

Stark Spectroscopy of Photosynthetic Systems

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Summary

The effects of applied electric fields on the absorption or emission spectrum of a molecule is known as Stark spectroscopy. This method probes the movement of charge associated with optical excitation, and is thus sensitive to features which are important for chromophore systems that carry out charge separation, such as photosynthetic systems. This chapter updates a review written about 2 years ago which also described electric field effects on reaction dynamics, notably electron transfer reactions (Boxer, 1993). Several recent extensions of the method such as higher order Stark spectroscopy and vibrational Stark spectroscopy are discussed along with examples. Experimental methods and methods of analyzing spectra are discussed in great detail, along with a discussion of experimental and conceptual issues which complicate the analysis of Stark spectra.

Abbreviations: HOSS – Higher order Stark spectroscopy; LDAO – Lauryldimethylamine-N-oxide; RC – Reaction center; VSE – Vibrational Stark spectroscopy

I. Introduction

The effect of an applied electric field on an absorption or emission spectrum is known as the Stark effect. This term is used interchangeably with electroabsorption or electrochromism, both

of which have been widely used in the literature. The basic approach goes back to early in this century when electric fields were first used to perturb the energy levels of atoms in the gas phase. Extensive work was published on simple molecules, often as guests in host crystals, primarily as a test of the predictions of molecular orbital calculations (Hochstrasser, 1973). Recent developments in this area were reviewed by me two

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years ago with a specific emphasis on photosynthetic systems (Boxer, 1993). That review also described experiments on the use of applied electric fields to perturb reaction dynamics, notably the rate of electron transfer reactions in photosynthetic reaction centers (RCs), measured either directly (Lockhart et al., 1990; Franzen et al., 1990; Franzen and Boxer, 1993) or indirectly via the effect of the electric field on the fluorescence which competes with electron transfer (Lockhart and Boxer, 1988; Lockhart et al., 1988). The emphasis in the following is on recent developments of absorption Stark spectroscopy and its application to understanding the excited states of photosynthetic pigments. I have particularly stressed experimental and conceptual complications, as well as the successes.

The change in transition frequency $\Delta\nu$, due to an externally applied field F , is given by:

$$h\Delta\nu = -\Delta\mu \cdot F - F \cdot \Delta\alpha \cdot F/2 \quad (1)$$

where $\Delta\mu$ and $\Delta\alpha$ are the change in dipole moment and polarizability, respectively, between the states involved. The most desirable method for obtaining Stark effect spectra is for uniaxially oriented molecules. If the first term dominates, as is often the case, this interaction gives rise to a linear shift in the absorption as the applied field strength is increased (the so-called linear Stark effect); the second term depends on the square of the applied field (the so-called quadratic Stark effect). In principle, it is possible to obtain information on the components of the difference polarizability tensor by varying the orientation of the molecule in the applied electric field. Unfortunately, uniaxial orientation is only rarely achieved in practice for complex molecules, and I am not aware of an example of a biological molecule where the Stark spectrum has been analyzed quantitatively.

For membrane proteins, the sample can be uniaxially oriented across a lipid bilayer (no orientation in the plane of the bilayer). It is then possible to create an electric field by varying the ionic strength on the two sides of the bilayer. This gives rise to spectral shifts, known historically as electrochromic shifts, and, at least at a qualitative level, these shifts are very widely used to probe changes in transmembrane potential using voltage sensitive dyes. A somewhat related effect in-

volves creating the field internally, rather than externally. For example, if charge is separated in response to light, as in the RC, the electric field due to these charges causes shifts in both the electronic absorption and vibrational bands of other spectator chromophores within the RC. This internal, transient electric field has a fixed spatial relationship with the spectator chromophores, so the observed Stark or electrochromic bandshifts can be treated as if the sample were completely oriented. Of course in this case it is not possible to vary the applied electric field strength systematically. Another possibility is that the protein containing a chromophore can be biaxially oriented, e.g., the protein is oriented across the bilayer, but the orientation does not distinguish one side of the bilayer from the other. If the absorption spectrum of a chromophore in such a system were very narrow, then the application of a transmembrane electric field would split the absorption due to the interaction of the fixed field direction with the two antiparallel projections of the chromophore difference dipole moments on the membrane normal. In practice, the absorption spectrum of biological chromophores is invariably inhomogeneously broadened, and the linewidth is typically several hundred cm^{-1} which is considerably larger than the interaction energy between $\Delta\mu$ and the applied field (for reasonable values of either). In this case, the effect of the field is to broaden the absorption spectrum, and, so long as the interaction energy is small relative to the inhomogeneous linewidth, the effect depends quadratically on applied electric field. In this case the distinction between effects due to $\Delta\mu$ and $\Delta\alpha$ can not be made on the basis of the field dependence, but must rather be made by analyzing the lineshape of the Stark effect spectrum. The same situation applies to non-oriented, immobilized samples which are simple to prepare and are the only cases to be discussed further in the next sections.

II. Methods

A. Analytical Methods

It is straightforward to show that for an isolated absorption band in a non-oriented, immobilized sample when $\Delta\nu$ is smaller than the inhomogene-

ous line width, the change in absorption upon application of a electric field, F , is given by (Lip-tay, 1974; Mathies, 1974):

$$\Delta A(\nu) = (fF)^2\{A_x A(\nu) + (B_x \nu / 15hc) d[A(\nu)/\nu] d\nu + (C_x \nu / 30h^2 c^2) d^2[A(\nu)/\nu] d\nu^2\} \quad (2)$$

where A_x depends on the transition polarizability and hyperpolarizability; $B_x = (1/2)\{5\text{Tr}(\Delta\alpha) + (3\cos^2\chi - 1)[3(\mathbf{p}\cdot\Delta\alpha\cdot\mathbf{p}) - \text{Tr}(\Delta\alpha)]\}$ (neglecting the contribution from the cross term of the transition polarizability and $\Delta\mu$); and $C_x = |\Delta\mu|^2[5 + (3\cos^2\zeta - 1)(3\cos^2\chi - 1)]$. ζ is the molecular angle between $\Delta\mu$ and the transition moment \mathbf{p} . ζ is obtained from the second derivative coefficients of the Stark spectra obtained at different values of χ , the experimental angle between F and the electric vector of the linearly polarized light used to probe ΔA . f is the local field correction which is discussed further below. In order to obtain information on the desired electro-optical properties, the coefficients A , B , and C in Eq. (2), the observed change in absorption is analyzed in terms of contributions from the zeroth, first and second derivatives of the absorption band.

We have recently extended the conventional method to include the higher harmonic responses of the absorption to the applied AC field, a technique we have called higher-order Stark spectroscopy (HOSS) (Lao et al., 1995a). The field-induced change in absorbance by an externally applied sinusoidal electric field $F(\omega) = F_0 \sin(\omega t)$ is given by:

$$\Delta A(\nu, F) = \Delta A(\nu, F^2, 2\omega) + \Delta A(\nu, F^4, 4\omega) + \Delta A(\nu, F^6, 6\omega) + \dots \quad (3)$$

Changes in absorbance, $\Delta A(\nu, F^2, 2\omega)$, $\Delta A(\nu, F^4, 4\omega)$, $\Delta A(\nu, F^6, 6\omega)$, etc. are recorded using lock-in detection at the 2nd, 4th and 6th-harmonic frequencies, respectively, of the field modulation frequency ω . The n th-order spectrum depends on the n th power of the applied field, F^n . Like the conventional or 2ω Stark spectrum, the higher order Stark spectrum can be fit to sums of derivatives of the absorption lineshape. For example, in the case of the 4ω spectrum, $\Delta A(\nu, F^4, 4\omega)$ is fit to the sum of up to the 4th derivative of the absorption lineshape. Additionally, for each n th-order Stark spectrum, the n th

derivative component depends only upon $\Delta\mu$ and ζ_A . Thus, if the n th-order Stark spectrum is dominated by the n th derivative of the absorption spectrum, it is immediately evident that $\Delta\mu$ dominates $\Delta A(\nu, F)$. An even simpler diagnostic for this case is that the $(n + 2)$ -order Stark spectrum is the second derivative of the n th-order spectrum. As seen below this simple diagnostic is quite powerful because it is possible to obtain Stark spectra with good signal-to-noise, whereas it is quite difficult to obtain good quality higher derivatives of the absorption spectrum.

B. Sample Preparation and Dielectric Breakdown

The conventional Stark effect depends quadratically on applied field strength (Eq. 2) and the higher order Stark spectrum on higher even powers of the field (Eq. (3)). Because the higher order terms have coefficients that become (usually) much smaller with term order, it is very desirable to obtain the highest possible applied electric field strength. The ultimate limit is determined by dielectric breakdown. Several factors determine the field at which dielectric breakdown occurs. (1) Low temperatures are essential for achieving the highest applied field strengths. We typically perform our experiments with samples immersed in liquid nitrogen in a strain-free optical dewar (N_2 bubbling is minimized by blowing a thin stream of gaseous helium over the surface). In other cases, we have cooled samples by blowing cooled gaseous nitrogen or helium over the sample in an optical dewar, or by contacting the sample with a cold-finger. Heat transport is less efficient in these cases than when the sample is immersed in liquid nitrogen, so it is difficult to obtain the highest fields. Recently, in collaboration with MMR Technologies, Inc. (Mountain View, CA), we have adapted a miniature Joule-Thompson refrigerator for electric field experiments (Stanley and Boxer, 1995). The MMR refrigerator is so small that it can easily be translated during an experiment, e.g., when the sample is degraded by prolonged exposure to a high peak power laser. In this device the sample is in contact with a cold finger which is cooled by the expansion of pure nitrogen through a network of capillaries. The problem with this system, and

many others that use refrigerators, is that the samples are often liquids at room temperature, the cold-fingers operate under vacuum, and the sample cell is not vacuum tight. The entire MMR refrigerator can be put in a -20°C or -80°C freezer until the liquid sample (typically containing 50–75% glycerol) is very viscous. At that point the refrigerator can be evacuated without causing sample bubbling. Alternatively the same can be very rapidly cooled using methane. Obviously this problem is not present for polymer film samples (e.g. in polyvinylalcohol); however, we have found that much better results can be obtained with frozen glass samples because the inhomogeneous broadening is typically less, the sample is not dehydrated, and it is possible to work with extremely small quantities by avoiding the sample waste associated with the preparation of films. In some cases it is useful to work at pumped liquid helium temperatures because this produces the narrowest spectral lines. This is achieved using a standard helium cryostat. (2) By working with the thinnest possible sample it is possible to obtain the best glasses and the highest fields. Of course this requires that the sample be very concentrated in order to achieve sufficient optical density. There are various approaches to making thin samples. We typically use precision spacers made of Teflon which are available in thicknesses down to about 10 microns. (3) We have found that the highest fields can be obtained by reducing the concentration of detergents as much as possible. In the case of photosynthetic RCs, we typically work at the absolute minimum detergent concentration compatible with maintaining good solubility (about 0.01% LDAO). (4) We use a power supply which has a current fuse which quickly cuts off the applied field as soon as current flow is sensed. Dielectric breakdown is a cumulative effect, and the sample can be preserved if breakdown is halted as soon as it begins. This is also advantageous because valuable samples or samples in difficult conditions (e.g. pumped He) are not destroyed. It is also an important safety precaution. We can routinely obtain applied electric fields (AC modulation) of approximately 1 MV/cm on frozen aqueous glasses immersed in liquid nitrogen for 10–25 micron cells. This can be achieved even at room temperature in polymer films, and fields as high

as 3 MV/cm have been achieved for very thin polymer films (several micron) using pulsed electric fields (Lao et al., 1993).

C. Light Sources, Detectors and Power Supplies

For most photosynthetic pigments, the absorption of interest occurs in the visible and near-infrared regions of the spectrum. The change in absorption can be probed using a conventional high pressure xenon or tungsten-halogen lamp whose output is dispersed through a monochromator. Si photodiodes are excellent detectors from about 400 to 1100 nm and have extremely low noise. Because they are only moderately sensitive, relatively high probe light intensities are needed. This can create interesting complications for pigment systems like the RCs which undergo charge separation with high quantum efficiency. Thus, electric field effects on the kinetics (Lockhart et al., 1990; Franzen et al., 1990; Franzen et al., 1992; Franzen et al., 1993) and steady-state population of intermediates (Franzen and Boxer, 1993) may be combined or may compete with the Stark effect on absorption (Lao et al., 1995b) or emission (Lockhart et al., 1991) giving rise to lineshapes which bear little relationship with the sum-of-derivatives form of Eq. (2). Si avalanche photodiodes have the advantage of integrated amplification. Most Si detectors are overcoated to minimize degradation by UV light and cannot be used effectively in the near UV. We have found that a photomultiplier with good near-UV sensitivity, wired so that only a few stages of gain are used, offers a reasonable compromise between signal-to-noise and spectral sensitivity. This has recently been used in a detailed study of the Stark effect of tryptophan (Pierce and Boxer, 1995). The infra-red region has not been explored very much thus far. We have used a germanium photodiode for Stark effect measurements on weak mixed-valence transitions in the Creutz-Taube ion in the 1–2 micron range (Oh and Boxer, 1990; Oh et al., 1991) and on the 1250 nm absorption band which is characteristic of P' in *Rb. sphaeroides* RCs (Stocker et al., 1993). Recently, we have performed the first conventional Stark experiments in the 2–4 micron range on simple organic nitriles using an InSb detector (Chattopadhyay

and Boxer, 1995). Vibrational Stark effects (VSE) have only rarely been measured in condensed phases; however, this should be a rich area of investigation in the future. These measurements have recently been extended into the mid-IR using a mercury-cadmium-telluride (MCT) detector (Chattopadhyay and Boxer, to be published). A Nernst glower source is adequate for vibrational Stark measurements.

In most cases the signal from the detector is processed in a lock-in amplifier. Although any conventional lock-in works well for most cases, recently introduced digital lock-in amplifiers offer many advantages. In particular, these lock-ins have exceptional dynamic reserve so that very small signals can be reliably extracted. This is especially important for HOSS and VSE experiments. The power supply used to generate the applied field is a critical component in the set-up. A number of suppliers manufacture voltage amplifiers which can be used with a standard waveform generator to produce the desired output. In collaboration with Joe Rolfe in the Stanford Chemistry Department electronics shop we have developed several generations of power supplies which offer many practical advantages (convenient variation of amplitude and modulation frequency, DC offset, fast shut-off if current flows, etc.). HOSS experiments are especially sensitive to harmonic distortion in the power supply output as this can introduce higher-harmonic signals which are not related to the desired signals described in Eq. (3). We currently minimize this problem by using a digitally synthesized sine wave from the lockin amplifier which is then amplified. Detailed schematics and power supplies are available through the author.

III. Limitations and Conceptual Issues

A. Experimental Limitations

The sample thickness is one of the primary sources of uncertainty, as the field strength is obtained by measuring the thickness and the applied voltage (the latter can be obtained with high precision with a calibrated high voltage probe). There have been suggestions that the Stark spectrum itself is a useful method for calibrating the field. Of course this cannot be used as a primary

standard because it depends on knowing the field strength. Once the Stark effect has been measured using an independently calibrated field, then the Stark effect for a particular transition can be used as a secondary standard, so long as there are no other sources of uncertainty. We have found that small sample-to-sample variations in the linewidth (associated with different amounts of detergent, buffer, sample imperfections, etc.) can lead to variations in the magnitude of the change in absorption for a given field. Thus, it is hazardous to use the Stark effect itself as a field calibration. Another poor method is to use the sample capacitance to measure thickness. This not only depends on independent information about the dielectric properties of the sample (generally not known very accurately), but it is also a bulk measurement and can give deceptive results for samples with thickness variations. It is, however, very useful to measure the sample capacitance during an experiment as a monitor of sample integrity.

For frozen glass samples, the cell thickness can usually be accurately measured at room temperature, either by measuring interference fringes or by using solutions of known concentration. However, because the Stark effect is usually measured at low temperature, the absorption spectra of standard solutions may change, the sample may contract, reducing the thickness, and the contraction may not be homogeneous across the portion of the sample which is probed, especially if thin windows are used to construct the cell. Under the most favorable conditions, when the sample is thin and freezes homogeneously, interference fringes are observed at low temperature. Although these fringes interfere with the analysis of the absorption spectrum, they are entirely absent in the Stark spectrum which is obtained using lock-in detection at some multiple of the field modulation frequency. The sample absorption, needed for lineshape analysis, can be measured on a separate sample. It is possible to obtain very precise thickness measurements on very thin films which are prepared by spin-coating. We have used a device manufactured by Dektak which is commonly used in the semi-conductor industry for measuring the thickness of thin films. This device works by scratching the film through to the substrate and then passing a stylus across the

sample surface and into the scratch to determine the thickness. Using this device, thin films can be measured with an accuracy of about 1000 Å, and it is possible to obtain quantitative information on film flatness over large areas. Some groups have used polymer films pressed between glass plates. This is an especially unsatisfactory method in our experience because there are surface variations on the film, and it is difficult to avoid air gaps.

B. Analytical Limitations

The absorption spectrum from which the derivatives are obtained must have extremely good signal-to-noise, especially in the wings of the absorption. Because the derivatives are typically much noisier than the Stark effect data, simply using the derivatives to fit the data introduces considerable uncertainty into the analysis. A better approach is to fit the absorption to an arbitrary lineshape, e.g. a sum of Gaussians (with no physical meaning to the individual components), and then obtain the analytical derivatives of this best fit. Although an improvement, the best-fit to the absorption tends to be least accurate in the wings of the absorption. This is not a problem for fitting the absorption; however, it is a problem for fitting the Stark data as the wings of the absorption are important in the derivatives. The best approach we have developed to date is to simultaneously fit the absorption and the Stark data (Middendorf et al., 1993).

A fundamental assumption underlying the application of Eq. (2) is that the electro-optic parameters such as $\Delta\mu$ and $\Delta\alpha$ are constant across the absorption band. If the Stark spectrum can not be fit to a sum of derivatives, it may be that the electro-optical parameters vary across the inhomogeneous band width. A case of great relevance to some photosynthetic pigments (e.g. carotenoids (Gottfried et al., 1991a, b) and the dimeric special pair primary electron donor (Middendorf et al., 1993)) is when the chromophore intrinsically has a small $\Delta\mu$ but a large $\Delta\alpha$. When such a chromophore is inserted into an ordered environment, e.g. a protein matrix, then the internal matrix electric field due to the constellation of polar, charged and polarizable groups in the protein can induce a dipole moment in the chromophore.

There will always be small variations in these fields from protein to protein (inhomogeneous broadening), and thus a distribution of induced dipole moments. An interesting nonbiological example of this has been studied in detail by V.P. Wild and co-workers who examined the induced dipole moment in a centrosymmetric organic dye at different wavelengths within the inhomogeneous absorption by measuring the Stark effect on holes burned at different wavelengths at 1.5 K (Vauthey et al., 1994). They found that the value of the induced $\Delta\mu$ in a moderately polar polymer matrix varied by a factor of 3 across the absorption band. Stimulated by this work, we measured the conventional and higher order Stark effect spectra for the same dye in the same matrix (Moore, Bublitz and Boxer, unpublished results). As expected, it was not possible to fit the conventional Stark effect spectrum with any combination of derivatives of the absorption. Although such a failure is not automatically diagnostic that this is physically what is occurring, it is one possibility. The higher-order Stark spectra can provide further insight and constraint on the fitting process.

Another problem is that even a simple electronic transition is almost always accompanied by some vibronic structure, often poorly resolved. Because the intensity of these vibronic features depends on coupling with other states whose electro-optic properties may be different, it is possible that the Stark effect for these features will be intrinsically different from that of the (typically) dominant 0-0 transition. This has not been examined systematically in any detail. In one case, the carotenoid spheroidene in the B800-850 antenna complex from *Rb. sphaeroides*, a prominent vibronic progression is observed. The entire progression could be quite well fit to the second derivative of the absorption band (Gottfried et al., 1991a,b).

Multiple, overlapping electronic absorption bands, sometimes from the same molecular species, or, as is the case of RCs, from different chromophores are another difficulty. The obvious approach to this problem is to deconvolve the absorption features. Although this is a standard procedure for the analysis of complex absorption bands, the requirements for the analysis of the Stark spectrum are much more stringent because

the electrooptic properties of the bands may be quite different from each other and the wing of the Stark effect of a transition with a large dipole moment may, for example, swamp the Stark effect for a nearby absorption with a small dipole moment. One of the most insidious examples arises when there are partially overlapping absorption features which have very different Stark effects, as in photosystem II RCs. Even in the purest preparations, there is substantial overlap among all the absorption bands of different chromophores in the Q_y region. In an early attempt to analyze this spectrum, Lösche et al. (1988) concluded that P680 has a small $\Delta\mu$, comparable to or smaller than a monomeric chlorophyll. This might indicate that P680 is not dimeric. We analyzed the Stark spectrum of PSII RCs, and attempted to fit the data more accurately. Our best fit suggested that $\Delta\mu$ for P680 was at least as large or larger than for a monomeric chlorophyll (Steffen, 1994); however, because the band overlap was so great, we never felt confident enough to draw any further conclusion.

Finally, there is the possibility that the basic Liptay formalism underlying Eq. (2) is inappropriate. This may occur when the observed transition is strongly coupled to or degenerate with a dark state. General treatments have been developed by Reimers and Hush (1991) and Sacra et al. (1995), the latter for the special case of semi-conductor nanocrystals. To date, there is no evidence that a breakdown of the fundamental assumptions inherent in the Liptay formalism plays a significant role in the Stark spectra of photosynthetic pigments; however, this may well prove to be wrong, especially as the analyses become more refined.

C. Local Field Correction

The local field correction accounts for the difference between the actual field felt at the position of the chromophore whose Stark spectrum is being probed and the precisely known applied electric field. Lack of knowledge of the local field correction is a long-standing limitation in the quantitative comparison between observed Stark effects and theory. The local field correction is discussed in depth in classical texts, such as Böttcher (1973). The local field correction is not

to be confused with the matrix electrostatic fields due to the protein. The latter are always present and may be very large in an ordered medium such as a protein, or they can change in time, as in the case of the electric field due to the charges formed following light-induced electron transfer. The local field correction accounts for the difference in the field felt at the probe chromophore upon application of the external applied field. In general the local field correction is a tensor quantity. Taking the local field correction to be a scalar, $F_{\text{int}} = f \cdot F_{\text{ext}}$. The local field correction is not the dielectric constant. Various models for the local field correction, such as the Lorentz model (Böttcher, 1973), give expressions for f in terms of the dielectric constant, but the dependence is rather weak, e.g., for a spherical cavity the dependence is: $f = (\epsilon + 2)/3$. Thus, for typical values of the dielectric constant of frozen solutions, f is not much greater than unity. Irrespective of the form chosen for the cavity, the value of f is greater than 1. Thus, the internal field actually felt at the position of the chromophores is greater than the externally applied field, though not by very much for typical dielectrics at low temperature.

Expressions for the local field correction become still more complex for a chromophore inside a protein which is itself a dilute solute in a frozen glass (Lösche et al., 1988). However, because the great majority of the sample to which the external field is applied is the bulk frozen solvent and this is the same for different chromophores within a protein complex (e.g. the RC) or among different chromophore-protein complexes embedded in the same frozen solvent, differences in the local field correction for different chromophores in different local environments are likely to be small. Thus, although the absolute value of the electro-optic parameters may be systematically in error (likely by a small amount), the relative values are likely to be reliable. We have taken the approach of reporting electro-optic parameters in terms of the local field correction as a formality. Finally, the angle ζ between the change in dipole moment and the transition dipole moment used to probe the Stark effect does not depend on the local field correction factor (see Eq. 2). This angle is often of greater

interest than the precise magnitude of the change in dipole moment itself.

There are approaches to obtain quantitative information on the local field correction. For example, in the RC, the driving force for the recombination reaction $P^+Q_A^- \rightarrow PQ_A$ is quite well known. Upon application of a large external electric field, it should be possible to perform the reverse reaction, namely charge separation without photoexcitation, as the energy of the $P^+Q_A^-$ state is tuned below that of ground state PQ_A . By measuring the concentration of $P^+Q_A^-$ at equilibrium in an accurately calibrated external electric field, and comparing this with the equilibrium concentration expected given the magnitude of the $P^+Q_A^-$ dipole moment (whose magnitude does not depend to any appreciable extent on the details of the locations of the charges on either radical ion), it should be possible to obtain the actual field felt by the $P^+Q_A^-$ dipole in the RC. We attempted this experiment, and at the highest fields available were unable to detect any appreciable $P^+Q_A^-$ formation in *Rb. sphaeroides* RCs in a PVA film. Based upon what is known about these RCs in the absence of an external field, we could conclude that f in this system could be no greater than 1.3, i.e., if it had been greater, the field external would have produced a measurable concentration of $P^+Q_A^-$ (Franzen and Boxer, 1993). A possibly related effect involving charge separation between P and cytochrome has been reported in *Rps. viridis* RCs (Alegria et al., 1993). Extensions of this approach to other systems where the driving force is smaller are in progress.

IV. Examples of Recent Results for Photosynthetic Systems

As mentioned at the outset, results until late 1992 were summarized in detail in an earlier review (Boxer, 1993) and these will not be repeated. A few recent results are discussed briefly in the following.

A. The Special Pair

In the original papers describing the Stark effect for the special pair, it was obvious that $\Delta\mu$ for

its Q_y transition was larger than for a monomeric BChl, either the B bands in the RC or the pure isolated monomeric chromophores (Lockhart and Boxer, 1987; 1988; Lösche et al., 1987, 1988a,b). The quantitative analysis assumed that only the second derivative contributed to the lineshape, although it was clear that this was not rigorously true. By working at 1.5 K in a frozen glass, where the special pair absorption is narrower and some substructure is evident, it was possible to test the lineshape analysis with much greater precision (Middendorf, 1992). The Stark lineshape at 1.5 K is not well fit by the second derivative of the absorption, rather there appears to be a significant contribution from the first derivative and some zeroth derivative. The interpretation of this result, no matter how good the data and the fit, is predicated on the validity of the Liptay formalism, which may break down for this system. Assuming the validity of the Liptay formalism, we find that $\Delta\alpha$ for the special pair is huge compared to that of a monomer. The estimated magnitude of $\Delta\mu$ is little affected by this more refined analysis, though ζ increases from about 38° to about 45° . Although it is still the case that $\Delta\mu$ is several times larger for the dimer than the monomer, the really striking finding is that $\Delta\alpha$ is *much* larger for the dimer. This can be interpreted as being a natural consequence of mixing intra-dimer charge-transfer character into the lower exciton state of P (Middendorf et al., 1993). If this model is correct, then it might be expected that as a large field is applied, radically shifting the energies of the CT states, then the Stark lineshape should change. Model calculations demonstrate that this should be observed if the CT states are within about 2000 cm^{-1} of the exciton states (the precise value is model dependent). We have measured the lineshape in PVA films for fields up to nearly 3 MV/cm and find an excellent fit to the expected quadratic field dependence of the amplitude, with no change in lineshape. Thus, these data suggest that the CT states are several thousand cm^{-1} higher (or, in principle, lower) than the exciton states.

The value of $\Delta\alpha$ extracted from the lineshape analysis is even larger than for long polyenes which are known to be highly polarizable. Of course it is not possible to remove the special pair

from the RC while maintaining its structure and study its electro-optic properties in a simpler matrix, as is possible for polyenes, though we have studied the Stark spectra of a number of covalently linked special pair model systems (Middendorf, 1992). If we make the assumption that the intrinsic (gas phase) $\Delta\mu$ of the special pair is small, and the observed large $\Delta\mu$ for the special pair is induced by interaction between the matrix (protein) electrostatic field in the RC and the large $\Delta\alpha$ of the special pair, then we can estimate the matrix electric field to be at least several MV/cm. Given the absence of a dependence of the lineshape on applied field, this internal matrix field may be even larger. Furthermore, as discussed in detail in Middendorf et al. (1993), it is likely that $\Delta\alpha$ is highly anisotropic, with the dominant component along the long axis of the special pair. If the matrix electric field pointed along this direction, then a much larger value of $\Delta\mu$ would be observed. Therefore, we concluded that the matrix field is large and points roughly perpendicular to the long axis of the special pair. This is roughly along the local C_2 axis of the RC. Some recent electrostatics calculations support this suggestion (M. Gunner, D. Chandler, personal communication).

Higher-order Stark spectra have been obtained for monomeric BChl and BPheo, the special pair at 77 and 1.5 K, the heterodimer mutant ((M)H202L), and for the carotenoid spheroidene both pure in an organic glass and in the B800-850 antenna complex (Lao et al., 1995a). A key result is shown in Fig. 1 which compares the second and fourth order Stark spectra of the wild-type homodimer and mutant (M)H202L heterodimer. The absorption spectrum of the heterodimer consists of two features whose total oscillator strength is comparable to that of the homodimer (because the bands are so spread out, it appears to be weaker). In earlier work (Hammes et al., 1990), it was evident that there was a huge Stark effect for the lower energy band compared to that of the homodimer, and this is evident in Fig. 1. However, it proved difficult to analyze the Stark spectrum in terms of contributions of $\Delta\mu$ and $\Delta\alpha$ because it is difficult to obtain precise information on the absorption lineshape of the transition corresponding to the large Stark

effect. As seen in Fig. 1, the fourth order Stark effect on this lowest energy transition is likewise very strong, and it is very close to the second derivative of the 2nd-order conventional Stark spectrum. Thus, for this transition, $\Delta\mu$ dominates $\Delta\alpha$. The contrast between this result and the comparable data for the homodimer is striking. It is obvious by inspection that the fourth order Stark spectrum is not the second derivative of the 2nd order Stark spectrum for the native homodimer. This confirms the earlier conclusion that the Stark lineshape for the special pair is not dominated by $\Delta\mu$, but rather that $\Delta\alpha$ makes the dominant contribution (Middendorf et al., 1993). A general theoretical treatment of the higher order lineshape is in hand, and we are currently attempting to extract further information on the components of the polarizability tensor from this data.

B. Vibrational Stark Spectroscopy

Chromophores which absorb in the visible and near IR are not that common in most proteins; however, molecular vibrations are associated with every residue. Recently, with the advent of better FTIRs and time resolved IR methodology, there has been considerable interest in assigning some of the vibrational features of RCs (as well as many other proteins) and using changes in the vibrational frequencies or intensities to monitor the response of the protein to electron transfer events (e.g. Maiti et al., 1993). In order to evaluate these changes quantitatively, it would be useful to have an independent experimental calibration of the sensitivity of the vibrational frequencies to electric fields. Towards this end, we have performed the first measurements of the VSE for anisonitrile (chosen largely because it is a strong transition in a convenient spectral region) (Chattopadhyay and Boxer, 1995). The VSE spectrum is shown in Fig. 2. From this we obtain $|\Delta\mu| = 0.05 \text{ D/f}$, and $\zeta = 0^\circ$, i.e., $\Delta\mu$ is collinear with the transition moment, as expected. If an electric field were aligned with this bond direction, the Stark tuning rate is: $(0.8 \times 10^{-6} \text{ cm}^{-1}/(\text{V/cm}))$. Extensions to a wider variety of vibrational transitions are in progress. These data provide fundamental information on vibrational anharmonicity, as well as calibrating the sensitivity of vibrational spectra to local electric fields.

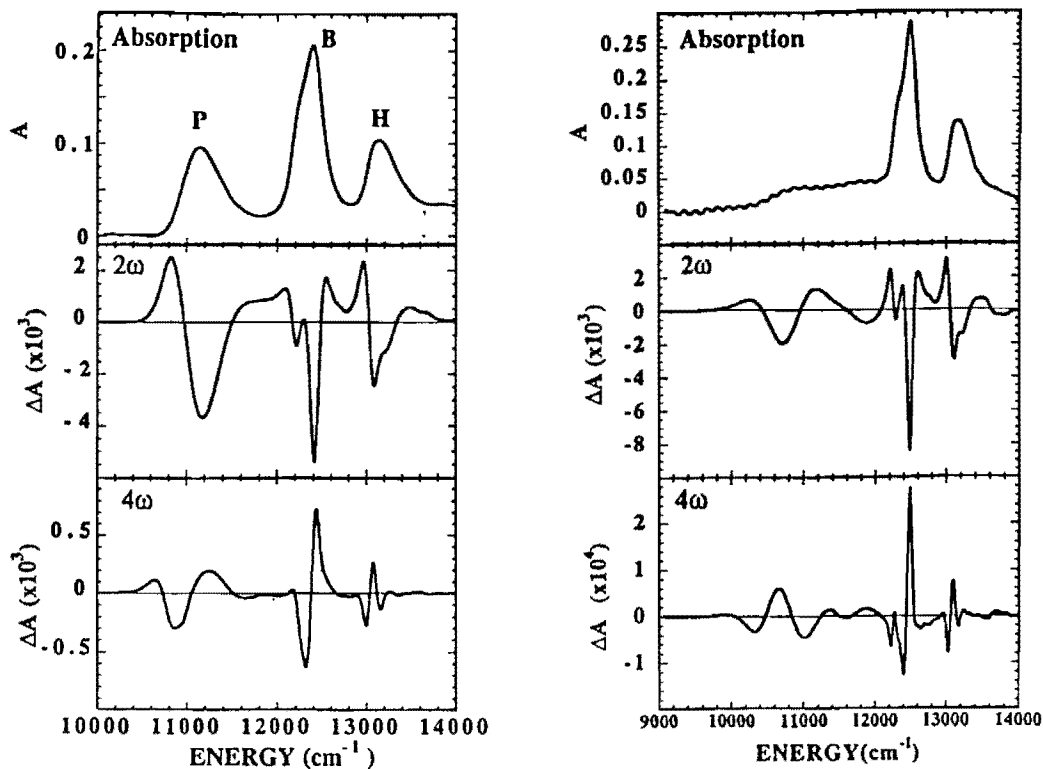


Fig. 1. Absorption (upper panel), conventional second order Stark (2ω , middle panel), and fourth order Stark (4ω , lower panel) spectra of homodimeric wild-type (left) and heterodimeric ((M)H202L) mutant (right) reaction centers from *Rb. sphaeroides*. (Lao et al., 1995). 77K in a glycerol/buffer glass; $|F| = 1.07$ MV/cm for the homodimer and 0.89 MV/cm for the heterodimer. Note the interference fringes in the heterodimer absorption spectrum which provide very accurate calibration of the sample thickness; these fringes are absent in the Stark spectrum.

C.- Unidirectional Electron Transfer in the RC

One useful application of the electronic Stark effect is to provide a calibration for electrochromic shifts due to the transient internal electric field produced by charge separation (Steffen et al., 1994). In particular, when charge is separated between P and Q_A , the resulting electric field is felt by the monomeric bacteriochlorophyll and bacteriopheophytin monomers (referred to as B and H, respectively). In order to calibrate these shifts, information on the sensitivity of monomeric pigments to electric fields is first obtained from conventional Stark spectroscopy. The B and H bands consist of overlapping absorptions from the monomeric BChls and BPheos, respectively, on the functional L and non-functional M sides. This limits the accuracy of the determination of

both the magnitude and direction of $\Delta\mu$ for these chromophores; however, from an analysis of the Stark spectrum the magnitude of the $\Delta\mu$ s are roughly the same on the functional and non-functional sides. The direction of $\Delta\mu$ is more problematic because the measurement of the angle dependence is compromised when bands overlap. We have no reason to believe that the direction of $\Delta\mu$ for the monomeric chromophores is appreciably different within the RC than when the pigments are extracted from the RC and studied in a simple organic solvent. Unfortunately, the angle ζ defines two projections of $\Delta\mu$ on the transition moment direction, i.e. two antiparallel cones. Upon formation of $P^+Q_A^-$ the B bands are observed to shift to higher energy and the H bands shift to lower energy. At a qualitative level this result immediately restricts the absolute direction of $\Delta\mu$ on each chromophore type to one of the

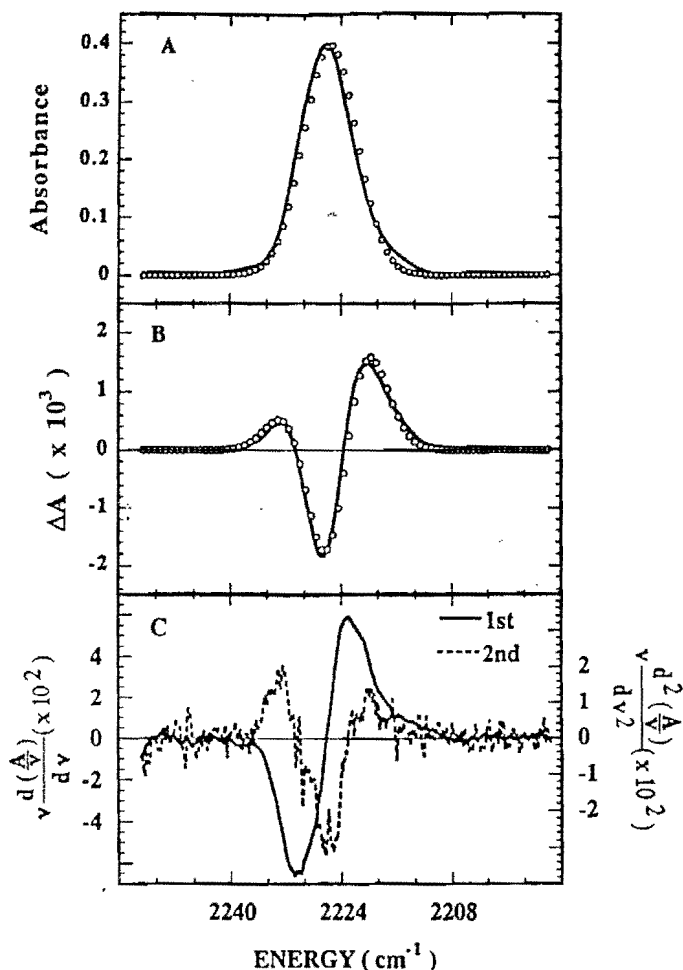


Figure 2. Vibrational Stark effect spectrum of anisonitrile in the region of the CN stretch (Chattopadhyay and Boxer, 1995). Top panel (A): IR absorption spectrum of anisonitrile in toluene at 77 K in the CN stretching region (solid line); middle panel (B): vibrational Stark effect spectrum for a field strength of 1.0 MV/cm, $\chi = 90^\circ$ (solid line); lower panel (C): decomposition of the VSE spectrum into first (solid line) and second (dashed line) derivatives of the absorption spectrum. The open circles on the absorption and VSE spectra are the simultaneous best fits using a single Gaussian for the absorption in the top panel and the derivative contributions in the lower panel, respectively.

two cones. Thus, both the magnitude and absolute direction of $\Delta\mu$ for all four spectator chromophores are known reasonably well.

The electrochromic bandshifts can be obtained by subtracting an absorption spectrum of a sample in the $P^+Q_A^-$ state from one in the ground state, normalized for the amount of bleach of P at its absorption maximum. The data at 1.5 K are shown in Fig. 3. Here too the overlap of the absorption bands causes some uncertainty; how-

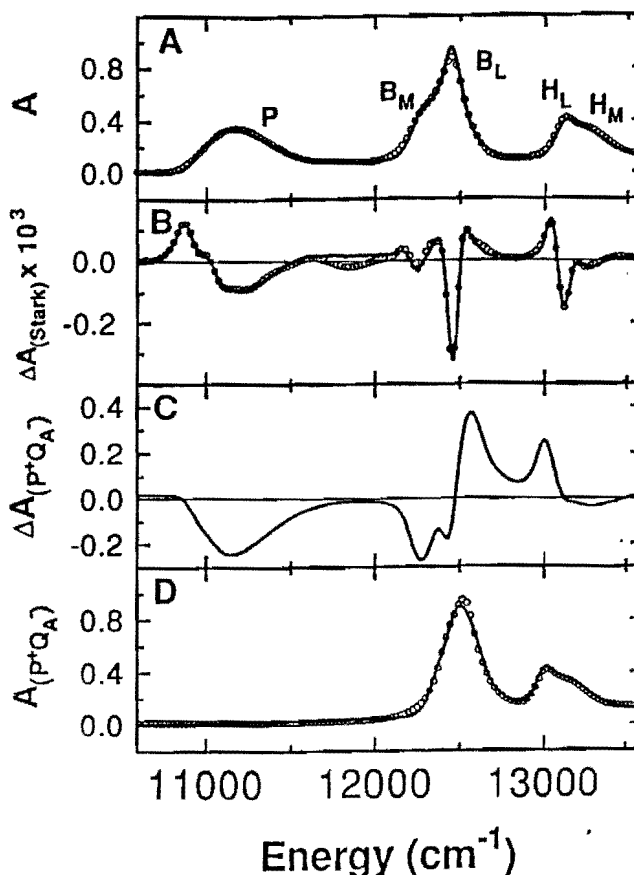


Fig. 3. (A) Absorption, (B) Stark effect, (C) $P^+Q_A^-$ -minus-P difference absorption, and (D) $P^+Q_A^-$ absorption spectrum in the Q_y region of wild-type *Rb. sphaeroides* RCs in a 50 percent (v/v) glycerol/buffer glass at 1.5 K (Steffen et al., 1994). The solid lines are the data and the circles indicate the fits to the data.

ever, it is evident by inspection that H_L and H_M shift by approximately the same amount, shown expanded in Fig. 4. Because the Stark effect spectrum in an external field demonstrates that their absorption bands are comparably sensitive to an electric field, this result suggests that the field at the H_L and H_M sites due to the transient *internal* $P^+Q_A^-$ electric field is about the same. However, it is obvious by inspection of the X-ray structure that the internal field due to $P^+Q_A^-$ should be substantially larger at H_L than at H_M because H_L is much closer to the charges on P^+ and Q_A^- than is H_M . Thus, we are forced to conclude that the dielectric screening on the L-side is considerably greater than on the M side. A quantitative analysis suggests that it is approximately three times greater on the functional side (Steffen et

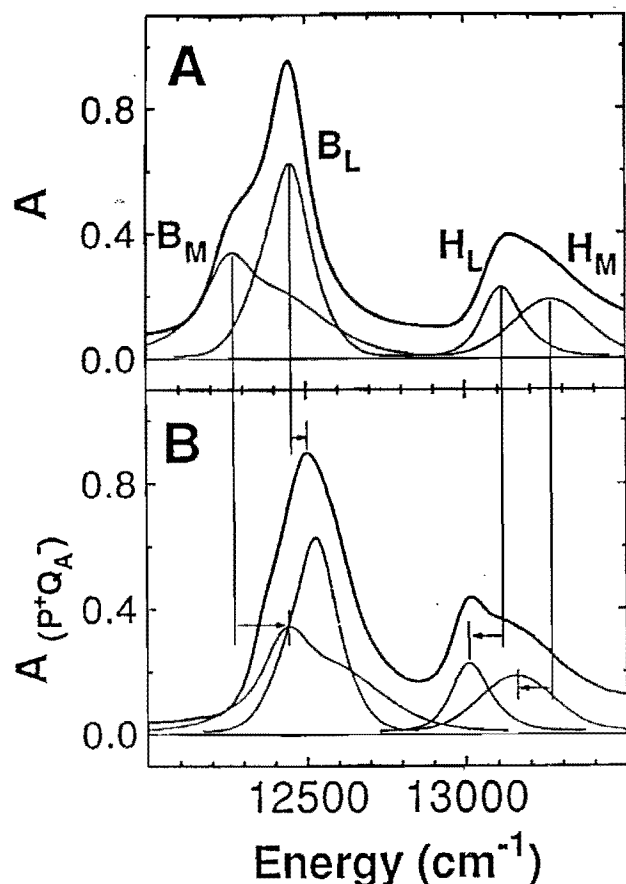


Fig. 4. (A) Ground state and (B) $P^+Q_A^-$ absorption spectra in the monomer Q_y region of wild-type *Rb. sphaeroides* RCs in a 50 percent (v/v) glycerol/buffer glass at 1.5 K showing the electrochromic bandshifts that occur when $P^+Q_A^-$ is formed (Steffen et al., 1994).

al., 1994). This dielectric asymmetry, probed functionally, may be a significant contributor to unidirectional electron transfer in the RC. Its molecular origin is not known; however, it is likely the result of long-range interactions from many amino acids. A similar analysis of the B bands gives nearly identical results for the dielectric asymmetry, even though some might question whether the B-band shifts upon $P^+Q_A^-$ are entirely electrochromic in origin (there is no evidence for appreciable exciton interactions between the P or B chromophores and the H chromophores). Our view is that the exciton interaction between P and B is considerably smaller than the shifts which occur upon $P^+Q_A^-$ formation. Furthermore, although the upper exciton band of P contributes to the absorption in the B-

band region of the spectrum, we believe that its intensity is quite small and the band is likely broad. These ideas are supported by more detailed analyses of the absorption spectra of modified RCs, including the Stark spectra (Moore and Boxer, to be published).

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