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RESONANCE RAMAN STUDIES OF THE QUATERNARY STRUCTURAL CHANGE IN CARP DEOXY HEMOGLOBIN

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1. Introduction

Interaction of hemoglobin (Hb) with oxygen is co-operative: i.e., the binding of oxygen to one heme facilitates the binding of the oxygen to the other heme groups. This increase in oxygen affinity, known as the heme–heme interaction, arises, at least in part, from a reversible transition between two alternative quaternary structures, the low affinity *T* structure and the high affinity *R* structure [1,2]. Resonance Raman spectroscopy (RR) is a powerful tool to study electronic and structural changes at the heme which accompany the *T–R* transition. Since visible radiation is absorbed only by the heme group of the hemoglobin molecule, it causes resonance enhancement of the vibrational modes associated only with this structure [3–5].

The RR spectra of human deoxyhemoglobin in the *T* (HbA) and *R* (NES-des-Arg_{141}-Hb) structure in the low frequency region, 100–500 cm^{-1}, differ in only one RR band that is due to the stretching of the Fe(II)–N_ϵ (His–F8) bond. In the *T–R* transition this RR line shifts from 215–221 cm^{-1}. From a Morse potential function, they conclude that the Fe(II)–N_ϵ (His–F8) bond is stretched by 0.01 Å in the *T* structure due to strain exerted by the globin, in qualitative agreement with [8]. In [8] it was suggested that the stronger interactions between the subunits in the *T* structure pull the proximal histidine away from the porphyrin plane, thus stretching the Fe(II)–N_ϵ (His–F8) bond. In the high frequency region of the spectrum, 1300–1700 cm^{-1}, there are RR bands which are sensitive to the oxidation state and to the spin state of the heme [4]. Using a technique for the measurement of Raman difference spectra which in some cases, where the signal-to-noise ratio is high, can detect frequency differences of some tenths of cm^{-1}, frequency differences as great as 2.2 cm^{-1} were found [9] in these marker lines for the *T* (HbA) and *R* structure (NES-des-Arg-HbA) of deoxyhemoglobin. It was concluded that the *R* structure has an effective increase in the electron density of the anti-bonding π^* orbitals of the porphyrin ring [9].

Beside the chemically modified Hbs, it is possible to reversibly induce the *T–R* transition hemoglobin of carp (*Cyprinus carpio*) for example, while keeping the protein in the deoxygenated state. This can be done by merely changing the pH [10,11]. At low pH, carp deoxy Hb adopts the *T* structure while at high pH it has the *R* structure. We have measured resonance Raman spectra of both of these states of carp hemoglobin. In the low frequency region of the spectrum we have found that only the RR line due to the stretching of the Fe(II)–N_ϵ (His–F8) bond is shifted as a result of the *T–R* transition. The shift is from 210–216 cm^{-1} in good agreement with results obtained on human Hb [6,7]. Using our technique it is difficult to detect the small shifts that occur in the RR bands in the high frequency region of the spectrum, similar to [12,13]. However, we demonstrate a shift in band A similar to that in [9] for human Hb.

2. Materials and methods

Carp blood was obtained as in [14]. The washed, packed erythrocytes were stored under liquid nitrogen. Lysis was achieved by thawing the cells and adding 2.5 vol. 1 mM Tris at pH 8. After 1 h the lysate was centrifuged at 15 000 rev./min in a Sorvall centrifuge

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for 20 min to remove the stroma. After that the HbO_2 was passed rapidly through a Sephadex G-25 column pre-equilibrated with 1 mM Tris at pH 8; it was then passed through a Dintzis deionizing column. The deionized HbO_2 was put in a DEAE-cellulose column pre-equilibrated with 10 mM Tris–HCl at pH 9. The hemoglobin was eluted with 10 mM Tris–HCl at pH 8.2. This last step of the purification procedure improved the quality of the Raman spectrum considerably, probably due to the removal of fluorescent semi-denaturated Hb, and was essential to observe weak Raman lines in the low frequency region. To obtain samples of deoxyHb, HbO_2 was deoxygenated by repeated evacuation and flushing with N_2 gas. After deoxygenation was complete a small amount of solid dithionite was added to remove any metHb. The concentration of the Hb samples, which in our RR measurements was ~1 mM in heme equivalents, was determined spectrophotometrically. Visible spectra were also used to detect metHb.

Resonance Raman spectra were obtained with a standard system, consisting of excitation by an argon ion laser, Spectra Physics model 166, a Jobin Yvon Ramanor HG 2S double monochromator and a c31034A02RCA low noise photon counting detector. Spectra were stored by a Spectroscope Modular 8000 multi-channel analyzer. For each Raman experiment, ~2 ml Hb solution was placed in a quartz cylindrical cell with 4.2 mm internal diameter. The power of the excitation beam was always <50 mW to prevent sample deterioration. The resolution ranged from 0.9–2 cm^{-1}.

3. Results

Fig.1 shows the resonance Raman spectra in the low frequency region, 180–350 cm^{-1}, of the *R* and *T* structures of carp deoxyHb. These spectra have been obtained using a 4579 Å excitation line of our Ar^+ laser.

This excitation is near the peak of the Soret band and enhances the total symmetric vibrational modes. Two of the 3 resonance Raman lines present in the spectrum do not change in frequency as a result of the *T–R* transition.

The third line, due to the stretching of the Fe(II)–N_ϵ (His–F8) bond, occurs at 210 cm^{-1} in the *T* structure and at 216 cm^{-1} in the *R* structure to give a shift between the two quaternary structures of 6 cm^{-1}. This RR line

Fig.1. Low frequency resonance Raman spectra of carp deoxyHb in the *R* structure (upper spectrum) in 30 mM Tris–HCl (pH 8.5) and in the *T* structure (lower spectrum) in 30 mM bis Tris–HCl (pH 5.8) + 1 mM IHP. Experimental conditions: 4579 Å excitation; at 50 mW incident power; 2.35 cm^{-1} slits; spectrum scan rate 0.08 $cm^{-1} \cdot s^{-1}$.

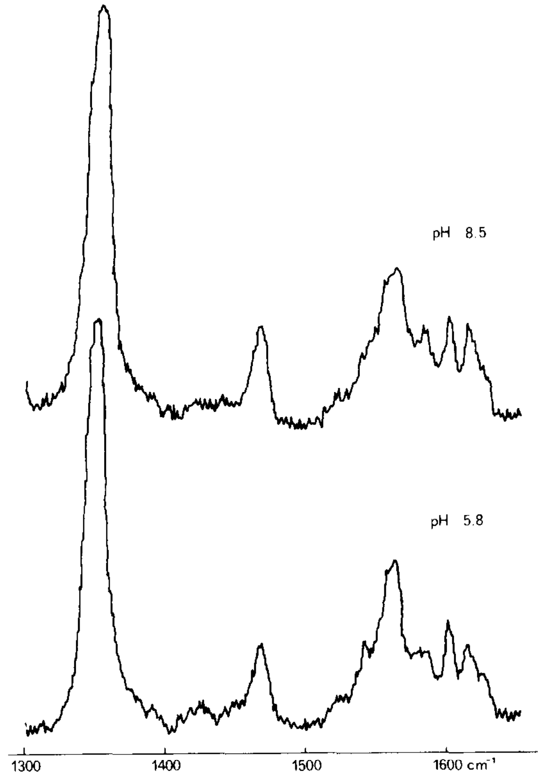

Fig.2. High frequency resonance Raman spectra of carp deoxyHb in the *R* structure (upper spectrum) in 30 mM Tris–HCl (pH 8.5) and in the *T* structure (lower spectrum) in 30 mM bis Tris–HCl (pH 5.8) + 1 mM IHP. Experimental condition: 4579 Å excitation; at 50 mW incident power; 2.35 cm^{-1} slits; spectrum scan rate 0.16 $cm^{-1} \cdot s^{-1}$.

Table 1
The vibrational frequencies in wave numbers (cm^{-1})[a] of the RR marker bands for carp deoxyHb in the *T* and *R* structures

Bands[b]	*T* state	*R* state	Intensity[c]
A	1352 cm^{-1}	1352 cm^{-1}	vs
E	1468 cm^{-1}	1467 cm^{-1}	m
B	1541 cm^{-1}	1542 cm^{-1}	w
D	1562 cm^{-1}	1562 cm^{-1}	s
F	1601 cm^{-1}	1600 cm^{-1}	m

[a] Accuracy in band position is ±1 cm^{-1}

[b] A and B bands, sensitive to oxidation state; D band, sensitive to spin state; E and F bands, sensitive to both oxidation state and spin state

[c] 4579 Å excitation: vs, very strong; s, strong; m, medium; w, weak

is also sharper and more symmetric in the *R* than in the *T* structure.

Fig.2 shows the RR spectra in the high frequency region 1300–1650 cm^{-1} of the carp deoxyHb *T* and *R* structures obtained at the same excitation wavelength of 4579 Å. Table 1 lists the frequencies and the relative intensities of the RR marker bands. The A and B lines are sensitive to the oxidation state; the D line is sensitive to the spin state; and the E and F lines are sensitive to both oxidation and spin state [4]. Within the experimental error of ±1 cm^{-1} in the peak positions there are no changes in frequency or intensity as a result of the transition between the two quaternary structures. Fig.3 shows the RR spectra of the carp deoxyHb *T* and *R* structure over 1340–1365 cm^{-1} obtained again with excitation at 4579 Å but with the 4579 Å interference filter removed. Without this filter these spectra contain a very sharp line which peaks at 1351 cm^{-1} which results from elastic scattering of a secondary spectral line of the laser. This line offers a very useful calibration for this region of the RR spectrum. In these two spectra we see that the A line in the *R* structure is completely hidden by the 1351 cm^{-1} laser line whereas in the *T* structure it is only partially hidden. We conclude that the RR A line is shifted toward lower frequency as a result of the *T*–*R* transition and is therefore sensitive to the change in the quaternary structure.

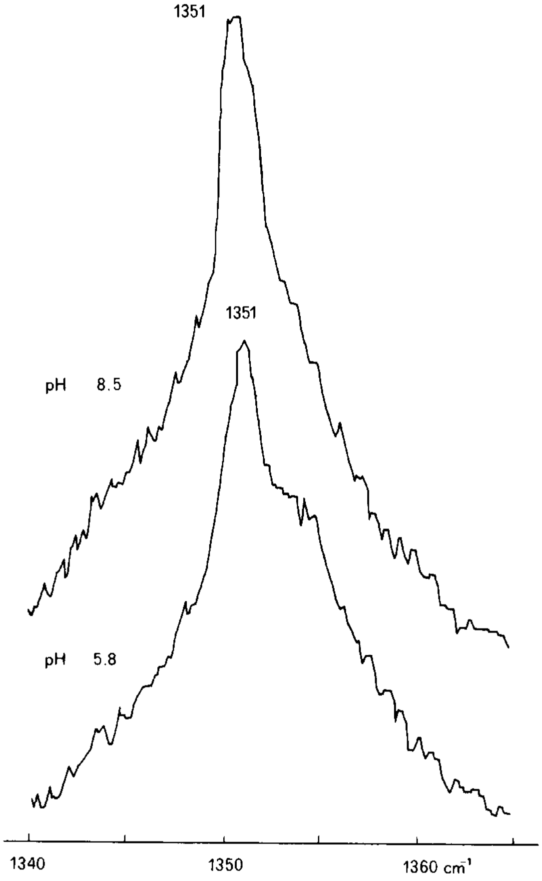

Fig.3. High frequency resonance Raman spectra of carp deoxyHb in the *R* structure (upper spectrum) in 30 mM Tris–HCl (pH 8.5) and in the *T* structure (lower spectrum) in 30 mM bis Tris–HCl (pH 5.8) + 1 mM IHP showing the RR A band along with the 1351 cm^{-1} line of the laser. Experimental conditions 4579 Å excitation without interference filter, 50 mW incident power, 0.95 cm^{-1} slits, spectrum scan rate 0.033 $cm^{-1} . s^{-1}$.

4. Discussion

The shift which we observe in the RR line due to the stretching of the Fe(II)–N_ϵ (His–F8) bond is precisely that found in [6]. Therefore, if we use the Morse potential formulation [6] to compute the change in the histidine–iron distance, we find a change of 0.008 Å, which clearly duplicates the estimate in [6]. Such a change in bond length would agree with the hypothesis [8] suggesting that the changes in optical absorption spectra in the visible and Soret region observed on *R*–*T* transition may be due to a stretching of this iron–histidine bond [15,16]. It would be interesting to determine whether in carp deoxyHb the transition produces the same change in the Fe(II)–N_ϵ (His–F8) bond in the α- and β-subunits.

The RR A line at 1352 cm^{-1} is the most intense RR line observed with 4579 Å excitation. It appears to be the most reliable marker of *cys* back-donation, i.e., the delocalization of charge from iron d_π orbitals to the porphyrin Eg (π^*) orbitals, because it is relatively insensitive to spin state changes of the iron. This Raman mode is due to the vibration that involves an in-phase displacement of 0.011 Å of the 4 pyrrolic nitrogen atoms toward the metal ion [18–20]. The shift observed in the A line as a result of the *T–R* transition is positive, i.e., in the *R* structure this line is situated at lower frequency and this indicates that the *R* state has an effective increase in the electron density of the anti-bonding π^* orbitals of the porphyrin ring, as concluded [9]. The change in the electron density of the anti-bonding π^* orbitals resulting from the *T–R* transition may be interpreted in terms of a general charge-transfer mechanism in which the charge in the π^* orbitals is increased in the *R* structure by transfer from an electron donor.

The donor of these electrons is a matter of controversy. It may be the iron atom and that the process is facilitated by the positioning of the iron atom in the plane of the porphyrin in the *R* state, as a result of the movement of the proximal histidine nearer the heme plane [7]. However, the donor could be the phenylalanine residues at CD1 and G5 [9]. Our results do not resolve this question. We obtain the same evidence for a shortening of the iron–histidine bond [7] but in carp hemoglobin aromatic residues are also found at both CD1 and G5.

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Note added in proof

We have recently learned that similar results on carp deoxy hemoglobin have been obtained at Bell Labs. [17].

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