# *Communications to the Editor*

## A UV Resonance Raman Monitor of Histidine Protonation in Proteins: Bohr Protons in Hemoglobin

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We report a new method for monitoring the protonation state of histidine residues in proteins, via ultraviolet resonance Raman spectroscopy (UVRR). This method complements NMR by quantitating the aggregate His protonation level, rather than protonation of individual residues. When applied to hemoglobin, the method gives results consistent with NMR;[1] a significant fraction of the alkaline Bohr effect is attributable to altered His protonation in the T vs R state.

When excited in the deep UV, Raman spectra of proteins contain resonance-enhanced bands of aromatic residues, which provide information about local structure and environment.[2] Such information would be particularly useful for histidine, but the scattering cross section is modest for histidine, even at the excitation profile maximums,[3] and His bands are not normally observed in protein UVRR spectra. However, protonated histidine gives a strong sharp band at 1408 $cm^{-1}$ when it is dissolved in $D_2O$ (Figure 1). The dominance of this band is due to the similarity of the 1408-$cm^{-1}$ imidazolium-$d_2$ ring mode eigenvector to the geometry change in the first $\pi-\pi^*$ excited state.[4] When the deuterons are replaced by protons, the participation of the N−H bending coordinates leads to a redistribution of the RR intensity into other ring modes. Imidazolium deprotonation also results in intensity redistribution, due to changes in the excited state, as well as in normal-mode compositions.[4] Intensity redistribution is consistent with the excitation profile of the 1408-$cm^{-1}$ band (Figure 1, inset), which is broad and double peaked; the shape is the same as previously reported for histidinium,[3] but the amplitude is much higher than for any of the histidinium-$h_2$ RR bands. The concentration of intensity into a single mode gives the 1408-$cm^{-1}$ histidinium-$d_2$ band as large a cross section as the tyrosine Y8a band, when measured at 218 nm.[5,6]

The 1408-$cm^{-1}$ band is readily detected in UVRR spectra of hemoglobin (Hb) in $D_2O$ (Figure 2), even at 229 nm, quite far from the excitation profile maximum, because the band position is free from interferences from other aromatic residues. The excitation profile in Hb tracks that of aqueous histidinium-$d_2$ (Figure 1, inset), indicating no shift in the excited-state energy between water and the average histidinium side-chain environment in Hb. The 229-nm excitation was chosen for quantitation of the intensity because it is the shortest wavelength line of the stable frequency-doubled $Ar^+$ laser. When the pD of the Hb solution is raised from 6.5 to 7.3, the 1408-$cm^{-1}$ intensity is lowered (Figure 2), reflecting deprotonation of histidinium side chains.

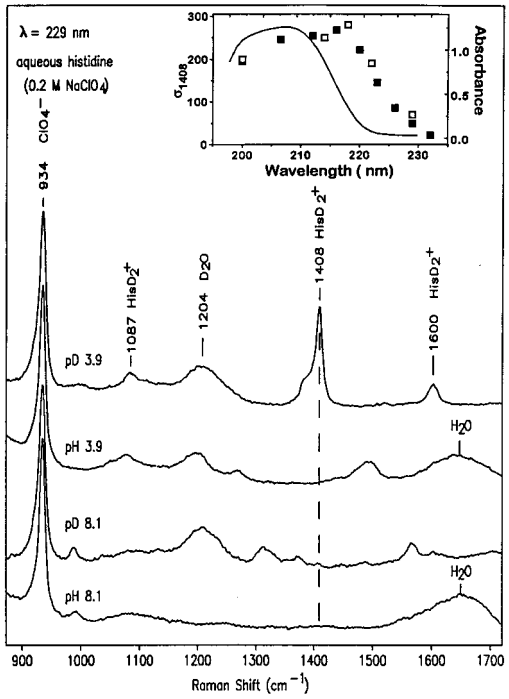


**Figure 1.** UV resonance Raman spectra of histidine (Sigma; 1 mM) at low and high pH in $H_2O$ and $D_2O$ with 0.2 M $ClO_4^-$ as internal intensity standard. The 229-nm cw laser excitation (∼0.35 mW at sample) was provided by an intracavity frequency-doubled FreD $Ar^+$ laser. The inset shows 1408-$cm^{-1}$ Raman excitation profile for $HisD_2^+$ (solid squares, in pD = 4.4 buffer) and for HbCO (open squares, in pD = 7.3 buffer); the solid line is the $HisD_2^+$ absorption spectrum. The HbCO cross section was normalized to that of $HisD_2^+$ in pD = 4.4 buffer upon excitation at 229 nm. Excitation at the indicated wavelengths was generated by an XeCl excimer-pumped dye laser, with frequency doubling and tripling in BBO.[15,16] The average power was ∼0.3 mW (∼1 $\mu$J/pulse at 300-Hz repetition rate). The UVRR spectra were acquired with a Spex 1269 single monochromator equipped with an intensified photodiode array.[15] Hemoglobin was prepared by established procedures.[15,17]

The 1408-$cm^{-1}$ band of protonated His in $D_2O$ was detected in nonresonance Raman spectra long ago,[7] and the assignment was subsequently confirmed via isotopic labeling of L-histidine by Tasumi et al.[8] Lord and Yu[7] actually suggested that the band might be useful to assess the His ionization state in proteins, but this suggestion was not pursued, presumably because of the spectral conjestion of nonresonance Raman spectra. Resonance Raman enhancement reduces spectral conjestion because of its chromophre selectivity.

Using the 934-$cm^{-1}$ band of 0.25 M sodium perchlorate as an internal standard, we calculated 13.4 histidinium residues per protein tetramer for deoxyHb and 12.4 for HbCO at pH 7.3 (Table 1), from the 1408-$cm^{-1}$ intensity ratio between Hb in $D_2O$ and aqueous histidinium-$d_2$. Thus, 35% of the 38 histidine residues in the Hb tetramer are protonated. When the pH is lowered to

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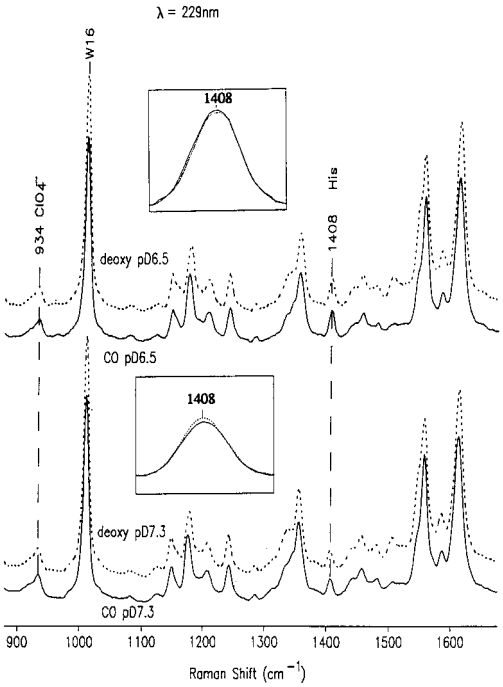


**Figure 2.** The 229-nm-excited UVRR spectra of 0.125 mM Hb (dotted line) and HbCO (solid line) with 0.25 M $ClO_4^-$ in $D_2O$ at pD = 6.5 and 7.3. The spectra were normalized with the $ClO_4^-$ band at 934 $cm^{-1}$. The inset shows the 1408-$cm^{-1}$ intensity differences. The spectra were coadded with the three 15-min measurements.

**Table 1.** UVRR Determination of His Residue Protonation

| pD | $ClO_4^-$ (M) | sample | $\sigma_{1408}/\sigma_{ClO_4^-}$ [a] | $HisD_2^+$ [b] |
|---|---|---|---|---|
| 7.3 | 0.25 | Hb | 1420 | 13.4 |
| 7.3 | 0.25 | HbCO | 1320 | 12.4 |
| 6.5 | 0.25 | Hb | 2080 | 19.6 |
| 6.5 | 0.25 | HbCO | 2140 | 20.2 |
| 7.3 | 0 | Hb | 1008[c] | 9.5 |
| 7.3 | 0 | HbCO | 820[c] | 7.7 |

[a] Molar cross section, relative to $ClO_4^-$, equals $(I_{1408}/I_{934})(C_{ClO_4^-}/C_{Hb})$. [b] Number of protonated histidines, based on the molar cross section of aqueous $HisD_2^+$, $\sigma_{1408}/\sigma_{ClO_4^-} = 106$. The estimated uncertainty is 5%, but the relative error between pairs of Hb/HbCO measurements is estimated to be 2%. [c] Obtained from the 1408-$cm^{-1}$ intensity relative to the W16 (1012 $cm^{-1}$) intensity, assuming that the intensity of the later is unaltered by 0.25 M $ClO_4^-$.

6.5, the histidinium count increases to 19.6 for deoxyHb and 20.2 for HbCO. Although the intensity differences between deoxyHb and HbCO are small, they are accurately measurable (Figure 2 inset). The shift from one proton released upon CO binding at pH 7.3 to 0.6 proton taken up at pH 6.5 is exactly as expected from the Bohr effect, which links proton and ligand binding to Hb.[1,9] The oxygen affinity rises with pH because ligand binding is accompanied by release of protons at alkaline pH and by uptake of protons at acid pH. The total number of Bohr protons released at pH 7.3 is somewhat less than 2.2,[1,9] depending on the nature and concentration of salt and buffer ions.[1,10]

The salt dependence is illustrated by the fact that we find the histidinium count droping by 41% to 9.5 for deoxyHb and by 61% to 7.7 for HbCO when the 0.25 M $NaClO_4$ internal standard is omitted from the pH 7.3 solution. Because of the loss of the reference peak, these numbers were calculated by taking the ratio of the 1408-$cm^{-1}$ band to the 1012-$cm^{-1}$ W16 band of the Trp residues, on the assumption that its intensity is unaltered in the presence or absence of the $NaClO_4$ internal standard (The relative intensities of the various Trp and Tyr bands are essentially independent of $NaClO_4$.). Perutz has recently emphasized the role of diffuse anion binding to the positively charged central cavity of the Hb tetramer, in controlling the Bohr effect.[11,12] The central cavity contains four His residues, and these could account for most of the augmentation of the 1408-$cm^{-1}$ band upon adding 0.25 M $NaClO_4$ at pH 7.3. Perchlorate binding induces proton uptake by about four histidines in both the R and T states, and the aggregate difference declines slightly, from 1.6 to 1.0 (Table 1).

Perutz[9] has emphasized the importance of the C-terminal $\beta$-chain residue, His $\beta$146, in the Bohr effect; its imidazole side chain has an elevated p$K_a$ in the T state because it forms an intrasubunit salt bridge with the carboxylate group of Asp $\beta$94, which is broken in the R state. Another Bohr contributor is the $\alpha$ chain N-terminus, Val $\alpha$1, whose amino group forms a salt bridge in the T state.[13] In addition, Ho and co-workers[1] have established p$K_a$ changes, both positive and negative, for several His residues, and Rao and Acharya[14] reported a significant Bohr contribution from a carboxylic acid side chain of Glu $\beta$43, which lies in the $\alpha_1\beta_2$ interface. The present technique can contribute to the eventual elucidation of the Bohr mechanisms, by providing a means of monitoring the total histidinium count under various solution conditions.

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