

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

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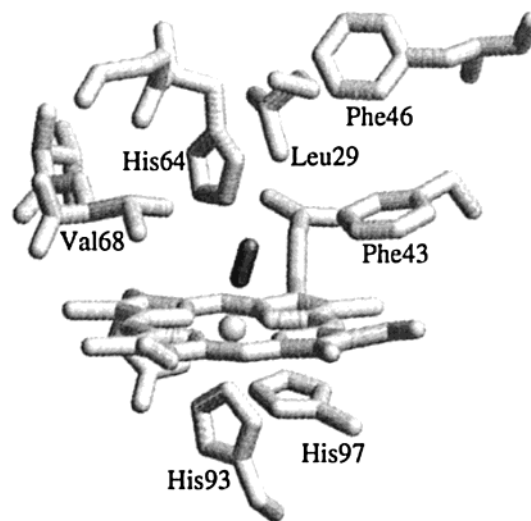
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We report the first measurement of the vibrational Stark effect in a protein, providing quantitative information on the sensitivity of a vibrational transition to an applied electric field. This can be used to interpret changes in the vibrational frequency 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  $(2.4/f) \text{ cm}^{-1}/(\text{MV}/\text{cm})$ , 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.<sup>1–8</sup> Electrostatic interactions can be probed by measuring  $\text{p}K_{\text{a}}$  shifts for titratable residues,<sup>9–11</sup> shifts in redox potential,<sup>12,13</sup> NMR chemical shifts,<sup>14</sup> and electrochromic band shifts (sometimes called internal Stark shifts).<sup>15,16</sup> 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  $F_{\text{matrix}}$ , 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.<sup>17</sup> The magnitude and direction of  $\Delta\mu$  can be obtained by Stark spectroscopy which quantifies the effect of an *externally* applied electric field,  $F_{\text{ext}}$ , on the transition. Stark spectroscopy of electronic transitions in proteins is now a standard method;<sup>18,19</sup> however, this technique is rarely applied to molecular vibrations.<sup>20,21</sup> 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



**Figure 1.** Schematic diagram of the heme pocket in MbCO derived from the crystallographic coordinates.<sup>36</sup>

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.<sup>22,23</sup> 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.<sup>22</sup> 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<sup>13</sup> and the  $\text{p}K_{\text{a}}$  for buried aspartic or glutamatic acid<sup>24</sup> have been measured, along with changes in  $\bar{\nu}_{\text{CO}}$  in the infrared.<sup>25,26</sup> It has been argued that the substantial variations observed for  $\bar{\nu}_{\text{CO}}$  in

a range of mutations to residues in the vicinity of bound CO on the distal side of the heme reflect differences in electric fields,<sup>22,26</sup> and similar arguments have been made for CO bound to the heme iron in cytochrome *c*.<sup>27</sup> 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  $\bar{\nu}_{\text{CO}}$  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.<sup>21</sup> As described in detail elsewhere,<sup>28</sup> the change in absorption,  $\Delta A$ , 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:<sup>19</sup>

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

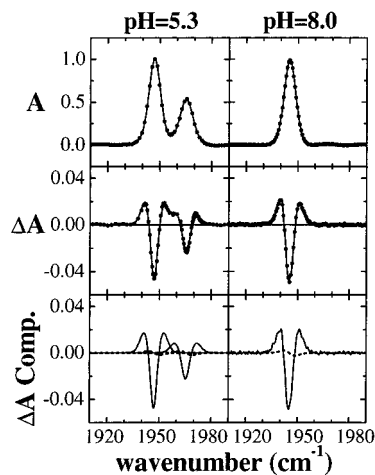
where  $F_{\text{ext}}$  is the external applied field,  $f$  is the local field correction,<sup>29</sup>  $h$  is Planck's constant, and  $c$  is the speed of light. The coefficients  $A_{\chi}$ ,  $B_{\chi}$ , and  $C_{\chi}$  are associated with molecular properties of the system.  $A_{\chi}$  is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

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

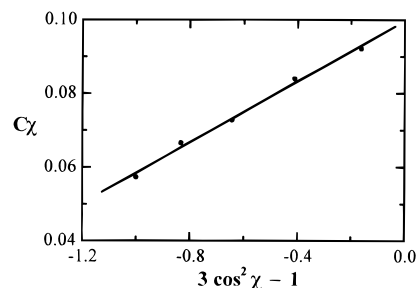
$C_{\chi}$  depends only on  $\Delta\mu$  and  $\zeta$ , the angle between  $\Delta\mu$  and the transition moment direction.  $\zeta$  is determined from the ratio of the Stark spectra taken at different experimental angles  $\chi$  between the direction of light polarization and the applied field direction. The coefficients in eq 1 are obtained by fitting the numerical derivatives of the absorption spectrum to the Stark spectrum.<sup>19</sup>

The absorption and Stark spectra of CO in wild-type MbCO at pH 5.3 and 8.0 are shown in Figure 2.<sup>30</sup> 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  $\text{cm}^{-1}$  (sometimes denoted  $A_1$ ) has the same  $\bar{\nu}_{\text{CO}}$  as the single band at pH 8; the second band is at 1965  $\text{cm}^{-1}$  (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.<sup>31</sup> Figure 3 shows the dependence of  $C_{\chi}$  on  $(3\cos^2\chi - 1)$  for the single peak at pH 8 (eq 1); a linear fit of the data gives  $\zeta = 0^\circ$  with an estimated error of  $7^\circ$ . 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<sup>13</sup> and  $\bar{\nu}_{\text{CO}}$ .<sup>25,26</sup> Both spectra involve multiple peaks. The Stark spectra line



**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<sup>48</sup> 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  $C_{\chi}$  to the Stark spectrum vs  $3\cos^2\chi - 1$ , where  $\chi$  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  $\zeta = 0^\circ$  and  $|\Delta\mu| = 0.14$  D/f. The data are for MbCO at pH 8 (right parts, Figure 2).

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 ( $\bar{\nu}_{\text{CO}} = 2131$   $\text{cm}^{-1}$ ) 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<sup>32</sup> in frozen 2-methyl-tetrahydrofuran ( $\bar{\nu}_{\text{CO}} = 1973$   $\text{cm}^{-1}$ ) and CO bound to the heme iron in cytochrome *c*<sup>33</sup> ( $\bar{\nu}_{\text{CO}} = 1963$   $\text{cm}^{-1}$ ) are very similar to those of Mb variants (Table 1).

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<sup>34</sup> and parallel to the CO bond axis,<sup>34–36</sup>  $\Delta\mu$  for CO bound to the heme is a sensitive and anisotropic detector of  $F_{\text{matrix}}$ . Third,  $|\Delta\mu|$  for wild-type MbCO is approximately the same at



correlation exists: at least for the cases we have studied thus far;  $\Delta\mu$  is a constant, and the observed variations in  $hc\Delta\bar{\nu}_{\text{CO}} = -\Delta\mu \cdot F_{\text{matrix}}$  reflect variations in  $F_{\text{matrix}}$ . The slope of the correlation between the calculated potential (interpreted here as  $F_{\text{matrix}}$ ) and  $\bar{\nu}_{\text{CO}}$  should give the Stark tuning rate. Taking results from ref 41, we obtain a Stark tuning rate of approximately  $1.5 \text{ cm}^{-1}/(\text{MV}/\text{cm})$ , though the scatter in the points is fairly large. This value is smaller than the value of  $(2.4/f) \text{ cm}^{-1}/(\text{MV}/\text{cm})$  based on the VSE data, possibly due to  $f > 1$ ,<sup>29</sup> 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  $\bar{\nu}_{\text{CO}}$  decreases as the electrostatic potential at the oxygen atom increases with respect to that at the carbon atom. Since  $hc\Delta\bar{\nu}_{\text{CO}} = -\Delta\mu \cdot F_{\text{matrix}}$ ,  $\Delta\mu$  should point from the oxygen atom toward the carbon atom.<sup>42</sup> Taking this information, we can then estimate the magnitude and direction of the change in  $F_{\text{matrix}}$  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.<sup>43,44</sup> It is generally agreed that the band at  $1965 \text{ cm}^{-1}$  is associated with a population of the sample in which His64 is protonated and swings out of the heme pocket.<sup>45</sup> If we associate the change in  $\bar{\nu}_{\text{CO}}$  of  $+20 \text{ cm}^{-1}$  with the electric field due to this protonation, i.e., treat it as an electrochromic band shift, then the change in  $F_{\text{matrix}}$  along the C–O axis is  $8f \text{ MV}/\text{cm}$  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  $F_{\text{matrix}}$ . 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.<sup>46</sup> 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,<sup>47</sup> and these can also be interpreted as resulting from changes in the electrostatic field associated with functionally important processes.

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- (30) Wild-type human Mb and the V68D and V68N variants were expressed and purified from *E. coli* as described elsewhere.<sup>24</sup> 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.<sup>26</sup> The samples contained  $\sim 3 \text{ mM}$  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 ( $\sim 50 \text{ \AA}$  thick Ni) sapphire windows separated by  $\sim 30 \mu\text{m}$  Teflon spacers. The cell containing the samples was immersed in a custom-built liquid  $\text{N}_2$  immersion cryostat that operates at approximately 74 K. Absorption and Stark spectra were obtained on a Bruker IFS 66V/S FTIR spectrometer ( $0.5 \text{ cm}^{-1}$  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  $+2.5 \text{ kV}$ ), 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.<sup>28</sup>
- (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)  $\alpha,\alpha,\alpha$ -tris(*o*-(2,2-dimethylpropionamido)phenyl)- $\beta$ -(*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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(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  $\bar{\nu}_{\text{CO}}$  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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