Band III Excitation Resonance Raman Spectra of Deoxymyoglobin. Implications for Heme Structure and Dynamics

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Band III is a weak, near-infrared-region charge-transfer absorption ($\lambda_{max} \sim 764$ nm, $\epsilon_{max} \sim 250$ M^{-1} cm^{-1}) of deoxymyoglobin (deoxyMb) and deoxyhemoglobin (deoxyHb)[1] that has been widely used to monitor the structure and dynamics of the heme prosthetic group and heme pocket.[2] The position and line shape of band III are highly dependent on the temperature and pH.[2a,d-k] Kinetic hole burning studies on deoxyMb and deoxyHb suggest that the inhomogeneous line shape of band III can be related to conformational disorder and/or subunit heterogeneity.[2f,i] Various models have been proposed to relate the peak position and line shape of band III to structural parameters of the heme and proximal histidine ligand.[2i,k,n] Most of these models assume that the line shape of the electronic transition is dominated by inhomogeneous contributions. In contrast, Champion and co-workers[2k] have proposed a model in which there is a significant homogeneous contribution to the line shape of band III arising from vibronic coupling of the charge-transfer transition to several low-frequency modes. These workers were able to account for the observed temperature dependence of the line shape by including modes at 69, 150, and 220 cm^{-1}. An important feature of the model is that the 69-cm^{-1} vibration is strongly coupled ($S \sim 0.8$) to the electronic transition. The frequency of this mode is close to the 50-cm^{-1} value predicted for the heme doming motion, γ_9,[3] which is associated with the ligand reaction coordinate in Mb and Hb,[2k] Champion and co-workers also proposed that a very low frequency mode is coupled to the B-state transition.[2k] In support of this proposal, these workers have recently observed oscillations characteristic of a mode at $\sim$50 cm^{-1} in femtosecond coherence spectroscopic studies of the B state.[4]

Resonance Raman (RR) spectroscopic studies of deoxyMb or deoxyHb that employ excitation into band III can in principal provide direct evidence concerning the coupling of vibrational modes to the electronic transition because the modes that appear in the RR spectrum are those most strongly coupled.[5] To date, however, band III excitation RR spectra have not been reported. This is in large part due to the fact that the extinction coefficient for band III is extremely low[1] and the energy of the electronic transition is in a region that is not particularly amenable to RR studies. The problems associated with band III excitation RR studies are made more tractable by the routine availability of Ti:sapphire lasers and CCD detectors. In this communication, we report the results of preliminary band III excitation RR studies on deoxyMb. These studies strongly suggest that the charge-transfer transition is in fact strongly coupled to a very low frequency vibrational mode ($\sim$60 cm^{-1}).

The low-frequency (100–800-cm^{-1}) region of the band III excitation RR spectrum of deoxyMb at pH 6.9 is shown in Figure 1a (upper trace).[6] For comparison, RR spectra obtained with B- and Q-state excitation are also shown in the figure (lower and middle traces). The 30–100-cm^{-1} region of the band III (and B-state) spectrum was also examined; however, a reliable analysis of these data is precluded by strong scattering due to the Rayleigh wing. Comparison of the RR spectra shown in Figure 1a reveals that the band III spectrum is qualitatively different from those obtained with either B- or Q-state excitation. Prominent bands are observed with band III excitation at 115, 168, 265, 331, and 449 cm^{-1} that have no clear analogs in either the B- or Q-state spectra. These features are due to RR scattering from the charge-transfer state and cannot be attributed to preresonant scattering from the Q or B states because their intensities diminish and eventually vanish as the excitation wavelength is tuned to the blue of band III. The band III excitation spectrum also differs from both the Q- and B-state spectra in that the RR scattering is confined to low-frequency vibrations. No features with significant intensity are observed above 450 cm^{-1}. The absence of features cannot be attributed to instrumental limitations because the throughput of the spectrometer and sensitivity of the detector are extremely high in the 800–1000-nm range.[6]

Although the band III excitation RR spectrum is different from those observed with either B- or Q-state excitation, there are some features in common among the spectra. In particular, RR bands are observed in the band III spectrum at 134, 155, 235, and 369 cm^{-1} which have apparent analogs in the B- and/or Q-state spectra. A band is also observed at 224 cm^{-1} in the band III spectrum which could correspond to the Fe–histidine stretching vibration, ν_{Fe-His}. The ν_{Fe-His} mode is observed at 218 cm^{-1} in the

(1) (a) Eaton, W. A.; Hanson, L. K.; Stephens, P. J.; Sutherland, J. C.; Dunn, J. B. R. *J. Am. Chem. Soc.* **1978**, *100*, 4991–5003. (b) Makinen, M. W.; Churg, A. K. In *Iron Porphyrins*; Lever, A. P. B., Gray, H. B., Eds.; Addison-Wesley: Reading, MA, 1983; Vol. I, pp 141–236.

(2) (a) Iizuka, T.; Yamamoto, H.; Kotani, M.; Yonetani, T. *Biochim. Biophys. Acta* **1974**, *371*, 1715–1729. (b) Eaton, W. A.; Hofrichter, J. *Methods Enzymol.* **1981**, *76*, 175–261. (c) Agmon, N.; Hopfield, J. J. *J. Chem. Phys.* **1983**, *79*, 2042–2053. (d) Fiamingo, F. G.; Alben, J. O. *Biochemistry* **1985**, *24*, 7964–7970. (e) Sassaroli, M.; Rousseau, D. L. *Biochemistry* **1987**, *26*, 3092–3096. (f) Campbell, B. F.; Chance, M. R.; Friedman, J. M. *Science* **1987**, *238*, 373–376. (g) Agmon, N. *Biochemistry* **1988**, *27*, 3507–3511. (h) Cordone, L.; Cupane, A.; Leone, M.; Vitrano, E. *Biopolymers* **1990**, *29*, 639–643. (i) Chavez, M. D.; Courtney, S. H.; Chance, M. R.; Kiula, D.; Nocek, J.; Hoffman, B. M.; Friedman, J. M.; Ondrias, M. R. *Biochemistry* **1990**, *29*, 4844–4852. (j) Xie, X.; Simon, J. D. *Biochemistry* **1991**, *30*, 3682–3692. (k) Srajer, V.; Champion, P. M. *Biochemistry* **1991**, *30*, 7390–7402. (l) Dunn, R. C.; Simon, J. D. *Biophys. J.* **1992**, *60*, 884–889. (m) Nienhaus, G. U.; Mourant, J. R.; Frauenfelder, H. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 2902–2906. (n) Gilch, H.; Schweitzer-Stenner, R.; Dreybrodt, W. *Biophys. J.* **1993**, *65*, 1470–1485. (o) Ansari, A.; Jones, C. M.; Henry, E. R.; Hofrichter, J.; Eaton, W. A. *Biochemistry* **1994**, *33*, 5128–5145.

(3) Li, X.-Y.; Zgierski, M. Z. *Chem. Phys. Lett.* **1992**, *188*, 16–20.

(4) Zhu, L.; Li, P.; Huang, M.; Sage, J. T.; Champion, P. M. *Phys. Rev. Lett.* **1994**, *72*, 301–304.

(5) Myers, A. B.; Mathies, R. A. In *Biological Applications of Raman Spectroscopy*; Spiro, T. G., Ed.; Wiley: New York, 1987; Vol. 2, pp 1–58.

(6) Sperm whale Mb (Sigma type II) in the met form was solubilized in 0.01 M phosphate (pH 6.9) or acetate (pH 3.1) buffers and further purified for RR studies. The pH of the samples was measured with an Orion pH meter (Model 420A). DeoxyMb was prepared in deoxygenated buffers under strictly anaerobic conditions by addition of a slight excess of buffered sodium dithionite using a syringe–septum technique. The B- and Q-state RR spectra were acquired by using a Spex triple spectrograph (Spex 1877) equipped with a holographically etched 2400 groove/mm grating in the third stage. The band III excitation spectra were acquired by using a red-optimized Spex triple spectrograph (Spex 1877). The filter stage of this spectrograph has 600 groove/mm gratings, and the spectrograph stage has 600 or 1200 groove/mm gratings all blazed at 750 nm. The samples were contained in a spinning cell (band III excitation) or a capillary tube (Q- and B-state excitation). The scattered light was collected in a 90° configuration by using a 50 nm f/1.2 Nikon camera lens. The excitation wavelengths were provided by the discrete outputs of a krypton ion (Coherent Innova 200-K3) or argon ion (Coherent Innova 400-15UV) laser or a Ti:sapphire laser (Coherent 890) pumped by the visible multiline output of the argon ion laser. A 1152 × 298 pixel, UV-enhanced front-illuminated charge-coupled device was used as the detector (Princeton Instruments, LN/CCD equipped with an EEV1152-UV chip) for the B- and Q-state experiments. A 512 × 512 pixel, back-thinned CCD (Princeton Instruments, LN/CCD equipped with a Tektronix chip) was used as the detector for the NIR experiments. The quantum efficiency of this CCD is 50 and 20% at 0.85 and 1 μm, respectively. The laser powers were typically 5 mW or less. Low laser powers, which necessitate long integration times, were used as a precaution against protein denaturation. Even small amounts of denaturation produce sufficient Rayleigh scattering to compromise the very low frequency region of the spectrum. The frequencies were calibrated by using the known frequencies of indene and CCl_4. The frequencies are accurate to ±1 cm^{-1} for strong and/or isolated bands. The slit widths were set for a 2-cm^{-1} resolution at a Raman shift of 500 cm^{-1}. No smoothing was performed on any of the spectra. A standard data acquisition protocol for the band III experiments consisted of the coaddition of 8–10 1-h (30 × 120 s) scans. Cosmic spikes were removed prior to coaddition of the data sets.

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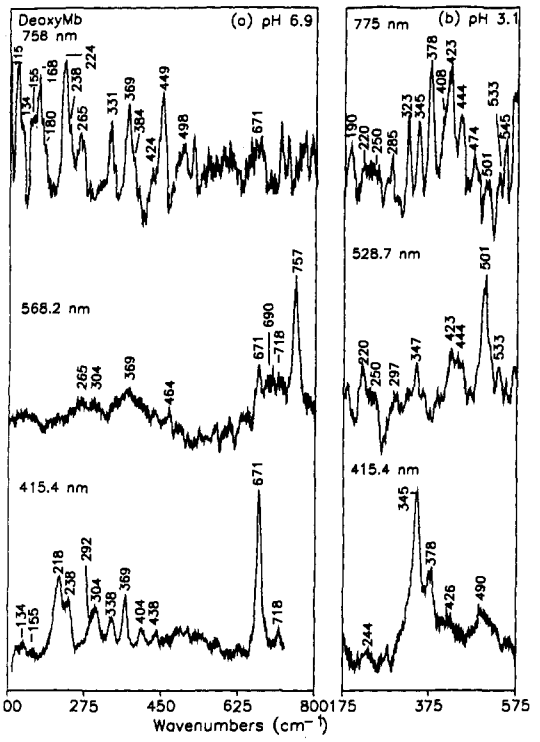

Figure 1. Low-frequency regions of RR spectra of deoxyMb obtained by using band III (top traces), Q-state (middle traces), and B-state (bottom traces) excitation: (a) pH 6.9, 0.01 M phosphate buffer; (b) pH 3.1, 0.01 M acetate buffer. The protein concentrations are ~1 mM (band III excitation), ~250 μM (Q-state excitation), and ~100 μM (B-state excitation).

B-state excitation RR spectrum[7] but is not observed with Q-state excitation.[8] It should be noted, however, that the 6-cm^{-1} frequency difference observed in the band III versus B-state RR spectra is outside the limit of experimental uncertainty (±1 cm^{-1}). This observation suggests that the 224-cm^{-1} band observed with band III excitation might not be due to ν_{Fe-His}.

In order to gain further insights into the possible identity of the 224-cm^{-1} band, the band III excitation RR spectrum was also acquired at pH 3.1. This spectrum along with the B- and Q-state analogs is shown in Figure 1b. At low pH, the Fe–His bond is ruptured and band III red shifts to ~780 nm.[9] The breakage of the Fe–His bond is confirmed by the absence of the 218-cm^{-1} band in the B-state-excitation RR spectrum. At low pH, the 224-cm^{-1} band is also absent from the band III spectrum. This observation is qualitatively consistent with a contribution of ν_{Fe-His} to band III RR scattering in the 220-cm^{-1} region at neutral pH. We were unable to determine whether the prominent low-frequency bands observed at 115 and 168 cm^{-1} at pH 6.9 also disappear at low pH because scattering from the Rayleigh wing obscured the spectral region below 175 cm^{-1}. Lowering the pH also results in a number of other changes in the band III excitation RR spectrum. New features appear in the 300–500-cm^{-1} region, some of which have apparent analogs in the B- and Q-state spectra (345, 378, 444, and 501 cm^{-1}) and other of which do not (285, 323, and 474 cm^{-1}). Additional studies are required in order to fully assess the effects of the pH-induced changes.

The most striking aspect of the band III excitation RR spectrum observed at pH 6.9 is the relatively strong RR scattering from the 115- and 168-cm^{-1} modes. Bands with these frequencies and intensities are unprecedented in the B- and Q-state RR spectra of heme proteins. The 115 and 168 cm^{-1} could be due to fundamental out-of-plane deformations of the macrocycle that are strongly coupled to the charge-transfer transition. On the other hand, the two bands could be the first and second overtones of a very low frequency vibration. If the 115- and 168-cm^{-1} bands are assumed to be overtones, the fundamental is calculated to be at ~59 cm^{-1}.[10,11] This value is intermediate between the 50-cm^{-1} frequency calculated for $\gamma_9{}^3$ and the 69-cm^{-1} frequency used by Champion and co-workers to model the band III absorption contour.[2k] The third and fourth overtones are calculated at ~218 and ~265 cm^{-1}, respectively. Accounting for the experimental errors in the measured RR frequencies (±1 cm^{-1}), the 224- and 265-cm^{-1} bands observed in the band III spectrum at pH 6.9 could correspond to these overtones. The relative intensities of the 115-, 168-, and 265-cm^{-1} bands decrease monotonically ($I_{115} > I_{168} > I_{265}$). This pattern is qualitatively consistent with that expected for a vibrational progression in a mode with $S < 1$.[5] Electron–phonon coupling strengths in this range are in general agreement with the S value of ~0.8 required for the 69-cm^{-1} vibration in Champion and co-workers' vibronic model for band III.[2k] The intensity of the 224-cm^{-1} band deviates from the monotonic pattern observed for the 115-, 168-, and 265-cm^{-1} bands. It is conceivable that the increased intensity of the 224-cm^{-1} band arises because this feature is a superposition of bands due to both the third overtone of γ_9 and ν_{Fe-His}. Fermi resonance interaction between the vibrational levels could also skew the intensity and frequency of the band(s). These issues could be addressed via isotopic labeling of the axial histidine ligand.

In conclusion, band III excitation RR spectra of deoxyMb have been observed for the first time. These spectra are qualitatively different from those observed with either B- or Q-state excitation. The observation of a number of relatively intense low-frequency modes indicates that these modes are strongly coupled to the resonant charge-transfer transition. Quantitative intensity measurements of the band III excitation RR features should provide detailed insights into the strength of the vibronic coupling and the excited state dynamics. These studies are currently in progress.

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(7) Kitagawa, T. In *Biological Applications of Raman Spectroscopy*; Spiro, T. G., Ed.; Wiley: New York, 1988; Vol. 3, pp 97–131.

(8) Dasgupta, S.; Spiro, T. G.; Johnson, C. K.; Dalickas, G. A.; Hochstrasser, R. M. *Biochemistry* **1985**, *24*, 5295–5297.

(9) (a) Han, S.; Rousseau, D. L.; Giacometti, G.; Brunori, M. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 205–209. (b) Sage, J. T.; Morikis, D.; Champion, P. M. *Biochemistry* **1991**, *30*, 1227–1237.

(10) This frequency was calculated using $E_v = (v + 1/2)\bar{\omega}_e - (v + 1/2)^2 \overline{\omega_e x_e}$. Using 115 and 168 cm^{-1} for the first and second overtones, $\bar{\omega}_e = 62$ cm^{-1} and $\overline{\omega_e x_e} = 1.5$ cm^{-1}.

(11) The frequency of the fundamental of the four-coordinate heme formed at pH 3.1 cannot be predicted with certainty because scattering from the Rayleigh wing precludes identification of a clear vibrational progression. However, if the 190- and ~250-cm^{-1} bands are assumed to be the third and fourth overtones, $\bar{\omega}_e = 35$ cm^{-1} and $\overline{\omega_e x_e} = -2.5$ cm^{-1}, and the fundamental is predicted at ~40 cm^{-1}. Note that this choice of assignments requires that the sign of the anharmonicity constant is opposite that of the five-coordinate species.