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Comment

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Comment on "Transient Conformational Changes of Sensory Rhodopsin II Investigated by Vibrational Stark Effect Probes"

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I am writing to draw attention to a serious flaw in the method used to evaluate the data in "Transient Conformational Changes of Sensory Rhodopsin II Investigated by Vibrational Stark Effect Probes".[1]

This paper describes the response of thiocyanate vibrational probes to *changes* in the protein electric field associated with the photocycle of sensory rhodopsin II, also known as vibrational Stark effects or Stark shifts. This concept is valid and is similar to work from many laboratories using these and related probes in response to mutations, pH changes, protein folding, etc. It is closest in concept to work from our lab in which a ligand bound in the active site of an enzyme was photoexcited to mimic the change in charge distribution associated with the enzyme's catalytic mechanism.[2] This was probed by visible pump-IR probe spectroscopy; a similar approach has also been reported[3] (ref 43 in ref 1). As far as I can tell, the experimental light-minus-dark difference spectra and their kinetics in Mohrmann et al. are carefully done.

The framework for understanding the observed spectral shifts is the vibrational Stark effect. As reviewed in ref 4, the correct way to interpret the observed vibrational frequency shift, $\Delta\bar{\nu}_{obs}$ (in cm^{-1}), in response to changes in the electric field the probe experiences from a protein (or any other organized environment), $\Delta\vec{F}_{protein}$ (in MV/cm), is through its vibrational difference dipole, $\Delta\vec{\mu}_{probe}$ as

$$hc\Delta\bar{\nu}_{obs} = -\Delta\vec{\mu}_{probe}\cdot\Delta\vec{F}_{protein} \quad \text{(A)}$$

where h is Planck's constant and c is the speed of light. $\Delta\vec{\mu}_{probe}$ is also known as the linear Stark tuning rate [units of cm^{-1}/(MV/cm)]. The magnitude of $|\Delta\vec{\mu}_{probe}|$ is obtained in a *separate* calibration experiment in which a known external electric field, $\vec{F}_{ext}$, is applied to an *isotropic immobilized* sample. The methods for evaluating $|\Delta\vec{\mu}_{probe}|$ from these vibrational (or electronic) Stark spectroscopy measurements, a calibration step, have been described in great detail.[5,6] The direction of $\Delta\vec{\mu}_{probe}$ for a high-frequency vibrational probe like the nitriles in this paper is parallel to the bond axis; thus, it can be estimated from X-ray crystallography if the nitrile is well-ordered. From eq A it is evident that spectral shifts accompanying a perturbation can be either to higher or lower energy, or if $\Delta\vec{F}_{protein}$ happens to be perpendicular to $\Delta\vec{\mu}_{probe}$, then no shift is expected. If the magnitude of the frequency shift is greater than the line width, then the difference spectrum will contain a bleach at the initial state wavenumber and a positive feature at the shifted wavenumber.[2,3] If the magnitude of the frequency shift is comparable to or less than the line width, then the difference spectrum will have a first-derivative-type line shape, either positive or negative depending on the sign of the dot product in eq A. The idea of the analysis is to obtain information on the projection of $\Delta\vec{F}_{protein}$ on $\Delta\vec{\mu}_{probe}$ (not actually evaluated in Mohrmann et al. although implied from the title) and its time dependence.

In contrast to correct eq A, Mohrmann et al. used an incorrect equation (eq 1 in ref 1) related to the form that describes the effect of an *external* electric field, $\vec{F}_{ext}$, on an *isotropic immobilized* sample. As described in detail in refs 5 and 6, complex lineshapes occur in field-on minus field-off difference spectra for an isotropic immobilized sample in an *external* electric field because the probe has no fixed orientation with respect to the external field; an analysis of these lineshapes gives $|\Delta\vec{\mu}_{probe}|$, the change in polarizability (typically small for vibrational transitions), and other electro-optic parameters. This is *not* the case for the internal field changes when a protein is perturbed, which tend to have well-defined orientations with respect to a probe and therefore give rise to spectral shifts as described by eq A. Of course, if the probe becomes more disordered, then both shifts to higher and lower energy are possible leading to more complex shapes in the difference spectrum (e.g., differences in line widths, populations, or environment), and this may have been what misled Mohrmann et al. Mohrmann et al. further compound the error by replacing $\vec{F}_{ext}$ in their equation with something they call F_{int}, meant to describe the field in the protein, but which has no place in this equation as it is meant to be synonymous with $\Delta\vec{F}_{protein}$, as in eq A. This is incorrect both because the observed effects are due to a *difference* in field (light-minus-dark, not an absolute field) and because the equation used by these authors has nothing to do with the projection of $\Delta\vec{F}_{protein}$ on the probe's difference dipole in the protein which gives rise to observed frequency shifts. An entire section of Supporting Information is dedicated to incorrect eq 1 where $\vec{F}_{ext}$ is replaced by F_{int}. Thus, the analysis using the equation in Mohrmann et al. is incorrect, and needs to be redone using the correct physics. Note that the time dependence of intensity changes (kinetics), even if analyzed using this incorrect formalism, presumably does reflect the underlying kinetics.

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Notes

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