Published in final edited form as:

Methods Enzymol. 2019 ; 620: 215–250. doi:10.1016/bs.mie.2019.03.012.

Measuring electronic structure properties of flavins and flavoproteins by electronic Stark spectroscopy

Robert J. Stanley^{*}, Cornelius J. van Galen

Department of Chemistry, Temple University, Philadelphia, PA, United States

Abstract

The optical spectrum of a flavoprotein is one of its signature properties. No two flavoprotein absorption spectra are exactly alike as each encodes the details of the interaction of the flavin cofactor electronic structure with the specific protein binding pocket. Electronic Stark spectroscopy has the potential to elucidate these interactions with high sensitivity, at low cost, and requiring minimal technical sophistication. In this chapter we will outline the theoretical basis for Stark spectroscopy and describe the construction of the Stark spectrometer. Step-by-step instructions are given for acquiring and interpreting Stark spectra to retrieve difference moments of the flavin ground versus excited state charge distributions.

1. Introduction

1.1 Motivation

Flavoproteins perform a vast array of biochemical reactions in and out of the cell (Lienhart, Gudipati, & Macheroux, 2013; Macheroux, Kappes, & Ealick, 2011). Of paramount significance is the fact that flavins can transfer either one or two electrons, making them more versatile than nicotinamide cofactors (e.g. NADPH). Flavin reduction potentials are modulated by proteins to accommodate a wide variety of chemistries (Stankovich, 1991). It is widely agreed, at least for noncovalently bound flavins, that the flavin binding site in the protein is how this modulation is accomplished. Hence, the biochemical characterization of a flavoenzyme is incomplete without reporting the absorption spectrum of the flavoprotein, preferably in as many oxidation states as are accessible. Many studies have focused on how protein contacts, conserved water, etc., affect the flavin reduction potential and spectrum in a number of individual flavoenzymes. As of yet, there does not appear to be a way of predicting these properties by sequence or structure.

For the past 20 years, we have been focused on the light-driven function of photoflavoproteins (Gindt et al., 2016; Kodali, Siddiqui, & Stanley, 2009; Lee, Kodali, Stanley, & Matsika, 2016; MacFarlane IV & Stanley, 2001, 2003; Narayanan, Singh, Kodali, Moravecic, & Stanley, 2017; Pauszek, Kodali, Siddiqui, & Stanley, 2011, 2016; Stanley & MacFarlane IV, 2000), and flavin dyads (Pauszek et al., 2013; Yu et al., 2012). In either case, it is not the ground state electronic structure that is of interest but, rather, the excited

^{*}Corresponding author: rstanley@temple.edu.

electronic states. Measuring this *excited* state charge distribution affords a rationality for how light-activated flavins interact with substrates or electron donors/acceptors.

Stark spectroscopy affords a determination of difference dipoles $\Delta\vec{\mu}_{eg} = \vec{\mu}_e - \vec{\mu}_g$, and difference polarizabilities $\Delta\vec{\alpha}_{eg} = \vec{\alpha}_e - \vec{\alpha}_g$ that characterize the (g)round- and (e)xcited-state permanent moments of the flavin charge distribution, $\rho(\vec{r})$. Moving toward richer fields, we hypothesize that the ground state UV/vis absorption spectrum of all flavoproteins has imprinted on it the effect of the flavin binding-cavity *electrostatic field*, $\vec{E}_{cav}$, and that Stark spectroscopy can be used to compute $\vec{F}_{cav}$. This will require Stark and absorption spectra taken in several oxidation/protonation states. We have already accomplished this for *E. coli* DNA photolyase (Kodali et al., 2009).

In this chapter, we provide protocols to build a Stark spectrometer as well as how to interpret the data obtained from it. To this end we start with some basic electrostatics.

1.2 Solute charge distributions

To approach the problem, consider a dipolar ellipsoidal molecule as solute in a simple dipolar solvent. We want to explore the effect of solvation on the energy of the solute through optical spectroscopy, so we assume that the molecule absorbs visible light. We also assume that the dipolar ellipsoidal molecule is a “point-dipole.” That is, we view (or measure) the electrostatic properties of the molecule at sufficient distance such that the details of molecular structure (bond length, angle, etc.) are negligible.

The electrostatic effects of the electronic structure (or charge distribution, $\rho(\vec{r})$) of the molecule are transmitted at some distance $\vec{r}$ from the molecule by the electric field generated by its charge distribution, $\vec{E}_{solute} \cdot \vec{E}_{solute}$ produced by $\rho(\vec{r})$ is the divergence of the electrostatic gradient, ϕ , as

$$\vec{E}_{solute} = -\vec{\nabla} \cdot \phi_{solute} \quad (1)$$

where the electrostatic gradient arises from the charge distribution of the molecule,

$$\phi_{solute} = \frac{1}{4\pi\epsilon_0} \int_V \frac{\rho(\vec{r}')}{|\vec{r} - \vec{r}'|} dv' \quad (2)$$

Here ϕ can be expanded in terms of the monopole, dipole, and quadrupole moments of $\rho(\vec{r})$, giving

$$\phi_{solute}(\vec{r}) = \frac{1}{4\pi\epsilon_0} \left[\frac{Q}{r} + \frac{\vec{\mu} \cdot \vec{r}}{r^3} + \sum_{i=1}^3 \sum_{j=1}^3 \frac{1}{2} \frac{|\vec{r}_i||\vec{r}_j|}{r^5} \alpha_{ij} \right] \quad (3)$$

where the first term is the gradient arising from a monopole or ionic species ($Q = 0$ for a neutral molecule), the second term is the gradient due to the dipole moment of the charge distribution $\vec{\mu}$, and the third term is the gradient contribution from the quadrupole moment

or polarizability (Reitz, Milford, & Christy, 1979). The individual elements of the polarizability tensor $\vec{\alpha}$ represent the degree to which the molecule can be polarized by an external electric field. $\vec{\alpha}$ is a 3×3 tensor,

$$\vec{\alpha} = \begin{pmatrix} \alpha_{xx} & \alpha_{xy} & \alpha_{xz} \\ \alpha_{yx} & \alpha_{yy} & \alpha_{yz} \\ \alpha_{zx} & \alpha_{zy} & \alpha_{zz} \end{pmatrix} \quad (4)$$

representing the polarizability along duples of the Cartesian coordinates ($ij = xx, xy, xz$, etc.). All these moments are defined relative to the center of mass of the molecule.

The dipolar electric field at some point in space, r , generated by the molecule is given by (Reitz et al., 1979)

$$\vec{E}_{dipole} = \frac{1}{4\pi\epsilon_0} \left[\frac{3\vec{r} \cdot \vec{\mu}}{|\vec{r}|^5} \vec{r} - \frac{\vec{\mu}}{|\vec{r}|^3} \right] \quad (5)$$

It is instructive to consider the magnitude of these effects. The computed ground state dipole moment of flavin is about $\vec{\mu}_g = 7$ Debye (7 D) and points roughly along the long axis of the molecule (Hall, Orchard, & Tripathy, 1987) (two opposite charges separated by $0.21 \text{ \AA}$ gives 1 D or $3.36 \times 10^{-30} \text{ C}\cdot\text{m}$).

All the field contributions to $\vec{E}_{solute} = \vec{E}_{ion} + \vec{E}_{dipole} + \vec{E}_{polarizability}$ are “imprinted” on the solvent. This signature of the charge distribution can be observed in the absorption spectrum as shown next.

1.3 Solvent effects on the absorption spectrum: Solvatochromism

In a solvent, the volume and electric field of the solute will push and pull solvent molecules generating a roughly ellipsoidal cavity (Liptay, 1969). The electric field of the molecule(s) create(s) an oriented solvent shell(s). We assume that the total interaction of the solute with the solvent can be represented by the sum of pairwise interactions (ignoring hydrogen bonds for the moment). If there are no ions present, then the strongest interaction will be two interacting dipoles, A and B , the energy of which is given by (Cantor & Schimmel, 1980):

$$E_{dipole} = \frac{1}{4\pi\epsilon_0} \left(\frac{\vec{\mu}_A \cdot \vec{\mu}_B}{|\vec{r}_{AB}|^3} - \frac{3(\vec{\mu}_A \cdot \vec{r}_{AB})(\vec{\mu}_B \cdot \vec{r}_{AB})}{|\vec{r}_{AB}|^5} \right) \quad (6)$$

where, again, distance and orientation play a major role. The solvent dipoles will align around the dipolar solute to minimize the overall energy. The more polar the solvent, the greater this solvent electric field. If the solute were removed instantaneously, then this anisotropic solvent electric field represents the reaction field. This is the basis for the phenomenon known as solvatochromism, where the solvent reaction electric field, $\vec{F}_R$, interacts with the dipolar solute leading to a change in solute energy levels relative to the

vacuum. Bear in the mind that above the freezing point of the solvent, there is no one orientation, but a fluctuating solvent cavity that gives rise to an average electric field.

The reaction field due to the oriented solvent is responsible for solvatochromic band shifts,

$$E_{shift}, \text{ where } \Delta E_{Shift} = -\vec{\mu}_e \cdot \vec{F}_R - (-\vec{\mu}_g \cdot \vec{F}_R) = -\Delta\vec{\mu}_{eg} \cdot \vec{F}_R, \text{ where}$$

$\Delta\vec{\mu}_{eg} = \vec{\mu}_e - \vec{\mu}_g$. An underlying assumption of this model is that the reaction field $\vec{F}_R$ is unchanged during the photon absorption event. $\Delta\vec{\mu}_{eg}$ is a very important quantity, as it tells us about the *change* in dipole moments between ground and excited states. In photobiology, it is the excited state electronic structure of the chromophore that dictates reactivity. $\Delta\vec{\mu}_{eg}$ supplies not only the magnitude of charge displacement, but also the direction as well. Examples of this will be shown below. Even so, as ground state flavinologists, why should we care about $\Delta\vec{\mu}_{eg}$ since most flavoproteins are not photoactivated.

To answer this let's look again at solvatochromism, an effect known to most biochemists. The color-shifting of a biological chromophore is often noted in the literature. Taking oxidized flavin (Fl_{OX}) as the most frequent example, when the Fl_{OX} spectrum shifts to the red in a more polar solvent we ascribe this to a larger solvent reaction field producing a lower flavin energy. The magnitude of this shift depends mostly on $|\Delta\vec{\mu}_{eg}|$. In contrast, a less polar solvent produces a blue shift. Why not stop here. Isn't this all the information we can glean from optical absorption spectroscopy?

The cubic term of this expansion (Eq. 3) gives the polarizability of the molecule. As chemists we think of polarizability as the ease with which charge can be polarized or redistributed by an external electric field. This field could be the reaction field, an externally applied electric field, or it could be due to charges in the solvent (an ion, a charged amino acid residue, etc.) that are close enough to the solute to induce a dipole through

$\vec{\mu}_{ind} = \vec{\alpha} \cdot \vec{F}_R$, where the energy of interaction is $E_{ind} = \vec{F}_R \cdot \vec{\alpha} \cdot \vec{F}_R$ vectoral nature of this interaction is always stabilizing ($E_{ind} < 0$). When the chromophore absorbs light energy, we can expect that the polarizability of the excited state is larger than the ground state, $\vec{\alpha}_e > \vec{\alpha}_g$. Intuitively this makes sense since the electronic structure of the ground state is the best compromise between electrons' attraction to the nuclei and their mutual repulsion (the highest occupied molecular orbital or HOMO). Any increase in electronic energy will place electrons in more dispersed orbitals (LUMO, LUMO+1, etc.). Being further from the nuclei they will more easily influenced by external electric fields. Therefore, the difference polarizability, $\Delta\vec{\alpha}_{eg} = \vec{\alpha}_e - \vec{\alpha}_g$ will always lead to a lower transition energy of the molecule (a red shift).

1.4 Application of an external electric field: Electrochromism

So far, we have seen how the solvent reacts to a dipolar solute, and that energy shifts that can be expected depending on the polarity of the solvent. These *solvatochromic* effects have been well studied and there is a firm theoretical basis for their interpretation. However, there is no way to measure the direction of the solvent reaction field, only its magnitude. Further,

other interactions, most notably hydrogen bonding between solvent and solute, can produce specific spectral perturbations that are difficult to separate from reaction field effects.

If an *external* electric field, $\vec{F}_{ext}$, is applied then all of the above arguments are in force, with the significant advantage that the direction of $\vec{F}_{ext}$ can be changed by the experimenter to separate out effects due to the reaction field and specific interactions. This effect is termed *electrodichroism*, in that the transmittance of the sample as a function of wavelength will change depending on the magnitude and direction of the applied external field. Most importantly, $\vec{F}_{ext}$ will provide information about the magnitude and direction of the change in charge distribution in the solute.

Fig. 1 illustrates the basic principle for a solute with ground and excited state dipole moments $\vec{\mu}_g$ and $\vec{\mu}_e$. With no external field ($\vec{F}_{ext} = 0$) absorption of a photon of wavelength λ_{ex} will lead to an absorption band. The band width will depend on a number of factors, primarily solvent-induced conformational inhomogeneous broadening and temperature.

Application of the electric field will cause the dipolar ground and excited states to change in energy. Because $\Delta E = -\vec{\mu} \cdot \vec{F}_{ext}$ molecules whose $\vec{\mu}$ is aligned parallel with the field will shift to lower energy while those antiparallel to the applied field will shift up in energy. For an unoriented ensemble this will produce a broadening of the absorption band, the width of which depends on the magnitude of the solute dipoles. A similar argument can be made for molecules where $\vec{\mu}_g$ and $\vec{\mu}_e = 0$ but $|\vec{\alpha}_g|$ or $|\vec{\alpha}_e| > 0$ then molecules will also energy shift in the electric field. Since this is an induced effect the shift will always be to lower energy. Finally, if the molecules are free to rotate during the application of $\vec{F}_{ext}$ and if $|\vec{\mu}_g| > 0$ then the molecules will pole in the field to achieve the lowest energy. Since the permanent and transition dipoles are fixed relative to each other in the molecular frame, this poling could result in an apparent increase or decrease in extinction coefficient.

The effect of the applied field on the absorption spectrum for these three cases is shown in Fig. 2.

It is critical to remember that these permanent moments of the charge distribution, whether in the ground or excited state, are locked (referenced) to the *transition dipole moment* of the molecule, $\vec{m}_{eg}$ connecting these states. Quantum mechanically, $\vec{m}_{eg} = \int \vec{\Psi}_e^* (-e \vec{r}) \vec{\Psi}_g ds$, and $|\vec{m}_{eg}|^2$ is proportional to familiar molar decadic extinction coefficient, ϵ . As the definition suggest, $\vec{m}_{eg}$ is a transient dipole moment much like a dipolar antenna found in common electronic devices. The probability, P , of absorption of radiation will depend on the relative orientations of the polarization vector of the light, $\vec{p}_0$, and $\vec{m}_{eg}$ such that

$$P \propto (\vec{p}_0 \cdot \vec{m}_{eg})^2 = (|\vec{p}_0| |\vec{m}_{eg}| \cos \theta)^2 \text{ where } \theta \text{ is the angle between these two vectors.}$$

Electromagnetic radiation polarized the in the direction of $\vec{m}_{eg}$ will cause the ground state charge distribution to oscillate at the Bohr frequency, $\nu_{eg} = (E_e - E_g)/h$, while

radiation polarized perpendicularly to $\vec{m}_{eg}$ will not be coupled (detected) into the molecular charge cloud. This process is termed *photoselection* and is particularly relevant to non-oriented ensembles of molecules interacting with polarized light, as described below. In essence, the $\cos^2\theta$ term confers a p-orbital-like spatial probability distribution for the absorption of a polarized photon, selecting out those molecules most closely oriented along $\vec{p}_0$.

This photoselected population will have a defined orientation to the externally applied electric field, $\vec{F}_{ext}$. The angle between these two directions is $\chi = \angle \vec{p}_0, \vec{F}_{ext}$ and it affords the transition from the laboratory frame of reference into the molecular frame through $\vec{p}_0 \cdot \vec{m}_{eg}$ (photoselection) and $\zeta_A = \angle \vec{m}_{eg}, \Delta\vec{\mu}_{eg}$. The final reference to the difference dipole is set by the experimentalist as shown in Fig. 3.

Once this connection from the laboratory frame to the molecular frame is made, $\vec{m}_{eg}$ becomes our molecular reference vector. Fig. 4 shows how the transition dipole moment relates to the permanent and difference dipole moments for a flavin in a simple solvent. The extraction of these vectors is the outlined in the next section.

1.5 Liptay analysis of Stark spectra

The Liptay formalism (Liptay, 1974) treats the electric field as a perturbation to the electronic states of the molecule through dipolar and induced dipolar couplings, as described above. If it is assumed that the electric field-induced perturbation does not affect the wave function of the molecule appreciably then the line shape of the spectrum, $S(\bar{\nu})$ (e.g. a Gaussian function as used in Fig. 2), may be expanded in a Taylor series in terms of derivatives of the zero-field line shape,

$$\Delta S(\bar{\nu}; \vec{F}_{ext}) = S(\bar{\nu}; \vec{F}) - S(\bar{\nu}; 0) = S(\bar{\nu}) - \frac{dS}{d\bar{\nu}} \Delta\bar{\nu} + \frac{1}{2} \frac{d^2S}{d\bar{\nu}^2} (\Delta\bar{\nu})^2 + \dots \quad (7)$$

Note that the transition energy, $\bar{\nu} = 1/\lambda$, is defined as wavenumbers in reciprocal centimeters, or cm^{-1} . This equation is generally truncated after the third term, resulting in the sum of the zeroth, first and second derivatives of the absorption spectrum. These terms are weighted by coefficients A_χ , B_χ , and C_χ , which are the connection between the observed line shape changes and the desired quantum-mechanical properties of the solute. A_χ , B_χ , and C_χ reflect the degree of electric field poling, polarizability response, and dipolar response to $\vec{F}_{ext}$. Since the amplitude of the line shape is proportional to the molar decadic extinction coefficient as a function of energy, $\epsilon/\bar{\nu}$, the electric field-induced change in these moments of the charge distribution can be

$$\left(\frac{\Delta\epsilon}{\bar{\nu}} \right) = \left(\vec{f} \vec{F}_{ext} \right)^2 \left\{ A_\chi \left(\frac{\epsilon(\bar{\nu})}{\bar{\nu}} \right) + \frac{B_\chi}{15ch} \frac{\partial \left(\frac{\epsilon(\bar{\nu})}{\bar{\nu}} \right)}{\partial \bar{\nu}} + \frac{C_\chi}{30c^2h^2} \frac{\partial^2 \left(\frac{\epsilon(\bar{\nu})}{\bar{\nu}} \right)}{\partial \bar{\nu}^2} \right\} \quad (8)$$

where c is the speed of light, h is Planck's constant, f is the local field correction factor (see below), and $\epsilon/\bar{\nu}$ is the energy-weighted extinction (absorption spectrum) in wavenumbers.

The first term A_χ leads to a change in the intensity of the band. For immobilized molecules, as is typical in a Stark experiment, A_χ is negligible since the molecules are not able to orient with respect to the applied field. A_χ contains terms (not shown) including the transition moment polarizability and hyperpolarizability where, as the name implies, $\vec{m}$ has significant polarizability as well as dipolar character. Cases exist in which A_χ cannot be ignored, such as the Stark analysis of conjugated polyenes (Bublitz, Ortiz, Marder, & Boxer, 1997) or, charge-transfer flavin conjugates (Pauszek, Kodali, Caldwell, et al., 2013). As an example however, for a molecule with a typical transition polarizability of $100\text{\AA}^3$ in a typical applied field of $3 \times 10^5\text{ V/cm}$, the induced transition dipole $\vec{m}_{eg}^{ind} = A_{eg} \cdot \vec{F}_{ext} \sim 0.03D$.

The B_χ term is the degree of shift in the absorption band due to the *induced* dipole moment by $\vec{F}_{ext}$, resulting in a first derivative line shape. This is due to the difference polarizability, $\Delta\vec{\alpha}$, of the molecule. The B_χ coefficient is, approximately:

$$B_\chi \approx \frac{5}{2} Tr \Delta\vec{\alpha} + (3\cos^2\chi - 1) \left(\frac{3}{2} \hat{m} \cdot \Delta\vec{\alpha} \cdot \hat{m} - \frac{1}{2} Tr \Delta\vec{\alpha} \right) \quad (9)$$

where $Tr \Delta\vec{\alpha}$ is the average difference polarizability (the sum of the diagonal elements of $\Delta\vec{\alpha} = \vec{\alpha}_e - \vec{\alpha}_g$, see Eq. 4) and $\hat{m} \cdot \Delta\vec{\alpha} \cdot \hat{m}$ is the component of $\Delta\vec{\alpha}$ along the direction of the transition dipole moment $\hat{m}$, here given as a unit vector. B_χ also contains terms related to the transition polarizability. For the same reasons listed above these terms are generally neglected.

The third term C_χ is a second derivative line shape caused by a broadening of bands due to positive and negative energy shifts of the isotropically oriented molecules with respect to the field. C_χ is related to the difference dipole moment $\Delta\vec{\mu}_{eg}$ and, in most cases, dominates the Stark spectrum. C_χ has the form:

$$C_\chi = |\Delta\vec{\mu}|^2 \{ 5 + (3\cos^2\chi - 1)(3\cos^2\zeta_A - 1) \} \quad (10)$$

where ζ_A is the angle between the transition dipole moment and the difference dipole moment as described above.

To bring all of this into focus for the flavinologist, consider the flavin *N*(3)-methyl,*N*(10)-isobutylisoalloxazine (a.k.a. *N*(3)-flavin, a generous gift of Prof. Vincent Rotello, U.niv. of Mass., Amherst). This flavin is very useful because it is soluble over a wide range of polarities. The low temperature absorption spectra of *N*(3)-flavin in ethanol, *n*-butanol, 2-MTHF, and toluene are shown in Fig. 5.

As a guide, the two dotted lines in Fig. 5 approximately mark the low energy edge $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$ transitions of the oxidized flavin in ethanol, respectively. The effects of the solvent reaction field on the electronic energies of the molecule are evident though not easily

interpreted. The general rule is that as the polarity of the solvent decreases the transition energy should increase ($\vec{F}_R$ is decreasing with polarity). Simple solvents such as ethanol and butanol engage in hydrogen bonding (H-bonding) and are not really that simple. Even 2-MTHF has H-bonding capacity. As a solvatochromic reference for nonpolar solutes toluene is useful. Its interactions with most molecules are minimal, and it forms an optically clear glass reliably.

A comparison of numerical derivatives of the absorption spectrum with a Stark spectrum will serve to illustrate the information content inherent in Stark spectroscopy. Consider Fig. 6 which replots the 77K absorption spectrum of *N*(3)-methyl-TPARF in a toluene glass, this time plotted by wavelength rather than energy. In panels (B) and (C), the first and second numerical derivatives of A are plotted. In panel (D), the Stark spectra are plotted for two different values of χ . All the plots are *energy-weighted* (the extinction is divided by the wavenumber, $\bar{\nu}$, as required by the Liptay analysis).

The second derivative of the absorption spectrum matches the Stark spectrum in shape indicating that charge redistribution is mostly dipolar (C_χ , Eq. 10) in nature. The lack of agreement in the amplitudes for the two different transitions highlights the ability of Stark spectroscopy to separate transitions with different charge redistributions. Here $|\Delta\vec{\mu}_{20}| > |\Delta\vec{\mu}_{10}|$ since the Stark spectrum for the S_{20} transition is much larger in amplitude than the corresponding range in panel (C), while the S_{10} transition is of the small relative magnitude. This part of the second derivative spectrum enlarged eightfold, i.e. $C_\chi^{20} \sim 8C_\chi^{10}$ so that $|\Delta\vec{\mu}_{20}| \sim \sqrt{8}|\Delta\vec{\mu}_{10}|$.

We exploited this sensitivity to different overlapping transitions to demonstrate unequivocally that the one-transition assumption that the near-UV/visible absorption band of reduced anionic flavin was actually two overlapping transitions (Pauszek et al., 2016). This result has made conclusions drawn on the one-band assumption in need of revision (Kao et al., 2008; Zhao et al., 2011; Zhong, 2015), including our 2003 ultrafast study on the mechanism of DNA repair by photolyase (MacFarlane IV & Stanley, 2003).

It is also worth noting that vibronic structure in the absorption spectrum is amplified by the derivative nature of Stark spectroscopy. In fact, we have been able to resolve two overlapping progressions in the S_{10} transition as “dimples” at c. 500 and 478nm superimposed on the more obvious progression.

Nowhere is this more evident than in the Stark spectra of flavoproteins. Since flavin binding sites are generally nonpolar and conformational freedom is substituted by distinct amino acid contacts, a remarkable unique report of this environment is *imprinted* on the Stark spectrum. This can be seen by comparing the spectra of old yellow enzyme (Hopkins & Stanley, 2003) with that of DNA photolyase (Kodali et al., 2009).

In Fig. 7(A) and (C), the 77K ground state absorption spectra are plotted vs wavenumber (energy). The oxidized proteins show characteristic transitions from $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$ ($\sim 20,000$ and $21,200\text{cm}^{-1}$ for OYE and PL, respectively). Overlapping vibronic progressions as also evident, splitting the major features into doublets. These features are more evident in

the Stark spectra (normalized to field strength of 1 MV/cm) as shown in Fig. 7(B) and (D). The apparent second derivative nature of the signal suggests that the $\Delta\vec{\mu}_{f0}$ dominates charge redistribution and that $\Delta\vec{\mu}_{20} > \Delta\vec{\mu}_{10}$. The amplitude of the $S_0 \rightarrow S_1$ is significantly larger than that obtained for $S_0 \rightarrow S_1$.

What is also intriguing is the difference in band splitting for $S_0 \rightarrow S_2$ transition. In OYE a series of doublets are seen around 25,000–28,000 cm^{-1} . In DNA photolyase these bands appear to merge together. By comparison, the band splitting pattern in the $S_0 \rightarrow S_1$ transition are nearly identical. We believe that these unique Stark spectra reflect differences in contacts between the protein and its flavin, as well as the protein reaction field interacting with the dipolar flavin. A long-term goal of our work is to use flavoprotein Stark spectroscopy to “read out” this information and correlate it with structure and function.

2. Electronic Stark spectroscopy: Materials and methods

2.1 Solvent selection

2.1.1 Overview—The first step in the performance of an electronic Stark spectroscopy experiment is the selection of an adequate solvent in which to dissolve the chromophore to be studied. In addition to fully solvating the desired chromophore, the selected solvent must form an optically transparent glass at the cryogenic sample temperature of 77K characteristic of spectral acquisition. For free organic chromophores such as riboflavin, FMN, or FAD, a number of non-polar, aprotic organic solvents are available which adequately solvate chromophore molecules, form an optically transparent glass at 77K, and are of very low dielectric constant. Furthermore, such solvents are limited in their ability to form hydrogen bonds with solute chromophores, reducing the multiplicity of environmental electrostatic or dispersive perturbations contributing to inhomogeneous broadening of chromophore absorption bands. Organic solvents used in our group, with advantages and disadvantages, are as follows.

2.1.2 2-Methylpentane—This alkane is used primarily to dissolve chromophores possessing extended aliphatic moieties making dissolution in more polar organic solvents difficult. It is totally unable to hydrogen bond with solute chromophores, possesses a low dielectric constant, and forms an acceptably transparent glass at 77K. The primary disadvantage of this solvent is its high vapor pressure. Bubbles form in the sample almost immediately following loading, necessitating rapid immersion in the LN2 dewar cavity immediately following sample loading. If bubbles do form in the cuvette, the absence of dielectric media in the space occupied by the bubbles will allow shorting of the system and prevent Stark signal acquisition.

2.1.3 Toluene—Toluene is more viscous than 2-methylpentane, allowing more careful, if not leisurely, sample preparation, is unable to form hydrogen bonds, and possesses a low dielectric constant. Significant disadvantages, however, include absorption below 300nm, and the fact that toluene must be thoroughly dried by distillation onto hot molecular sieves in order to permit formation of a transparent glass. Aside from the drying requirement, toluene is an excellent solvent for chromophores with negligible absorption below 300nm.

2.1.4 2-MTHF—2-MTHF is capable of hydrogen bonding, possesses a vapor pressure approaching that of 2-methylpentane, and is of a higher dielectric constant than either 2-methylpentane or toluene, but reliably forms nearly perfectly transparent optical glasses at 77K even at large path lengths, up to 1mm for a low temperature absorption spectroscopy experiment. An important caveat when working with 2-MTHF is that it is often shipped containing the peroxide-formation inhibitor butylated hydroxytoluene (BHT). BHT absorbs at 280nm, and must be removed prior to use with chromophores absorbing into the UV.

2.1.5 Ethanol, 1-butanol—These two solvents are of high dielectric constant, but may be used when solubility considerations necessitate a solvent that is either of greater polarity or able to form hydrogen bonds. The greater extent of 1-butanol's alkane component contributes to a lower vapor pressure and lower dielectric constant relative to ethanol, but ethanol has been observed to permit formation of optically transparent glasses of greater path length than 1-butanol. If a path length of 25 μm is to be used, then 1-butanol is preferable, but for path lengths beyond 25 μm ethanol must be used.

2.1.6 Solvents for biological macromolecules—Enzymes and DNA require the use of aqueous solvents in order to limit denaturation of the macromolecular native structure. The most significant disadvantage of aqueous solvents is their high dielectric, necessitating the use of small path length cuvettes to recover field magnitude. Aqueous buffers typical to biochemistry are incapable of forming optically transparent glasses at 77K. One option for adapting biochemical aqueous buffers to use in Stark spectroscopy is to combine the buffer solution with 8M lithium chloride. 8M LiCl solutions hold decent fields, form high quality glasses, and are easy to work with. The high ionic strength of the solvent, however, perturbs the electrostatic interactions that stabilize the structures of biological macromolecules, potentially denaturing the native structures of the species desired to be studied.

Perturbation of native structural states may be avoided by using a composite solvent consisting of a 35:65 volume-to-volume mixture of aqueous buffer and glycerol. The glass formed using this solvent is of inferior quality to that formed by 8M LiCl, and glycerol may disrupt pi-pi interactions, but the reduced relative dielectric constant of the aqueous glycerol solution allows higher field magnitudes to be generated within the cuvette, contributing to sharper Stark signals.

2.2 Cuvette construction

2.2.1 Overview—The Stark cuvette is an optically transparent parallel plate capacitor, with the space between the two plates filled with the solvated chromophore to be studied. The plates are approximately 2.5cm square pieces of glass or quartz slides of thickness 0.5 to 0.7mm surfaced on one side by a coating that is transparent and conductive. The plates are separated from one another by strips of an insulating material, typically kapton. The distance between the plates is set by the thickness of the insulating material, for example, 25 to 50 μm . Greater path lengths hinder formation of optical glasses of sufficient transparency. By staggering the plates, it is possible to connect each plate to a separate electrical lead, permitting required charging of the plates during Stark spectrum acquisition.

Construction of high quality Stark cuvettes requires patience, dexterity, and practice. Until familiarity with cuvette construction is gained, it is advisable to practice making a few cuvettes with just solvent in order to identify difficult steps and arrive at a personalized method for cuvette construction, especially if the chromophore to be studied is expensive or difficult to obtain. Cuvette construction is accomplished in the following manner.

2.2.2 Cuvette materials—The first step in cuvette construction is to obtain the necessary materials. The two plates of the cuvette capacitor are slides of approximate dimensions $2.5\text{cm} \times 2.5\text{cm} \times 0.7\text{mm}$. The height and length of the slides to be used may vary depending on the inner diameter of the cryostat to be used in a particular instrumental set-up. The width of the slides, on the order of $0.5\text{--}0.7\text{mm}$, is not customizable in the lab. Thinner slides transmit light better but are more fragile, making use of thicker slides the safer and less frustrating choice. The slides are typically purchased in larger sections that may cut to the desired size and shape in the lab using a diamond scribe. The material of the slide to be used in a particular Stark experiment depends on the range of wavelengths required to be transmitted through the cuvette and sample. If the studied chromophore absorbs solely in the visible range (i.e. above 320nm), then float glass slides are the most economical choice. If transitions between 300 and 320nm will be interrogated spectroscopically, then boro-aluminosilicate glasses will suffice.

For chromophores absorbing in the UV range to a lower limit of 200nm , more expensive quartz slides must be used. In order to permit charging of the capacitor plates, the slides must be coated on the inside surface of the cuvette with a conducting and optically transparent material. For chromophores absorbing above 260nm , a $15\text{--}30\text{nm}$ coating of indium tin oxide (ITO) is the conductive coating of choice. Although a thicker coating of ITO would improve conductivity and lower resistance, thus maximizing the frequency response of the electric field applied to the sample, diminished transmission at shorter wavelengths of the accessible range reduces sensitivity below 300nm . For chromophores absorbing in the UV range down to and beyond 260nm , inconel-coated fused silica neutral density filters transmit light with constant attenuation of approximately 50% to below 260nm .

Non-adhesive kapton tape of thickness $25\mu\text{m}$ or $50\mu\text{m}$ must be obtained to form the insulating spacers separating the two slides of the cuvette. The spacers should be cut to a width that is approximately 20% that of the slides, and a length that is perhaps 4mm longer than the slides.

2.2.3 Cuvette assembly—The next step in cuvette construction is to clean the slides and insulating spacers and verify the side possessing the conductive surface. Wearing clean, powderless examination gloves, hold each slide or spacer in one corner and, with a folded optical cleaning wipe saturated with acetone and held using a plastic hemostat or tweezers, wipe each side of the slide or spacer lightly in a single motion. Change the corner by which the slide or spacer is held and repeat. After cleaning, place each cuvette component onto a clean optical wipe and verify the conductive side using a multimeter set to measure resistance. The surface of the slide coated with the conductive surface will register a non-

zero, non-infinite resistivity, while the non-conducting side will register an infinite resistivity that may be reported on the device by a zero (Figs. 8 and 9).

After verifying the sides that are conductive, place one slide on the optical wipe with the conductive surface up. Position one spacer on top of the slide such that the center of the spacer is positioned immediately upon the right edge of the slide. Position the second spacer on top of the slide such that the center of the spacer is approximately 30% of the length of the slide from the left edge of the slide.

Place the second slide onto the spacers with the conductive surface facing down. The left edge of the top slide should be positioned immediately above the center of the left spacer, and the right edge of the top slide should project over the right edge of the bottom slide by approximately 20% of the length of the top slide. The initially assembled cuvette may then be picked up carefully using a hemostat or tweezers and placed onto the immobilized cold finger platform (Fig. 10). If the components shift while moving them, their relative positions may be adjusted before attaching the cuvette to the cold finger platform using a small binder clip. The arms of the binder clip may then be removed, and the extended surfaces of the top and bottom slide connected to the cold finger's electrical leads.

At this point a micrometer can be used to measure the path length of the cuvette if the slide thicknesses are known. A multimeter should then be used to determine if the cuvette has capacitance. For ITO-coated boro-aluminosilicate slides with a plate separation of 50 μ m, the measured capacitance is typically on the order of approximately 300pF. If the cuvette possesses capacitance, it may be filled using a syringe by capillary action (fill volume approximately 50 μ L) and immersed in the liquid nitrogen bath of the cryostat.

2.2.4 Samples in organic solvents—Depending on the volatility of the organic solvent used, filling should be done as near to the cryostat as possible. If bubbles form in the cuvette between filling and immersion, the capacitor will likely fail upon application of the electric field and have to be reassembled.

2.2.5 Samples in aqueous solvents—Aqueous samples are sufficiently viscous to preclude loading the cuvette via capillary action. Instead, the sample must be applied as a droplet using a syringe to the bottom plate before the top plate of the cuvette is positioned. After the sample is applied, cuvette construction proceeds in the same manner described above, with the exception that care must be taken when attaching the cuvette to the cold finger platform so that the space occupied by the sample is not perturbed to the point that the sample “spills,” and that the outer surfaces of the cuvette may need to be wiped prior to immersion in the case that the sample has spilled to a small extent.

2.3 Acquiring the Stark spectrum: From lamp to lock-in amplifier

2.3.1 Overview—The Stark spectrometer currently in use in our group consists of a number of discrete electronic devices and optical elements able to be optimized for study of specific chromophores. They are laid out in Fig. 11.

2.3.2 Light sources—The first component of the spectrometer is a 300W xenon arc lamp (Hamamatsu L-2479), power supply/housing (Oriel 66485/69911, see Fig. 11A). A xenon arc lamp is used because of its stable intensity throughout the wavelength range from 200 to 800nm, covering the UV/vis absorption of flavins. We have also used Hg lamps, but their spectral output is more “jagged” than Xe and the intrinsic stability is lower. New Xe sources have been explored as well (e.g. EQ series from Energetiq Inc.) and are recommended for those setting up a spectrometer *de novo*.

In general, the higher the power output of the lamp, the larger the arc size, thus limiting the coupling efficiency into most monochromators. Short-arc lamps are preferred for their low noise and smaller image formation on the slit of the monochromator. However, lamps with output power below 150W will probably not produce sufficient light on the sample to obtain high S/N spectra. A light intensity controller (Oriel 68945) is used to stabilize beam intensity on the scale of minutes to hours in order to limit lamp drift.

2.3.3 Monochromator—The second component of the spectrometer is a 1/8-m monochromator (CM110, Spectral Products Inc.). Almost any computer-controllable monochromator will work, as the absorption bands of flavin at 77K are typically tens of nanometers broad (cf. Figs. 5 and 6). For example, bands in the Stark spectra of OYE (Fig. 7B) have a FWHM ~7nm. We typically use 0.6–1.2mm slits (bandpass of 2–4nm, depending on the grating) to maximize light intensity while sufficiently resolving the line shape of the transitions.

Most monochromators scan in equal wavelength increments (e.g. $\lambda = 2\text{nm/step}$), but the scanned transitions are linear in energy, not wavelength. Therefore, we have chosen to scan in equal wavenumber increments (e.g. from 300 to 600nm with $\Delta\bar{\nu} = 111\text{cm}^{-1}$). In this case, the step size in wavelength at 300nm will be $\lambda(300\text{nm}) = 1\text{nm}$ while $\lambda(600\text{nm}) = 4\text{nm}$. A careful simulation of this approach suggests that any artifacts are below the level of detection under typical conditions (data not shown).

2.3.4 Optics—The third, fourth, and fifth components of the spectrometer are a fiber optic cable (400 μm , UV/SR-VIS High OH Content Ocean Optics OOS-006872-09, Fig. 12C) connected to the exit port of the monochromator, a collimating telescope, a 1cm quartz Glan–Taylor polarizer (Fig. 12D), and a simple fused silica plano-convex focusing lens (Fig. 11E). The fiber optic cable connects to the exit port of the monochromator by means of a Spectral Products F/# Matching Fiber Optic Adapter (CVI AF-CML) and depolarizes the light exiting the monochromator. A collimating telescope at the exit of the fiber (Fig. 12C) must be fused silica as well and produces a ~5mm uniformly illuminated beam. This combination removes much of the spatial “structure” inherent in the lamp-monochromator. The light passes through the Glan–Taylor polarizer set to the desired polarization. The polarized light is then focused by the plano-convex lens ($F/\# < 2$) onto the sample.

It is desirable to have as small a spot size on the frozen glass as possible. Often frozen glasses fracture and a smaller probe beam diameter will allow good spectra to be obtained without the scattering produced by these fractures. Using an optical fiber with a diameter of

400 μm , about the width of the exit slit, and a low F/# focusing lens (Fig. 12E) will produce this condition.

2.3.5 Dewar or cryostat—The sixth component of the spectrometer is the sample assembly within the cryostat. We have used several dewars over the past 20 years, including an inexpensive H.S. Martin quad-window immersion dewar that can provide an optically quiet environment so long as the space above the LN2 is purged with helium. Our current two chamber cryostat is based on the design of Andrews and Boxer (2000) and is manufactured by JANIS Research. The unit has four fused silica windows on each chamber (Fig. 13F). The outer chamber contains a sacrificial LN2 reservoir used to reduce thermal currents in the inner immersion chamber of the dewar, thus minimizing optical noise generated by LN2 bubbling or swirling across the optical path. The standard operating procedure for filling the cryostat is as follows.

2.3.5.1 Filling the cryostat

- a. Purge the inner and outer dewars with anhydrous nitrogen gas for at least 40min prior to filling with LN2 (Fig. 14A). We use a length of tygon tubing to carry the purge gas to the bottom of the inner dewar to ensure effective purging.
- b. While purging the inner dewar the outer dewar is filled with LN2 via gravity from an external transfer dewar. The filling apparatus (Fig. 14B) consists of a plastic funnel connected to a thin metal tube extending to the bottom of the outer reservoir. A conical piece of filter paper is placed in the funnel to prevent ice from entering the outer dewar. As the outer dewar fills, the cryostat as a whole cools slowly, and vaporized LN2 escapes from the outer dewar exit port. When the outer dewar is filled with LN2, excess LN2 will spurt from this port. After filling is completed the outer dewar's filling hole should be stoppered.
- c. The inner dewar is filled using a custom piece of glassware utilizing a glass frit filter to draw ice-free LN2 into the inner dewar under vacuum (Fig. 14C). After the inner dewar is filled, it is essential to stopper the top of the inner dewar with a two-holed stopper and continue to purge the inner dewar to prevent snow formation in the LN2.

2.3.5.2 Sample immersion: The cuvette is mounted on the cold finger/flange (Fig. 15) that consists of a long steel rod with an in-house made platform attached to the end (Fig. 15D). The cuvette is attached to the nylon platform using binder clips, as discussed above.

The cold finger can be rotated to select the angle χ using a rotation stage (top of support rod in Fig. 11, Newport Inc.). Copper wire leads insulated using glass and ceramic tubes (Fig. 15B–E, Kimball Physics Inc.) are attached to the rod and connect to the cuvette via small flat-tipped alligator clips. A small amount of indium foil is applied between the clips and cuvette to maximize contact (not shown). The copper leads are attached to the rod in such a manner as to limit relative movement of the alligator clips and cuvette, limiting the probability of disconnect upon rotation to a new angle (Fig. 15C). *Warning: A high voltage hazard is present depending on the connectors used between the cold finger flange and the*

HVA (cf. Fig. 15A and E). *Double check that the HVA is off before connecting or disconnecting the cold finger.* Following immersion of the cuvette in the cryostat LN2 reservoir, the sample glass should be allowed to anneal for ~45min to attain good glass quality and stability.

2.3.6 Photodiode and current preamplifier—There are many photodiodes available. Our current favorite is the Hamamatsu S1226–44BQ, operating in photocurrent (low noise) mode. A Si photodiode is used for measurements from 190 to 1100nm while a SiC photodiode (SGLux) is used for measurements from 220 to 360nm as it conveniently rejects visible light without a loss of sensitivity. A low F/# fused silica double convex lens (Fig. 11G) focuses light transmitted by the sample onto a photodiode (Fig. 11H).

The photocurrent is amplified 10^5 – 10^7 -fold using a low noise current amplifier (Stanford Research Systems SR570 or Keithley 427) in direct current or low pass mode. Some photodiodes however, can be purchased with an integrated current preamplifier (e.g. OSI UDT-455UV). Care must be taken in setting the gain of the amplifier so that signal components above $2f$ are amplified without distortion. A good way to check for this is to verify that the LIA output voltage of the Stark signal exactly scales with the amplifier gain.

Since the HVA produces significant through-space background noise at the applied frequency we have enclosed the photodiode in a makeshift Faraday cage (a wooden box insulated with aluminum foil). This will block AC pickup from the HV leads. An integrated photodiode/amplifier is also an excellent way to avoid pickup.

2.3.7 High voltage amplifier and sine wave sources—A high voltage amplifier (HVA) must have sufficient current and bandwidth to generate a sine wave without distortion. The HVA sees the cuvette as a capacitive load, and the peak current can be calculated using $I_{peak} = \pi(C_{load} + C_{HVA})fV_{p-p}$, where the peak current is a function of the load capacitance C_{load} , C_{HVA} is the HVA internal capacitance, and V_{p-p} is the peak-to-peak voltage desired from the HVA at the desired frequency f . For example, the TREK 609E-6 HVA has $C_{HVA} = 50$ pF. The coaxial cable contributes another 100pF. Typical cuvette capacitance is $C_{load} = 400$ pF. Field strengths of 4×10^5 V/cm are usually adequate to obtain good S/N Stark spectra for molecules having $|\mu| > 1$ D. For a 25 μ m path length cuvette $V_{p-p} = 2000$ V. Therefore $I_{peak} = \pi(400pF + 50pF)(10^{-12}F/1pF)(1.5 \times 10^3 s^{-1})(2000V) = 4.2$ mA. The Trek HVA can deliver up to 10mA and has a large signal bandwidth of >6kHz with only 1% distortion. For the kinds of cuvettes and solvents we use it is important to have $f > 1.5$ kHz. The higher the frequency the lower the noise, $N(N \propto 1/f)$.

A sine wave generator is necessary as the input to the HVA. The fidelity and stability of the generator are very important, since variations in the generator will generate noise as $|\vec{F}_{ext}|^2$. We have found that the SR830 LIA provides a pure sine wave that can be adjusted either manually or under computer control.

2.4 Acquisition of the Stark spectra

The acquisition of Stark data is facilitated using an in-house computer program written in Labview (LV 2015, National Instruments). The LV program interfaces with the LIA amplifier, as well as a 16-bit data acquisition board (NI PCI-6036E). This program will be described later but first the physical components of the data acquisition are outlined.

2.4.1 Lock-in amplifier—The LIA (SR830) is used as the central clock and detects and digitizes both the signal and quadrature of the signal (X and Y, respectively). With proper phasing only the signal is important, but recording/displaying both X and Y channels will expedite setting the LIA phase. The internal sine wave generator is manually set to control both f and the amplitude of the sine wave (in volts rms, V_{RMS} , such that the zero-peak voltage $V_{0-Peak} = \sqrt{2}V_{rms}$). This variable sine wave is amplified by a TREK 609E-6 high voltage amplifier and connected as described using the cold finger's copper leads to the two plates of the cuvette capacitor. Light transmitted by the sample is modulated at f ; however, the resultant linear Stark effect will cancel due to symmetry, and therefore the signal is detected at $2f$, allowing the LIA to isolate the Stark signal with a sensitivity of 10^{-6} .

Signal to noise is maximized during Stark spectrum acquisition by maximizing the frequency and voltage of the applied electric field. We operate our Stark experiments at a frequency of 3.5kHz derived from the LIA sine wave generator. Because the modulation of the sample transmittance due to the electrochromism of the chromophore is small, even at high chromophore concentrations, a single cuvette affords both the electrochromic signal,

$$\Delta A(\vec{F}_{ext}) = \Delta \epsilon(\vec{F}_{ext}) c \ell \text{ where } \Delta \epsilon(\vec{F}_{ext}) = \frac{2\sqrt{2}I(\vec{F}_{ext})}{2.303I(\vec{F}_{ext}=0)c\ell} \text{ and } I_{REF}(\lambda), \text{ as long as}$$

$K \ll I_{REF}$. $I_{REF}(\lambda)$ is obtained using a NI DAQ analog-digital converter (ADC) with 16-bit digitization. Since LIA signals rarely rise above 10 μ V for a 4–5V amplified photocurrent, this requirement is met. The differential extinction can be computed using Eq. (11):

$$\Delta \epsilon(\vec{F}_{ext}) = 2\sqrt{2}I(\vec{F}_{ext}) = \frac{2\sqrt{2}I(\vec{F}_{ext})}{2.303I(\vec{F}_{ext}=0)c\ell} \quad (11)$$

2.4.2 Phasing the LIA signal—Phasing of the LIA is challenging because the signal can be positive or negative depending on whether the transition is mostly dominated by changes in μ or α (see Fig. 2). The SR830 LIA affords measurement of both X and Y components of the signal. It is strongly recommended that a preliminary scan be performed to set the proper phase angle.

- a. Using the room or low temperature absorption spectrum as a guide, set the monochromator to the maximum absorbance, λ_{max} , of the red-most absorption band. Doing so will minimize signal cancelation artifacts due to overlapping bands.
- b. If the LIA has an auto-phase function then set the LIA time constant to $3-5 \times$ the time constant to be used for data acquisition. Auto-phase after the signal has

settled. This will give the maximum positive X output. If no auto-phase is available, then find the phase angle that gives the maximum positive signal.

- c. Run a scan and verify that the quadrature is indeed randomly wandering about $Y = 0$. All the signal should now be in the X component.
- d. Once this condition has been met examine the spectrum carefully. If μ dominates the Stark spectrum then the maximum in the signal will occur at the $\lambda_{\max}$ (see Fig. 2C). However, the signal should be negative so change the LIA phase by $\pm 180^\circ$ so that a maximum negative signal is achieved. If instead the maximum signal has red-shifted compared to $\lambda_{\max}$ then α dominates the signal which should be positive (see Fig. 2B) and the LIA phase is correctly set.

2.4.3 Optimizing the signal—Because the magnitude of the Stark signal is proportional to the square of the magnitude of the applied field, the magnitude of the applied field should be maximized to improve signal to noise of acquired Stark spectra. After immersion and annealing, the HV is slowly applied to the cuvette. Fields of up to $F_{\text{ext}} = 0.5$ MV/cm can be achieved in the best samples. The applied V_{RMS} required from the lock-in sine generator for this field and a 50 μm cuvette is

$V_{\text{RMS}} = V/(\sqrt{2}g) = (5 \times 10^5 \text{ V/cm})(5 \times 10^{-3} \text{ cm})/(\sqrt{2} \times 10^3) = 1.768 \text{ V}$ where g is the gain of the HVA ($1000 \times$ in our case).

2.5 Acquiring the low temperature absorption spectrum

2.5.1 Overview—As discussed above, interpreting Stark spectra via the Liptay formalism requires an absorption spectrum measured under the same conditions as the Stark spectrum. The absorption spectrum is calculated as:

$$A(\lambda) = \log \frac{\langle I_0(\lambda) \rangle}{\langle I(\lambda) \rangle} \quad (12)$$

where $\langle I_0(\lambda) \rangle$ and $\langle I(\lambda) \rangle$ are the averaged reference and sample transmittances. Because of the use of separate sample and reference cuvettes, the acquisition of LTA spectra is the most technically difficult component of the Stark experiment. As such, signal to noise of the low temperature absorption spectrum must be maximized by limiting noise introduced by differential scattering of light by the sample and reference cuvettes through especial effort to obtain high quality glasses that are free of bubbles and cracks, and to limit the accumulation of snow within the inner dewar. To reduce the likelihood of artifacts entering into the data the Stark spectrometer is modified to acquire the low temperature absorption (LTA) spectrum in the following manner.

2.5.2 The absorption cuvette—The sample solution should be at the same concentration used for Stark spectrum acquisition. The cuvette is modified by eliminating the extending portions of the two plates used during Stark spectrum acquisition to connect the copper leads. While this modification serves no technical purpose, it makes the cuvette more compact and thus less likely to be snagged and disrupted during lowering into the cryostat. Short-path fused silica cuvettes can also be used. It is advantageous to extend the

optical path length when working with weakly absorbing solutes. For example, a 50 μm cuvette filled with 1mM flavin will only have an absorbance of 0.055 OD. Measuring the LTA in path lengths of 0.1–0.5mm is a workaround for this. Following immersion of the cuvette in the cryostat LN2 reservoir, the reference and sample glasses should be allowed to anneal for 45 min prior to interrogation to maximize glass quality.

2.5.3 Configuring the absorption spectrometer—The Stark spectrometer is modified by removing the Glan–Taylor polarizer to increase the incident light intensity. The cuvette is positioned normal to incident light. A kHz optical chopper is added at the output of the fiber optic. The blade spacing for high frequency choppers is small, ~5mm, which puts constraints on the width of the probe beam. This highlights an advantage of using the fiber optic described above. The chopper is run at its maximum frequency given the constraints imposed by the beam size. The LIA Amplifier is operated in “**R**” mode ($R = \sqrt{X^2 + Y^2}$). The amplified signal from the spectrometer is The detected at the chopper frequency ω (not 2ω). The downstream components are otherwise identical to the Stark spectrometer set-up.

2.5.4 Data acquisition parameters—We typically acquire and average three separate sample and reference spectra at the maximum achievable LIA sensitivity. The choice of time constant always depends on field frequency, but also depends on the frequency of the noise spectrum. For the absorption spectrum it is often advantageous to use a shorter time constant (e.g. 100ms) but increase the number of scans so the same data acquisition time is obtained as with a smaller number of scans using a 1s time constant. The reason for this is that there are fluctuations in the dewar on a longer time period than 1s, and increasing the number of scans while reducing the LIA time constant will reduce this “slow” noise through averaging.

3. Calculations

3.1 Overview

Absorption spectra must be converted to extinction, $\epsilon(\lambda) = A(\lambda)/(c_{\text{corr}} \times l)$ (see Eq. 11). Here c_{corr} is the solute concentration corrected for solvent contraction. Most organic solvents contract 15–25% during immersion, as determined by measuring the height of solvent in a standard 1mm quartz cuvette before and after freezing.

The calculation of ϵ as a function of applied field and χ has been reviewed elsewhere, as have the fitting procedures (Pauszek et al., 2016; Pauszek, Kodali, & Stanley, 2013; Pauszek & Stanley, 2014; Stanley, 2001).

3.2 Least squares fitting of the Stark and absorption spectra

Having acquired the low temperature absorption and Stark spectra, electro-optical charge redistribution parameters are determined by simultaneously fitting all measured spectra using a custom MATLAB executable that is freely available upon request. The general strategy employed by our method proceeds as follows:

The low temperature absorption spectrum is modeled as a superposition of Gaussian functions that lacks physical meaning but is easily differentiated analytically. First and second analytical derivatives of the low temperature absorption spectrum are computed and their Gaussian parameters (three per band) are optimized to fit the LTA.

The collected Stark and LTA spectra are then fitted simultaneously. After determining sets of A_χ , B_χ , and C_χ for Stark spectra measured at 2 χ -values (and perhaps multiple field strengths as well) it is possible to find unique values to the electro-optical charge redistribution parameters described in Eqs. (8)–(10). The solute does not experience the full strength of the applied external field due to the solvent cavity shape and dielectric properties. This attenuation is termed the *local field correction factor*, f . Several methods have been employed to estimate this factor and have been referenced previously. When the shape of the solvent cavity is difficult to estimate then it is still useful to report the Stark parameters in terms of f . Ratios of difference dipoles can then lead to use comparisons.

3.3 The ζ_A degeneracy problem

Because the Liptay formalism permits determination of only the relative orientation of the difference permanent dipole and transition dipole moments, and not the absolute orientations of the two dipole moments within the structural context of the chromophore, theoretical calculations are needed to determine the absolute orientations of the difference permanent dipole and transition dipole moments. This conundrum is illustrated in Fig. 16. Quantum calculations and chemical intuition must be employed to find the most likely ζ_A .

DFT and TD-DFT calculations at the TD/B3LYP/6–311+G(d,p) level of theory are typically sufficient to determine the absolute orientations of the fitted ζ_A and $\Delta\vec{\mu}_{eg}$ within the molecular frame of reference. For flavins though, higher levels of theory are sometimes useful. These approaches and results have been described in detail in previous papers (Kodali, Kistler, Matsika, & Stanley, 2008; Kodali, Kistler, Narayanan, Matsika, & Stanley, 2010; Kodali, Narayanan, & Stanley, 2012; Kodali et al., 2009; Pauszek III, Kodali, & Stanley, 2014; Pauszek, Kodali, Caldwell, et al., 2013; Pauszek et al., 2016; Rohwer et al., 2018). We believe that we are on the threshold of elucidating how the unique electrostatic cavity fields in proteins affect flavin energetics and catalysis.

4. Summary

Stark spectroscopy can be implemented in almost any laboratory at relatively low cost. It is unique in the information it can retrieve about cofactors in proteins and the impact of the environment surrounding them (Boxer, 2009; Bublitz & Boxer, 1997; Fried, Bagchi, & Boxer, 2014; Fried & Boxer, 2015; Wu & Boxer, 2016).

Acknowledgments

We wish to thank Professor Ronald Koder (CUNY, City College, NY) for the generous gift of N(3)-TPARF. This work was supported, in part, by a NASA Exobiology grant 80NSSC17K0033.

Abbreviations

2-MTHF	2-methyl-tetrahydrofuran
AC	alternating current
BHT	butylated hydroxytoluene
DFT	density functional theory
DNA	deoxyribonucleic acid
FAD	flavin adenine dinucleotide
Fl_{ox}	oxidized flavin
FMN	flavin mononucleotide
FWHM	full width at half maximum height
HOMO	highest occupied molecular orbital
HV	high voltage
HVA	high voltage amplifier
ITO	indium tin oxide
LIA	lock-in amplifier
LN₂	liquid nitrogen
LT	low temperature
LTA	low temperature absorption
LUMO	lowest unoccupied molecular orbital
NADPH	reduced nicotinamide adenine dinucleotide phosphate
OYE	old yellow enzyme
PL	photolyase
S/N	signal to noise
TD-DFT	time-dependent density functional theory
TPARF	tetraphenylacetyl-riboflavin
UV	ultraviolet

References

- Andrews SS, & Boxer SG (2000). A liquid nitrogen immersion cryostat for optical measurements. *Review of Scientific Instruments*, 71(9), 3567–3569.

- Boxer SG (2009). Stark realities. *Journal of Physical Chemistry B*, 113(10), 2972–2983.
- Bublitz GU, & Boxer SG (1997). Stark spectroscopy: Applications in chemistry, biology, and materials science. *Annual Review of Physical Chemistry*, 48, 213–242.
- Bublitz GU, Ortiz R, Marder SR, & Boxer SG (1997). Stark spectroscopy of donor/acceptor substituted polyenes. *Journal of the American Chemical Society*, 119(14), 3365–3376.
- Cantor CR, & Schimmel PR (1980). *Biophysical chemistry*. Vol. 2 New York: W.H. Freeman and Company.
- Fried SD, Bagchi S, & Boxer SG (2014). Extreme electric fields power catalysis in the active site of ketosteroid isomerase. *Science*, 346(6216), 1510–1514. 10.1126/science.1259802. [PubMed: 25525245]
- Fried SD, & Boxer SG (2015). Measuring electric fields and noncovalent interactions using the vibrational stark effect. *Accounts of Chemical Research*, 48(4), 998–1006. 10.1021/ar500464j. [PubMed: 25799082]
- Gindt YM, Edani BH, Olejnikova A, Roberts AN, Munshi S, & Stanley RJ (2016). The missing electrostatic interactions between DNA substrate and *Sulfolobus solfataricus* DNA photolyase: What is the role of charged amino acids in thermophilic DNA binding proteins? *Journal of Physical Chemistry B*, 120(39), 10234–10242. 10.1021/acs.jpcb.6b07201.
- Hall LN, Orchard BJ, & Tripathy SK (1987). The structure and properties of flavins: Molecular orbital study based on totally optimized geometries. I. Molecular geometry investigations. *International Journal of Quantum Chemistry*, 31, 195–216.
- Hopkins N, & Stanley RJ (2003). Measurement of the electronic properties of the flavoprotein old yellow enzyme (OYE) and the OYE:P-cl phenol charge-transfer complex using stark spectroscopy. *Biochemistry*, 42(4), 991–999. [PubMed: 12549919]
- Kao Y-T, Saxena C, He T-F, Guo L, Wang L, Sancar A, et al. (2008). Ultrafast dynamics of flavins in five redox states. *Journal of the American Chemical Society*, 130(39), 13132–13139. [PubMed: 18767842]
- Kodali G, Kistler KA, Matsika S, & Stanley RJ (2008). 2-Aminopurine excited state electronic structure measured by stark spectroscopy. *Journal of Physical Chemistry B*, 112(6), 1789–1795.
- Kodali G, Kistler KA, Narayanan M, Matsika S, & Stanley RJ (2010). Change in electronic structure upon optical excitation of 8-vinyladenosine: An experimental and theoretical study. *Journal of Physical Chemistry A*, 114(1), 256–267.
- Kodali G, Narayanan M, & Stanley RJ (2012). Excited-state electronic properties of 6-methylisoxanthopterin (6-MI): An experimental and theoretical study. *Journal of Physical Chemistry B*, 116(9), 2981–2989. 10.1021/jp2110083.
- Kodali G, Siddiqui SU, & Stanley RJ (2009). Charge redistribution in oxidized and semiquinone E. coli DNA photolyase upon photoexcitation: Stark spectroscopy reveals a rationale for the position of Trp382. *Journal of the American Chemical Society*, 131(13), 4795–4807. [PubMed: 19292445]
- Lee W, Kodali G, Stanley RJ, & Matsika S (2016). Coexistence of different electron transfer mechanisms in the DNA repair process by photolyase. *Chemistry—A European Journal*, 22(32), 11371–11381. 10.1002/chem.201600656.
- Lienhart WD, Gudipati V, & Macheroux P (2013). The human flavoproteome. *Archives of Biochemistry and Biophysics*, 535(2), 150–162. 10.1016/j.abb.2013.02.015. [PubMed: 23500531]
- Liptay W (1969). Electrochromism and solvatochromism. *Angewandte Chemie International Edition*, 8(3), 177–188.
- Liptay W (1974). Dipole moments and polarizabilities of molecules in excited electronic states In Lim EC (Ed.), Vol. 1 *Excited states* (pp. 129–229). New York: Academic Press.
- MacFarlane AW IV, & Stanley RJ (2001). Evidence of powerful substrate electric fields in DNA photolyase: Implications for thymidine dimer repair. *Biochemistry*, 40(50), 15203–15214. [PubMed: 11735403]
- MacFarlane AW IV, & Stanley RJ (2003). Cis-Syn thymidine dimer repair by DNA photolyase in real time. *Biochemistry*, 42(28), 8558–8568. [PubMed: 12859203]
- Macheroux P, Kappes B, & Ealick SE (2011). Flavogenomics—A genomic and structural view of flavin-dependent proteins. *FEBS Journal*, 278(15), 2625–2634. 10.1111/j.1742-4658.2011.08202.x. [PubMed: 21635694]

- Narayanan M, Singh VR, Kodali G, Moravcevic K, & Stanley RJ (2017). An ethenoadenine FAD analog accelerates UV dimer repair by DNA photolyase. *Photochemistry and Photobiology*, 93(1), 343–354. 10.1111/php.12684. [PubMed: 27935052]
- Pauszek RF III, Kodali G, & Stanley RJ (2014). Excited state electronic structures of 5,10-methenyltetrahydrofolate and 5,10-methylenetetrahydrofolate determined by stark spectroscopy. *Journal of Physical Chemistry A*, 118(37), 8320–8328. 10.1021/jp501143u.
- Pauszek RF, Kodali G, Caldwell ST, Fitzpatrick B, Zainalabdeen NY, Cooke G, et al. (2013). Excited state charge redistribution and dynamics in the donor-pi-acceptor flavin derivative ABFL. *Journal of Physical Chemistry B*, 117(49), 15684–15694. 10.1021/jp406420h.
- Pauszek R, Kodali G, Siddiqui MS, & Stanley RJ (2011). Excited state charge redistribution in reduced flavins obtained by stark spectroscopy: One lump or two? In Paper presented at the proceedings of the 17th international symposium on flavins and flavoproteins, Berkely, CA USA.
- Pauszek RF, Kodali G, Siddiqui MSU, & Stanley RJ (2016). Overlapping electronic states with nearly parallel transition dipole moments in reduced anionic flavin can distort photobiological dynamics. *Journal of the American Chemical Society*, 138(45), 14880–14889. 10.1021/jacs.6b06449. [PubMed: 27686753]
- Pauszek RF, Kodali G, & Stanley RJ (2013). Stark spectroscopy of flavins In S. E & W. S (Eds.), *Methods in molecular biology*. New York: Humana Press.
- Pauszek RF, & Stanley RJ (2014). A “how-to” guide to the stark spectroscopy of flavins and flavoproteins. In Weber S & Schleicher E (Eds.), Vol. 1146 *Flavins and flavoproteins: Methods and protocols* (pp. 443–466).
- Reitz JR, Milford FJ, & Christy RW (1979). *Foundations of electromagnetic theory*. Addison Wesley Publishing Company.
- Rohwer EJ, Akbarimoosavi M, Meckel SE, Liu XS, Geng Y, Daku LML, et al. (2018). Dipole moment and polarizability of tunable intramolecular charge transfer states in heterocyclic pi-conjugated molecular dyads determined by computational and stark spectroscopic study. *Journal of Physical Chemistry C*, 122(17), 9346–9355. 10.1021/acs.jpcc.8b02268.
- Stankovich MT (1991). Redox properties of flavins and flavoproteins In Müller F (Ed.), Vol. I *Chemistry and biochemistry of flavoenzymes*. Boca Raton: CRC Press.
- Stanley RJ (2001). Advances in flavin and flavoprotein optical spectroscopy. *Antioxidants & Redox Signaling*, 3(5), 847–866. [PubMed: 11761332]
- Stanley RJ, & MacFarlane AW IV. (2000). Ultrafast excited state dynamics of oxidized flavins: Direct observations of quenching by purines. *Journal of Physical Chemistry A*, 104(30), 6899–6906.
- Wu YF, & Boxer SG (2016). A critical test of the electrostatic contribution to catalysis with noncanonical amino acids in ketosteroid isomerase. *Journal of the American Chemical Society*, 138(36), 11890–11895. 10.1021/jacs.6b06843. [PubMed: 27545569]
- Yu X, Eymur S, Singh V, Yang BQ, Tonga M, Bheemaraju A, et al. (2012). Flavin as a photo-active acceptor for efficient energy and charge transfer in a model donor–acceptor system. *Physical Chemistry Chemical Physics*, 14(19), 6749–6754. 10.1039/c2cp40073a. [PubMed: 22509495]
- Zhao RK, Lukacs A, Haigney A, Brust R, Greetham GM, Towrie M, et al. (2011). Ultrafast transient mid IR to visible spectroscopy of fully reduced flavins. *Physical Chemistry Chemical Physics*, 13(39), 17642–17648. 10.1039/c1cp22097g. [PubMed: 21887430]
- Zhong D (2015). Electron transfer mechanisms of DNA repair by photolyase. In Johnson MA & Martinez TJ (Eds.), Vol. 66 *Annual review of physical chemistry* (pp. 691–715).

$$\Delta E = h\Delta \nu \approx -\Delta \bar{\mu}_{ge} \cdot \vec{F}_{ext} - \frac{1}{2} \vec{F}_{ext} \cdot \Delta \bar{\alpha}_{ge} \cdot \vec{F}_{ext}$$

Fig. 1.

A simplified Jablonski diagram showing the effect of an external electric field on an optical transition. Both ground and excited states change energy in response to the field. Here only the extrema of the effect are drawn.

Fig. 2.

Simulations of the Stark signal assuming a Gaussian band shape. Panel (A) shows the effect of an applied field on an ensemble of molecules that are unoriented and mobile. A decrease in the extinction coefficient is observed. Panel (B) shows the field effect on the polarizability, $|\alpha| \neq 0$, is obtained. Panel (C) shows the effect if $|\mu| \neq 0$.

Fig. 3.

The orientation of the polarized probe light to the sample cuvette as seen from above with the probe light polarized in the plane of the page. χ is the angle between the polarization vector of the light $\vec{p}_0$ and the applied electric field $\vec{F}_{ext}$. At normal incidence, $\chi = 90^\circ$, $\vec{p}_0$ can have almost any orientation with respect to $\vec{F}_{ext}$. Note too that the path length changes as the sample is rotated from normal incidence. At 45° the path length increases by factor of $\sqrt{2}$.

Fig. 4.
Permanent dipoles and the transition dipole in the molecular frame of reference, where χ_A is the angle between $\vec{m}_{eg}$ and $\Delta\vec{\mu}_{eg}$.

Fig. 5. Low temperature (77 K) absorption spectra of *N*(3)-methyl, *N*(10)-isobutylisoalloxazine in solvents of decreasing polarity.

Fig. 6. LT energy-weighted absorption spectrum (A), with first (B), and second (C) derivatives for 3.3mM N(3)-TPARF in toluene. Panel (D) shows the $\chi = 55^\circ$ (—) and 90° (---) Stark spectra.

Fig. 7.

77K absorption (A/C) and Stark spectra (B/D) of OYE/DNA photolyase. The Stark spectra were taken with $\chi = 53 \pm 2^\circ$ and normalized to 10^6 V/cm for comparison.

Fig. 8.
(Left) The bottom slide with the Kapton spacers. (Right) The top slide.

Fig. 9.
The assembled cuvette.

Fig. 10.
The assembled cuvette attached to the cold finger platform and leads.

Fig. 11.

An overview of the Stark spectrometer in use in our group, including the (A) xenon arc lamp and light intensity controller, (B) 1/8-m monochromator, (C) fiber optic outlet, (D) Glan–Taylor polarizer, (E) plano-convex focusing lens, (F) cryostat, (G) double convex focusing lens, and (H) photodiode. The photodiode Faraday cage and current amplifier are not shown in this image.

Fig. 12.

A close-up of the first half of the Stark spectrometer set-up. The camera next to the lamp and mirror between the lamp and monochromator are components of the light intensity controller system. The plano-convex lens (E) is positioned so closely to the cryostat in order to focus the beam exiting the polarizer to a single point on the sample held within the cryostat.

Fig. 13.
A close-up of the second half of the Stark spectrometer set-up.

Fig. 14.

Three stages of filling the cryostat with LN2. (A) The tubing carrying anhydrous nitrogen gas into the inner and outer dewars. (B) The funnel used for filling the outer dewar and the continued purging of the inner dewar. (C) The stoppered outer dewar and the custom pyrex LN2 transfer funnel with frit positioned on top of the inner dewar.

Fig. 15.

Different views of the assembled cold finger. Panel (A) leads entering the external banana plugs are derived from a piece of partially stripped coaxial cable, with the cable core used to carry the output from the high voltage amplifier to one plate of the cuvette capacitor. The separated cable braid is used to ground the cuvette. (B) The partially stripped coaxial cable on the inner side of the cold finger flange. The copper connects shown in (B) above are used for convenience. Copper wires are jacketed completely in glass tubes. The ceramic pieces in (C) and (D) permit isolating rotational motion of the central rod from the transverse relative motion of the alligator clips, reducing the likelihood of disconnecting the cuvette. The black nylon platform is visible in (D) and was custom-machined.

Fig. 16.

The ζ_A degeneracy problem. The multiple cones represent mathematically correct solutions for the orientation of μ relative to $\vec{m}_{eg}$ ($\zeta_A = \angle \vec{m}_{eg}, \Delta\vec{\mu}_{eg}$).