Supplementary Information

**Tertiary Dynamics of Human Adult Hemoglobin Fixed in R and T Quaternary Structures**

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Detailed descriptions of the experimental setup and data acquisition for
time-resolved resonance Raman measurements

1. Experimental setup

Time-resolved resonance Raman measurements were performed with two
nanosecond pulse lasers operating at 1 kHz. Probe pulses at 436 nm were second harmonics of
power was as low as possible (0.5 µJ/pulse) in order to avoid ligand photodissociation by the
probe pulse. The pump pulses at 532 nm were generated with a diode-pumped Nd:YAG laser
(Megaopto, LR-SHG), the power of which was adjusted to 185 µJ/pulse. These pump and probe
beams were directed collinearly using a dichroic mirror and focused onto the sample cell with
spherical and cylindrical lenses. The timing between pump and probe pulses was adjusted with
a computer-controlled pulse generator (Stanford Research Systems, DG 535) via a GPIB
interface. The time delay of the probe pulse with respect to the pump pulse was determined by
detecting the two pulses with a photo-diode (Electro-Optics Technology, ET-2000) just before
the sample point and monitoring with an oscilloscope (Iwatsu, Waverunner DS-4262). Pulse
widths of the pump and probe pulses were 20 and 30 ns, respectively. Jitters in the delay time
were within ±10 ns. Sample solutions for time-resolved resonance Raman measurements were
contained in an airtight 10-mm φ NMR tube, and were spun with a spinning cell device rotating
at 1200 rpm at room temperature. Scattered light was detected with a liquid nitrogen-cooled
charge-coupled device camera (Roper Scientific, Spec-10:400B/LN) attached to a single
spectrograph (HORIBA, iHR550) equipped with a custom-made prism prefilter (Bunko Keiki).
The single spectrograph had a blazed-holographic grating with a groove density of 2400
grooves-mm⁻¹. The spectral slit width was 4.8-5.5 cm⁻¹. The wavenumber resolution was 0.5
cm⁻¹/CCD pixel. The spectra were calibrated using the standard Raman spectra of cyclohexane
and carbon tetrachloride.

2. Data acquisition

The time-resolved resonance Raman data acquisitions were conducted as follows. In
the forward scan of delay time, delay times were changed by increasing them. Measurements
in the backward scan of delay time were done following the same procedure except for the
direction of change in delay time. At each delay time, Raman signals were collected for three
20-second exposures with both the pump and probe beams present in the sample. This was
followed by equivalent exposures for pump-only, probe-only, and dark measurements.
Time-resolved Raman data were obtained by averaging the data for the 9–30 measurement
cycles described above depending on the signal-to-noise ratio and the amount of protein
sample. This method avoided the errors caused by a slow drift in laser power and allowed
quantitatively reproducible spectra from one day to the next, which was possible because of
the excellent long-term stability of the laser system. Neither smoothing nor binning of the CCD channels was employed for the spectra shown in the present paper.

**Data analysis**

The frequencies of $\nu$(Fe-His) and $\nu_4$ bands as well as the relative intensity of $\nu_6$ band are involved in Figure 3, Figure 6 and Figure S12. These data were obtained by the procedure illustrated as follows.

**1. $\nu_4$ frequency shift**

Raman difference spectroscopy was applied to determine the frequencies of $\nu_4$ bands in the time-resolved resonance Raman spectra. The principle of Raman difference spectroscopy was described in detail by Laane and Kiefer. Figure S1 gives an example. Spectra A and B have identical intensity ($I_A=I_B=I_0$) and width ($\Gamma_A=\Gamma_B=\Gamma$) except that their frequencies are separated by $\Delta$. In the difference spectrum (B-A), the peak-to-valley height of the derivative like curve is defined as $d$. Laane and Kiefer showed that $d/I_0$ and $\Delta$ exhibit a linear dependence when $\Delta$ is sufficiently small ($\Delta/\Gamma < 0.2$) regardless of the band shape. Therefore, the linear relationship between $d/I_0$ and $\Delta$ makes it possible to determine the sizes of frequency shift by measuring the peak-to-valley intensities of the Raman difference spectra.

![Figure S1. Example of Raman difference spectra. The bands in spectra A (green) and B (blue) are separated $\Delta$ by in frequency. The red trace is a difference spectrum (B-A).](image)

The calibration curve between $d/I_0$ and $\Delta$ was obtained by the method reported previously. Briefly, a background subtracted spectrum of WT rHb $[l(i)]$ was digitally shifted by $\pm j$ pixels to yield $[l(i\pm j)]$, as shown in Figure S2(A). The values of $d/I_0$ in the difference spectrum, $\Delta(l(i\pm j))= l(i, j)-l(i)$, were plotted against the values of $\Delta$, to which was converted from the pixel shift ($j$) as shown in Figure S2(B). For WT rHb at pH 8.0, we calculated the difference spectra of the time-resolved resonance Raman spectra with respect to the deoxy spectrum in Figure S2(C). We determined the sizes of the frequency shift by using the calibration curve. Spectra in Figure S2(C) were averages of three independent measurements. We calculated the standard deviations of the
frequency shift using the data of three independent measurements and found that the standard deviations were as small as 0.1-0.3 cm⁻¹. Thus, the Raman difference spectroscopy allowed us to determine the frequency shift of ν₄ band with the accuracy of 0.1-0.3 cm⁻¹.

![Figure S2](image1.png)

**Figure S2.** Illustration of the calculation method of the frequency shift of ν₄ band by Raman difference spectroscopy. (A) Observed (blue) and its digitally shifted spectra by +3 pixel (green). A difference spectrum between the green and red traces is shown in blue line. One pixel corresponds to 0.54 cm⁻¹ in the present experiments. (B) Plot of d/Δₒ in the difference spectra against the values of Δ, to which was converted from the pixel shift. (C) Time-resolved difference spectra of WT rHb in 50mM HEPES buffer at pH 6.4 containing 1 mM IHP. The difference spectrum at each delay time was obtained by subtracting the deoxy spectrum of WT rHb in the same buffer after normalizing the Raman spectral intensities with respect to the ν₄ band intensity.

2. ν(Fe-His) frequency

The peak positions of the ν(Fe-His) band gradually shifted downward as the delay time increased. We calculated the center of the ν(Fe-His) band in terms of the first moment of the bands, M₁, in the following way:

\[
M₁ = \frac{\sum_i I(ν_i)ν_i}{\sum_i I(ν_i)}
\]

The suffix i denotes the pixel number of the detector and I(ν_i) and ν_i represent Raman intensity and frequency at pixel i. Figure S3 shows the ν(Fe-His) band of WT rHb in the time-resolved resonance Raman spectrum at 100 μs. The moments were calculated for signal intensities above the baseline for each pixel.
**Figure S3.** Illustration of the calculation method of the first moment of $\nu$(Fe-His) band. In order to draw baselines, pixels where the band intensities were nearly zero were selected in both sides of the band as illustrated with a broken line. The averaged intensities over five pixels around the channel were calculated. The baselines were drawn by connecting the two points. Signal intensities above the baseline are shown in pale pink color. The green trace is a 100-μs time-resolved spectra of WT rHb in 50mM HEPES buffer at pH 6.4 containing 1 mM IHP.

**3. $\nu_8$ relative intensity**

In Figure S4, the calculation method of $\nu_8$ relative intensity is illustrated using the deoxy spectrum of WT rHb at pH 6.4 as an example. To separate the contribution of the $\nu_8$ band intensity, we decomposed the spectrum in the region of 325-392 cm$^{-1}$ in the following way. Baseline was subtracted from the time-resolved difference spectra. Then, the spectral intensity was normalized with respect to the intensity of the $\nu_7$ band. The baseline subtracted and normalized spectrum (green) is fitted with the sum of three Gaussian bands, which are due to the $\nu_8$, $\delta$(C$\beta$C$\gamma$C$\delta$), and an unassigned modes, respectively. The first component yields the $\nu_8$ intensity relative to the $\nu_7$ intensity.

**Figure S4.** Illustration of the calculation method of $\nu_8$ relative intensity. Deoxy spectrum of WT rHb in 50mM HEPES buffer at pH 6.4 containing 1 mM IHP is shown as an example. The baseline subtracted and normalized spectrum (green) is fitted with the sum of three Gaussian bands, which are due to the $\nu_8$, $\delta$(C$\beta$C$\gamma$C$\delta$), and an unassigned modes, respectively.
Vibrational modes of heme

Table S1. Assignments and vibrational characters of the resonance Raman bands of heme.

<table>
<thead>
<tr>
<th>Frequency / cm$^{-1}$</th>
<th>Deoxy form</th>
<th>CO-bound form</th>
<th>Mode</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1623</td>
<td>1635</td>
<td>$\nu_{10}$</td>
<td>$C_\alpha$--$C_m$ anti-symmetric stretching</td>
<td></td>
</tr>
<tr>
<td>1568</td>
<td>1586</td>
<td>$\nu_2$</td>
<td>$C_\beta$--$C_\beta$ stretching</td>
<td></td>
</tr>
<tr>
<td>1472</td>
<td>1499</td>
<td>$\nu_3$</td>
<td>$C_\alpha$--$C_m$ symmetric stretching</td>
<td></td>
</tr>
<tr>
<td>1357</td>
<td>1372</td>
<td>$\nu_4$</td>
<td>pyrrole half-ring stretching</td>
<td></td>
</tr>
<tr>
<td>673</td>
<td>675</td>
<td>$\nu_7$</td>
<td>pyrrole ring deformation</td>
<td></td>
</tr>
<tr>
<td>365</td>
<td>375</td>
<td>$\delta(C_\beta C_\gamma C_\delta)$</td>
<td>deformation of the propionate methylene group</td>
<td></td>
</tr>
<tr>
<td>342</td>
<td>353</td>
<td>$\nu_8$</td>
<td>metal–pyrrole stretching</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td></td>
<td>$\gamma_7$</td>
<td>out-of-plane methine wagging</td>
<td></td>
</tr>
<tr>
<td>217</td>
<td></td>
<td>$\nu$(Fe–His)</td>
<td>iron–His stretching</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Frequencies for WT rHb in 50 mM sodium phosphate buffer at pH 8.0.

$^b$ Designations of carbon atoms are shown in Figure S5.

Figure S5. Molecular structure of protoporphyrin IX and designations of carbon atoms.
Figure S6. Static resonance Raman spectra of the βD99N mutant and WT rHb in the CO-bound forms in 50 mM sodium phosphate buffer at pH 8.0 (A and C), and those of the βN102T mutant and WT rHb in 50mM HEPES buffer at pH 6.4 containing 1 mM IHP (B and D). In each panel, the blue, red, and green traces represent spectra of the βD99N and βN102T mutants, and wild type recombinant hemoglobin, respectively. The intensity of each spectrum in the 160-850 cm⁻¹ region was multiplied by 2 in Panels A and B. The spectra in the frequency regions of 160-900 and 1000-1700 cm⁻¹ were recorded in independent measurements. In all these panels, the accumulation time for each spectrum was 60 and 18 minutes for the low and the high frequency parts, respectively.
Figure S7. Expanded views of time-resolved resonance Raman spectra of the βD99N mutant (blue) and wild type recombinant hemoglobin (green) in 50 mM sodium phosphate buffer at pH 8.0 in the 180-260 (A), and 320-360 (B) cm$^{-1}$ region. All the spectra were normalized based on the intensity of the $\nu_7$ band.
Figure S8. Difference spectra between time-resolved resonance Raman spectrum at each delay time and that at 30-ns delay of the βD99N mutant (A) and of WT rHb (B) in 50 mM sodium phosphate buffer at pH 8.0. The accumulation time for each transient spectrum was 18 minutes.
Figure S9. Expanded views of time-resolved resonance Raman spectra of βN102T mutant (red) and wild type recombinant hemoglobin (green) in the 180-260 (A), and 320-360 (B) cm$^{-1}$ region. All the spectra were normalized based on the intensity of the $\nu_7$ band.
Figure S10. Difference spectra between time-resolved resonance Raman spectrum at each delay time and that at 30-ns delay of the βN102T mutant (A) and of WT rHb (B) in 50mM HEPES buffer at pH 6.4 containing 1 mM IHP. The accumulation time for each transient spectrum was 18 minutes.
Figure S11. Comparisons of the ν₃ bands of WT rHb and the Hb mutants. (A) The 30-ns and the deoxy spectra of the βD99N mutant (blue) and WT rHb (green) in 50 mM sodium phosphate buffer at pH 8.0. The difference spectra between the mutant and WT rHb were shown in grey color. Their intensities are magnified by 2. (B) The 30-ns and the deoxy spectra of βN102T mutant (red) and WT rHb (green) in 50mM HEPES buffer at pH 6.4 containing 1 mM IHP. The difference spectra between the mutant and WT rHb were shown in grey color. Their intensities are magnified by 2. (C) The time-resolved difference spectra between the βD99N mutant and WT rHb. (D) The time-resolved difference spectra between the βN102T mutant and WT rHb.
Figure S12. The inverse correlation between the frequencies of the $\nu$(Fe-His) and $\nu_4$ bands for the photodissociated form of rHb. Panel A shows the frequencies of the $\beta$D99N mutant (blue) and WT rHb (green) in 50 mM sodium phosphate buffer at pH 8.0. Panel B the $\beta$N102T mutant (red) and WT rHb (green) in 50mM HEPES buffer at pH 6.4 containing 1 mM IHP.
References