity that conforms more closely to the elliptical shape of the flavin. Unfortunately, these calculations were aimed at computing the energies of tautomeric forms of the various redox states of flavins; no predictions of electronic structure were given. In a related area, time-dependent density functional theory (TDDFT) [46] has been applied to calculating the properties of excited states for relatively large molecules of biological interest and may represent the best alternative to the INDO/s semiempirical method for elucidating our results further. We are initiating TDDFT calculations on flavins soon to refine our understanding of how flavin electronic structure influences reactivity. We have already begun Stark spectroscopic measurements on flavoproteins so that we can see how the internal electric field of the flavin binding pocket of this large class of redox proteins is used to modulate redox potentials and to direct electron transfer.

Conclusions

We have measured low-temperature Stark spectra for oxidized *N*(3)-methyl, *N*(10)-isobutyl-7,8-dimethylisoalloxazine in a variety of solvents. The change in dipole moment between the (S_0 and S_1) and (S_0 and S_2) states increases by a factor of 3, irrespective of solvent polarity. The angle between the difference dipole moment for the $S_0 \rightarrow S_1$ transition and the transition dipole moment for that transition is about 55°. From these values and from calculated values for the ground-state dipole moment direction, the direction of the S_1 dipole is roughly parallel with the S_0 dipole moment. The direction of the S_2 dipole moment is less clear because it is referenced to the $S_0 \rightarrow S_2$ transition dipole moment direction, but it is also likely within about 10° of the ground-state dipole moment direction. Insight from ab initio and semiempirical calculations suggests that $\vec{\mu}_2$ points toward N(10) and is roughly parallel with the ground-state dipole moment.

The polarizability change for these two transitions suggests that the S_0 and S_1 states have about the same polarizability, while the S_2 state is significantly more polarizable than the lower energy states. A solvent dependence of the average polarizability in the S_2 state was observed, but its origin is not well understood. This polarizability might be interacting with the solvent reaction field to generate an induced dipole moment, but no hard evidence for this inductive effect is available from the current measurements.

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