Vibrational Stark Spectroscopy in Proteins: A Probe and Calibration for Electrostatic Fields

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We report the first measurement of the vibrational Stark effect in a protein, providing quantitative information on the sensitivity of a vibrational transition to an applied electric field. This can be used to interpret changes in the vibrational frequency that are often observed when amino acids are changed or when a protein undergoes a structural change in terms of the change in the internal or matrix electric field associated with the perturbation. The vibrational Stark effect has been measured for the vibration of CO bound to the heme iron in myoglobin. The vibrational Stark effect is surprisingly large, giving a Stark tuning rate of $(2.4/f)$ cm^{-1}/(MV/cm), where f is the local field correction; this is nearly 4 times larger than for free CO. It is also found that the change in dipole moment is parallel to the transition moment; that is, the change in dipole moment is in the direction perpendicular to the heme plane. Vibrational Stark effect data are also reported as a function of pH, for various mutants, for a modified picket fence porphyrin, and for cytochrome *c*. The Stark tuning rate is found to be very similar in all cases, indicating that the CO stretch frequency for CO bound to the heme iron is a sensitive and anisotropic local detector of changes in the electrostatic field. This information is used to evaluate electrostatics calculations for heme proteins.

Electrostatic interactions are central to understanding the properties of molecules in the condensed phase and are especially important in complex organized systems such as proteins. A large body of theoretical work is directed at understanding the role played by electrostatics in folding, assembly, and catalysis.[1−8] Electrostatic interactions can be probed by measuring pK_a shifts for titratable residues,[9−11] shifts in redox potential,[12,13] NMR chemical shifts,[14] and electrochromic band shifts (sometimes called internal Stark shifts).[15,16] Electrochromic band shifts in a protein result from the interaction between a probe chromophore and the electric field due to the surrounding organized environment of the protein matrix and associated prosthetic groups and solvent. This field is collectively called the matrix electric field F_{matrix}, and the observed electrochromic band shift is $\Delta E = hc\Delta\bar{\nu} = -\Delta\mu\cdot F_{\text{matrix}}$, where $\Delta\mu$ is the change in dipole moment associated with a spectroscopic transition.

To interpret or calculate the electrochromic band shift in terms of the matrix electric field due to the protein or any other ordered environment, it is necessary to know $\Delta\mu$ as this gives the intrinsic sensitivity of the transition to an electric field.[17] The magnitude and direction of $\Delta\mu$ can be obtained by Stark spectroscopy which quantifies the effect of an *externally* applied electric field, F_{ext}, on the transition. Stark spectroscopy of electronic transitions in proteins is now a standard method;[18,19] however, this technique is rarely applied to molecular vibrations.[20,21] In the present communication we report the first measurement of the Stark effect for a vibrational transition in a protein. Since molecular vibrations are ubiquitous, this may prove to be a generally useful method for mapping electric fields in proteins.

The particular system we have investigated is the carbonyl stretch of CO bound to the heme iron in deoxymyoglobin MbCO, illustrated schematically in Figure 1. This system was chosen for several reasons. First, the CO stretch is found in a region of the spectrum that is not too congested and the oscillator strength is large. Second, there has been a large body of work on this particular transition due to widespread interest in MbCO as a model for conformational substates in proteins and as a probe for ligand-binding dynamics and discrimination.[22,23] Third, a large collection of single and multiple amino acid mutants has been prepared, and FTIR spectra have been collected and analyzed in terms of a variety of factors.[22] For example, one class of mutations involving a residue near the heme iron, valine 68 (ValE11) has been changed to potentially charged amino acids specifically to probe electrostatic interactions. The effects of these substitutions on the Fe(II)/Fe(III) redox potential[13] and the pK_a for buried aspartic or glutamatic acid[24] have been measured, along with changes in $\bar{\nu}_{\text{CO}}$ in the infrared.[25,26] It has been argued that the substantial variations observed for $\bar{\nu}_{\text{CO}}$ in

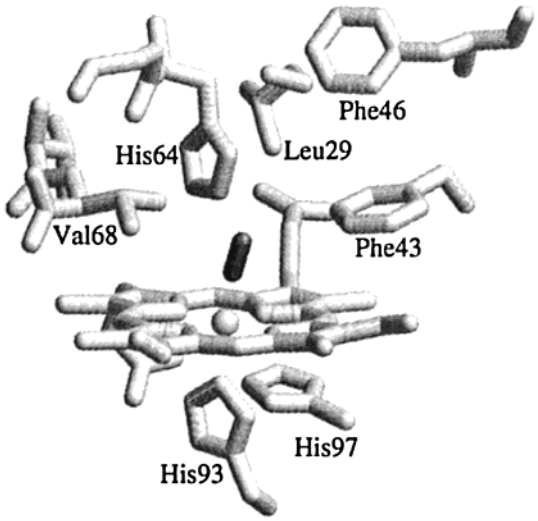

Figure 1. Schematic diagram of the heme pocket in MbCO derived from the crystallographic coordinates.[36]

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a range of mutations to residues in the vicinity of bound CO on the distal side of the heme reflect differences in electric fields,[22,26] and similar arguments have been made for CO bound to the heme iron in cytochrome *c*.[27] Because there are no data on the magnitude and direction of $\Delta\mu$ for the CO vibration at the active sites of these proteins, the analysis of the variation in $\bar{\nu}_{CO}$ has focused on correlations, not on a direct quantitative analysis.

The vibrational Stark effect (VSE) spectrum is obtained by applying a large static electric field to a frozen glass containing the sample of interest.[21] As described in detail elsewhere,[28] the change in absorption, ΔA, due to the application of the electric field, was obtained by subtracting the absorption with the field off from that with the field on using an FTIR spectrometer. For an immobilized, isotropic molecule, the Stark spectrum can be described as the sum of derivatives of the absorption spectrum:[19]

$$\Delta A(\bar{\nu}) = (F_{ext}f)^2\left\{A_\chi A(\bar{\nu}) + \frac{B_\chi}{15hc}\bar{\nu}\frac{d}{d\bar{\nu}}\left(\frac{A(\bar{\nu})}{\bar{\nu}}\right) + \frac{C_\chi}{30h^2c^2}\bar{\nu}\frac{d^2}{d\bar{\nu}^2}\left(\frac{A(\bar{\nu})}{\bar{\nu}}\right)\right\} \quad (1)$$

where F_{ext} is the external applied field, f is the local field correction,[29] h is Planck's constant, and c is the speed of light. The coefficients A_χ, B_χ, and C_χ are associated with molecular properties of the system. A_χ is related to the change in the transition moment upon application of an applied field; B_χ is associated with the change in polarizability $\Delta\alpha$ between the ground and excited states and the transition polarizability; and C_χ, the coefficient of the second derivative contribution to the Stark line shape, is given by

$$C_\chi = |\Delta\mu|^2[5 + (3\cos^2\chi - 1)(3\cos^2\zeta - 1)] \quad (2)$$

C_χ depends only on $\Delta\mu$ and ζ, the angle between $\Delta\mu$ and the transition moment direction. ζ is determined from the ratio of the Stark spectra taken at different experimental angles χ between the direction of light polarization and the applied field direction. The coefficients in eq 1 are obtained by fitting the numerical derivatives of the absorption spectrum to the Stark spectrum.[19]

The absorption and Stark spectra of CO in wild-type MbCO at pH 5.3 and 8.0 are shown in Figure 2.[30] The absorption spectra, shown in the top parts, consist of a single band at pH 8 and two bands at pH 5.3. At pH 5.3, the lower energy band at 1945 cm^{-1} (sometimes denoted A_1) has the same $\bar{\nu}_{CO}$ as the single band at pH 8; the second band is at 1965 cm^{-1} (sometimes denoted A_0). The VSE spectra of these species are shown in the middle parts of Figure 2; it is evident that the Stark spectra closely resemble the second derivative of the absorption. This is confirmed quantitatively by a decomposition of the Stark spectra into a sum of contributions from the first and second derivatives of the absorption shown in the lower parts of Figure 2. The fits to the absorption and Stark spectra are overlaid on the data in the upper and middle parts, respectively.[31] Figure 3 shows the dependence of C_χ on $(3\cos^2\chi - 1)$ for the single peak at pH 8 (eq 1); a linear fit of the data gives $\zeta = 0°$ with an estimated error of 7°. Using this value, $|\Delta\mu|$ and $|\Delta\alpha|$ were calculated using eqs 1 and 2, and the results are summarized in Table 1.

Figure 4 shows the absorption and Stark spectra for two mutants at position 68 (V68D and V68N) that lead to large changes in the redox potential of the heme iron[13] and $\bar{\nu}_{CO}$.[25,26] Both spectra involve multiple peaks. The Stark spectra line shapes of these mutants are also dominated by the second derivative of the absorption spectrum. The values of $|\Delta\mu|$ and $|\Delta\alpha|$, obtained from the quantitative analysis, are very similar to those for wild-type and are given in Table 1. We have also investigated three other systems for comparison with the data obtained for MbCO variants (data not shown; see Table 1). The VSE spectrum of free CO in frozen 2-methyl-tetrahydrofuran ($\bar{\nu}_{CO} = 2131$ cm^{-1}) is much weaker than for heme-bound CO giving $|\Delta\mu| = 0.04$ D/*f*. The VSE spectra of CO bound to a modified picket fence porphyrin[32] in frozen 2-methyl-tetrahydrofuran ($\bar{\nu}_{CO} = 1973$ cm^{-1}) and CO bound to the heme iron in cytochrome *c*[33] ($\bar{\nu}_{CO} = 1963$ cm^{-1}) are very similar to those of Mb variants (Table 1).

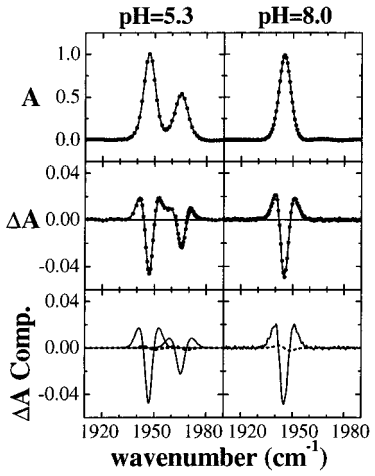


Figure 2. Absorption spectra (top parts, dotted curve), vibrational Stark spectra (middle parts, dotted curve), and (bottom parts) decomposition of the Stark spectrum into contributions from the second derivative (solid lines) and first derivative (dashed line) of the absorption for MbCO at pH 5.3 and 8.0 at 74 K in 50/50 glycerol/buffer. The solid lines in the absorption and Stark parts are the best fits using eq 1 from which $|\Delta\mu|$ and $|\Delta\alpha|$ are obtained (see text). The Stark spectra are scaled to 1 MV/cm applied electric field[48] and are normalized for unit absorbance at the absorption maximum to facilitate comparison. The actual absorbance at the peaks are approximately 0.05 and 0.1 for MbCO at pH 5.3 and 8.0, respectively.

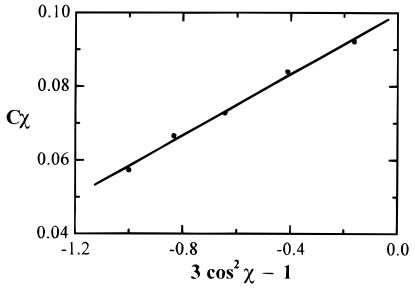


Figure 3. The second derivative contribution C_χ to the Stark spectrum vs $3\cos^2\chi - 1$, where χ is the experimental angle between the applied field direction and the electric vector of the polarized light used to probe the VSE. The solid line is the linear fit using eq 2 giving $\zeta = 0°$ and $|\Delta\mu| = 0.14$ D/*f*. The data are for MbCO at pH 8 (right parts, Figure 2).

We focus on three important results. First, the spectra in Figures 2 and 4 demonstrate that very high signal-to-noise VSE data can be obtained in a protein. Second, $|\Delta\mu|$ is very large when CO is bound to the heme iron as compared with free CO, and $\Delta\mu$ is parallel to the transition moment. Because the transition dipole moment is nearly perpendicular to the heme plane[34] and parallel to the CO bond axis,[34−36] $\Delta\mu$ for CO bound to the heme is a sensitive and anisotropic detector of F_{matrix}. Third, $|\Delta\mu|$ for wild-type MbCO is approximately the same at

TABLE 1: Vibrational Frequencies, $|\Delta\mu|$, and $|\Delta\alpha|$ for MbCO Variants, CO Bound to cytochrome *c*, a Heme Model Compound, and Free CO[a]

	$\bar{\nu}_{CO}$ (cm^{-1})	$\|\Delta\mu\|\cdot f$ (Debye)[b]	$\|\Delta\alpha\|\cdot f^2$ ($Å^3$)[c]
Wild-type Mb pH = 5.3	1945	0.136 ± 0.003	3.0
	1965	0.146 ± 0.004	4.7
Wild-type Mb pH = 8.0	1945	0.140 ± 0.002	2.5
V68D[d,e]	1973	0.133 ± 0.006	0.5
V68N[d]	1911	0.145 ± 0.008	3.9
	1925	0.156 ± 0.005	4.3
modified picket fence porphyrin[f]	1973	0.133 ± 0.004	0.2
free CO[f]	2131	0.04	
cytochrome *c*[d]	1963	0.156 ± 0.002	0.9

[a] All spectra were taken at 74 K. [b] Errors represent one standard deviation and are obtained from repeated experiments. [c] The first-derivative contributions to the Stark effects are all very small (see Figures 2 and 4), therefore the values of $|\Delta\alpha|$ are small, and we expect that the errors are quite large. [d] pH 7.0. [e] A deconvolution was not attempted. The whole band was treated as a single peak in the Stark analysis. [f] Solvent: frozen 2-methyl-tetrahydrofuran.

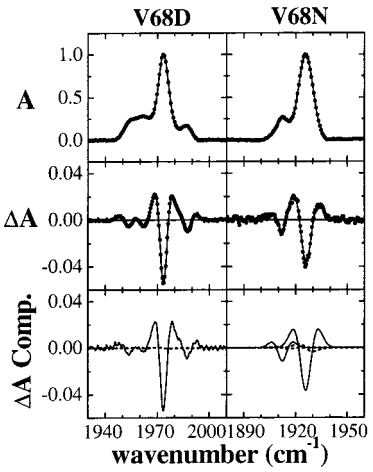
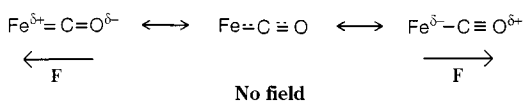


Figure 4. Absorption spectra (top parts, dotted curve), vibrational Stark spectra (middle parts, dotted curve) and (bottom parts) decomposition of the Stark spectrum into contributions from the second derivative (solid lines) and first derivative (dashed line) of the absorption for V68D and V68N MbCO at pH 7.0 at 74 K in 50/50 glycerol/buffer. The solid lines in the absorption and Stark parts are the best fits using eq 1 from which $|\Delta\mu|$ and $|\Delta\alpha|$ are obtained.

low and high pH, as well as for all of the mutants we have examined thus far, for the modified picket fence porphyrin, and for a different heme protein, cytochrome *c*, despite the fact that $\bar{\nu}_{CO}$ varies over a wide range (~60 cm^{-1}). Taking the average value, CO when bound to a heme iron has a Stark tuning rate of (2.4/*f*) cm^{-1}/(MV/cm); that is, for a matrix electric field parallel to the CO bond direction, $\bar{\nu}_{CO}$ will experience a electrochromic band shift $\Delta E = hc\Delta\bar{\nu}_{CO}$ of (2.4/*f*) cm^{-1} for a matrix electric field of 1 MV/cm.

The VSE can be observed only when the oscillator is anharmonic and/or the force constant of the oscillator depends on the applied electric field.[20,37] When CO is bonded to the heme iron, the bonding character can be changed more easily than in free CO by having different charge-separated resonance structures stabilized depending on the orientation of Fe−C−O fragment in the electric field:



It is likely that this mechanism contributes to the enhanced value of $|\Delta\mu|$ when CO is bound to the heme iron, but the Stark effect cannot distinguish this mechanism from enhanced bond anharmonicity.[38] The close similarity of $|\Delta\mu|$ for CO bound to the heme iron in Mb as a function of pH, in mutants, and in modified picket fence porphyrin despite the large variation in $\bar{\nu}_{CO}$ shows that the intrinsic sensitivities of the vibrational frequencies to an electric field are essentially the same. Therefore, the observed variations in $\bar{\nu}_{CO}$ at different pH's or in different mutants can be interpreted as electrochromic band shifts with the CO probe oscillator sensing variations in the electric field. It is also expected that the variation in $\bar{\nu}_{CO}$ should be much larger when CO is bound to the heme iron than for an equivalent environmental perturbation when it is photolyzed off and trapped in the protein matrix, as $|\Delta\mu|$ is approximately 3−4 times larger when the CO is bound; this is observed.[39]

With the direction and magnitude of $\Delta\mu$ in hand, it should be possible to analyze and interpret variations in $\bar{\nu}_{CO}$ for different mutants in terms of F_{matrix}, and make quantitative comparisons with electrostatics calculations. To do this, it is necessary to have some estimate for the magnitude and likely variation of the local field correction factor *f*.[29] This is a vexing issue which enters into many comparisons between theory and experiment. The measured value of $|\Delta\mu|$ for free CO in frozen 2-MeTHF is quite close to what is calculated[37] and measured for CO adsorbed on a nickel surface[20] if we set $f \approx 1$. Because the Stark effect for free CO is very small, the experimental uncertainty in $|\Delta\mu|$ is relatively large. The measured value of $|\Delta\mu|$ for CO bound to the picket fence porphyrin in the same solvent used to measure $|\Delta\mu|$ for free CO is approximately 3.5 times greater than that for free CO. Significantly, this value is, within the experimental error, the same as that measured for all the Mb variants and CO bound to the heme iron in cytochrome *c*. Finally, the local electrostatic perturbations in V68D and V68N compared with wild-type, or in wild-type at low pH are very large, as evidenced by the large variations in $\bar{\nu}_{CO}$; nonetheless, the measured value of $|\Delta\mu|$ is a constant within the experimental error. There are various models for estimating *f*;[29] however, it is not obvious how to apply them to large molecules, let alone proteins.[40] On the basis of the collection of data presented here, it seems reasonable to argue that the variation in *f* is likely to be quite small. Furthermore, a consistent argument can be made that the value is not very different from $f \approx 1$, building on the free CO data as outlined above, but this remains uncertain. Because high-level calculations are possible for simple vibrations, the vibrational Stark effect may turn out to be useful for a more quantitative analysis of *f*.

Laberge et al. have presented detailed calculations for CO bound to cytochrome *c* and analyzed the variations in $\bar{\nu}_{CO}$ in terms of electrochromic bandshifts.[27] These authors used a Stark tuning rate of 0.49 cm^{-1}/(MV/cm) based on the result of a Stark experiment for CO adsorbed at a known orientation on a nickel surface.[20] According to our results, the Stark tuning rate for CO bound to cytochrome *c* is substantially larger, (2.6/f) cm^{-1}/(MV/cm). As argued above, it seems very unlikely that the local field correction is about 5, thus we suggest that there may be a systematic error in the calculated field in ref 27.

Phillips and co-workers have recently calculated electrostatic potentials for a large number of Mb variants and correlated $\bar{\nu}_{CO}$ with the difference in electrostatic potentials between the O and C atoms.[41] These authors did not use the language of electrochromic band shifts developed here. However, on the basis of the data presented here, we would argue that this is the correct physical picture, and we can see the underlying reason such a

correlation exists: at least for the cases we have studied thus far; $\Delta\mu$ is a constant, and the observed variations in $hc\Delta\bar{\nu}_{CO} = -\Delta\mu \cdot F_{matrix}$ reflect variations in F_{matrix}. The slope of the correlation between the calculated potential (interpreted here as F_{matrix}) and $\bar{\nu}_{CO}$ should give the Stark tuning rate. Taking results from ref 41, we obtain a Stark tuning rate of approximately 1.5 cm^{-1}/(MV/cm), though the scatter in the points is fairly large. This value is smaller than the value of (2.4/*f*) cm^{-1}/(MV/cm) based on the VSE data, possibly due to $f > 1$,[29] and possibly because there is insufficient data to obtain a reliable correlation. These comparisons suggest on one hand that there may be sources of systematic error in the estimates of the electrostatic potential using current programs to calculate electrostatics and, at the same time, the value of direct measurements of $\Delta\mu$ to provide an experimental basis for refining such calculations.

Finally, we can obtain information on the absolute direction of $\Delta\mu$ from the sign of the slope of the correlation in ref 41 where it is shown that $\bar{\nu}_{CO}$ decreases as the electrostatic potential at the oxygen atom increases with respect to that at the carbon atom. Since $hc\Delta\bar{\nu}_{CO} = -\Delta\mu \cdot F_{matrix}$, $\Delta\mu$ should point from the oxygen atom toward the carbon atom.[42] Taking this information, we can then estimate the magnitude and direction of the change in F_{matrix} associated with protonation of the distal histidine, His64 (see Figure 1) by using the value of the magnitude of $\Delta\mu$ from our experiments and the absolute direction of $\Delta\mu$ from the slope of the correlation. The origin of the new band appearing at higher frequency in wild-type Mb at low pH has been discussed extensively in the literature.[43,44] It is generally agreed that the band at 1965 cm^{-1} is associated with a population of the sample in which His64 is protonated and swings out of the heme pocket.[45] If we associate the change in $\bar{\nu}_{CO}$ of +20 cm^{-1} with the electric field due to this protonation, i.e., treat it as an electrochromic band shift, then the change in F_{matrix} along the C−O axis is 8*f* MV/cm pointing from the carbon atom toward the oxygen atom. Site specific mutations of residues in the distal pocket that lead to spectral shifts, e.g., those shown for V68D and V68N in Figure 4, can also be treated this way.

It is the purpose of this paper to present the experimental information and strategy needed to relate observed band shifts to F_{matrix}. We hope that investigators who are doing electrostatics calculations can use this information to calibrate and refine their estimates. The example of the VSE spectrum of CO in MbCO presented here is a first example of the use of the VSE to calibrate the sensitivity of molecular vibrations to the electric field in complex systems such as proteins. Because this is a particularly sensitive transition, it is likely to be one of the easiest to measure.[46] However, this concept can be extended to any vibrational transition in a protein, such as amide bands which can be labeled with isotopes or probes that could be inserted. Many investigators are measuring time-dependent band shifts in the infrared,[47] and these can also be interpreted as resulting from changes in the electrostatic field associated with functionally important processes.

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(29) The actual field felt by the chromophore in response to the externally applied field is usually called the internal field $F_{int} = f \cdot F_{ext}$. F_{int} is not to be confused with the electric field due to the surrounding protein F_{matrix}. The local field correction *f* is in general a tensor quantity; it is treated here as a scalar and the value of *f* should be somewhat greater than 1 (see text). Due to uncertainty in the value of *f* we report the observed values of $|\Delta\mu|$ divided by *f*. For example, see: Böttcher, C. J. F. *Theory of Electric Polarization*; Elsevier: Amsterdam, 1973; Vol. 1.
(30) Wild-type human Mb and the V68D and V68N variants were expressed and purified from E. coli as described elsewhere.[24] All proteins also contain the surface mutation C110A to improve the yield upon purification; this mutation does not significantly perturb the structural and ligand binding properties. MbCO samples are prepared as published in detail elsewhere.[26] The samples contained ~3 mM Mb in 50/50 glycerol/buffer (100 mM phosphate buffer for pH 7.0 and 8.0; 100 mM citrate buffer for pH = 5.3). The samples were loaded into a homemade IR cell made from a pair of Ni coated (~50 Å thick Ni) sapphire windows separated by ~30 μm Teflon spacers. The cell containing the samples was immersed in a custom-built liquid N_2 immersion cryostat that operates at approximately 74 K. Absorption and Stark spectra were obtained on a Bruker IFS 66V/S FTIR spectrometer (0.5 cm^{-1} resolution) using an InSb detector. A horizontally transmitting IR polarizer was inserted between the detector and the cryostat, and the sample cell was rotated about the vertical axis for the polarization measurements. A single scan was obtained with the field off, then a scan with the field on (typically about +2.5 kV), then another scan with the field off, and finally a scan with the same magnitude of the field on but in the opposite direction. This cycle was repeated at least 512 times for each measurement. Further details of the setup are described elsewhere.[28]
(31) The Stark spectra are dominated by the second derivative contribution with a small first derivative contribution, and there is no measurable

contribution from the zeroth derivative. Numerical derivatives were usually obtained after smoothing the absorption spectrum. Complications arise if the absorption bands contain multiple peaks, as the line shapes of the different absorption bands need to be known accurately to get the individual values of $|\Delta\mu|$ and $|\Delta\alpha|$. The separation of the peaks in the absorption spectrum was performed by fitting the peaks with a sum of Lorentzians and Gaussians and their analytical derivatives were used in the analysis of the Stark data.

(32) Iron(II) α,α,α-tris(o-(2,2,-dimethylpropionamido)phenyl)-*β*-(*o*-3-(3-pyridyl)-propionamidophenyl)-porphyrin. This compound was prepared by the general synthetic procedure described for the chloroacetyl-picket fence porphyrin analogue. For example, see: Collman, J. P.; Broring, M.; Fu, L.; Rapta, M.; Schwenninger, R.; Straumanis, A. *J. Org. Chem.* **1998**, *23*, 8082.

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(38) It may be possible to distinguish these mechanisms by comparing the Stark effect for CN^- bound to Fe(II)Mb, which is isoelectronic with MbCO, with the Stark effect for CN^- bound to Fe(III)Mb. The bond anharmonicity is expected to be similar for both complexes, but such resonance structures are not available for CN^- bound to Fe(III)Mb.

(39) We expect that $|\Delta\mu|$ for free CO trapped in the protein matrix following photolysis of MbCO at low temperature (the so-called B states) should be very similar to what we have measured in 2-MeTHF since the CO stretching frequency for free CO in 2-MeTHF is about the same as that of B state CO. Also the B state CO does not make a covalent bond with the protein. The VSE spectrum for B state CO is hard to obtain using our current setup since there is a partial rebinding even at 74 K. There is an interesting dispersion in $\bar{\nu}_{CO}$ for the B states in different mutants; this will be reported in a subsequent paper and interpreted as electrochromic band shifts (Park, E. S., Boxer, S. G. In preparation.)

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