Electronic Supplementary Information

Time Scale of the Quaternary Structural Change in Hemoglobin Revealed by Transient Grating Technique

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Experimental Section

Sample Preparation

Human hemoglobin (Hb) purchased from Sigma-Aldrich was dissolved in 100 mM sodium phosphate buffer with pH 7.0 and the resulting solution was filtered to remove any aggregates (Whatman 0.2 μm). The protein concentration was adjusted to 300 μM. Hb solution was transferred to a quartz cuvette with a 2 mm optical path length and sealed tight by a rubber-top and plastic film. The solution contained in the cuvette was bubbled for 30 min by nitrogen gas to remove oxygen and then reduced by the injection of sodium hydrosulfite aqueous solution (1 M, 10 μL). CO gas was flowed in the reduced Hb solution for 30 min to ligate CO to Hb. The sample solution was freshly prepared and used immediately for each measurement.

Transient Grating (TG) Experiment

The experimental setup for TG measurement was similar to that reported previously.[1-3] The second harmonic (532 nm) of a Nd:YAG laser (Brilliant B, pulse width 6-7 ns) was used as an excitation beam and a photodiode laser (780 nm, Thorlabs) was used as a probe beam. The excitation beam was split into two equivalent beams, which intersected each other inside the sample. A signal was diffracted in the direction of the vector sum of the three beams. The diffracted signal (TG signal) was separated from the excitation beams by a glass longpass filter (Thorlabs FGL715S) and then was detected by a photosensor module (Hamamatzu H7732-10). The detected signal was digitized by a digital oscilloscope (Tetronix TDS 3052B). To get a sufficient signal-to-noise ratio, the signal was averaged 160 times. The repetition rate of the excitation beam was 2 Hz. The total energy of the excitation beams was adjusted under ~15 μJ to prevent multiphoton absorption. The size of the excitation beam was focused to ~1 mm diameter. The irradiated volume by the excitation laser was small compared to the total volume

of the sample. The TG signals were measured at various grating wavenumbers, q, which were varied by changing the angle between the two excitation beams. The rate constants of the thermal energy relaxation in each setup at various q were determined from the TG signal of the reference sample, $CoCl_2$, which releases the absorbed photon energy only as heat into the solvent without any photoreactions. By using the thermal decay rate and well-known thermal diffusion coefficient of the water, D_{th}, q values at each alignment were determined accurately.

Transient Absorption (TA) Experiment

The second harmonic (532 nm) of a Nd:YAG laser (Brilliant B, pulse width 6-7 ns) was used as an excitation beam and a continuous wave (cw) Xe-lamp (250 W) was used as a probe light source. The probe light was passed through the sample cell in perpendicular direction against the excitation laser beam. The TA signal was passed through a grating spectrometer (ACTON SpectraPro) and detected by an ICCD (Andor iStar). The observed wavelength region was from 400 nm to 460 nm and the time delays covered from 300 ns to 100 ms.

Singular Value Decomposition (SVD) was used for the analysis of TA spectra. The m by n matrix, **A** was composed by the measured TA spectra, where m is the number of the wavelength points in the absorption spectra and n is the number of the time delays. This matrix **A** could be decomposed to the separate matrices **U**, **S** and **V**. **U** is the left singular matrix of m by n dimension and each column vector of **U** is a time-independent spectrum. **S** is the singular value matrix and an n by n diagonal matrix. The diagonal value of **S** is called the singular values, which are related with the contribution of each singular vector **U** and **V** corresponding to the singular value. **V** is the right singular matrix of n by n and each column of **V** matrix is the temporal change corresponding to each time-independent spectrum. The large ith singular value, S_i means that the corresponding ith singular vector, U_i and V_i contributes to the TA spectra more dominantly. In our case, the first two singular values were large enough to be judged to provide reliable signal and used for the kinetic analysis. When we analysed the kinetics of the temporal change of TA spectra, two right singular vectors, V_1 and V_2 were multiplied by the corresponding singular values, S_1 and S_2, respectively, to correct the contribution of the each vectors. The resulting S_1V_1 and S_2V_2 were fitted together by using a sum of four exponentials and the resulting theoretical curves were well matched with the SVs, as shown in Figure S1. The decay time constants of 1.2 (±0.5) μs, 48 (±12) μs, 313 (±19) μs and 6.0 (±0.5) ms were determined by the fitting.

Theoretical Background of Transient Grating Method

The TG intensity (I_{TG}) is proportional to the sum of the squares of the refractive index (δn) and the squares of the absorption (δk) changes.[4, 5]

$$I_{TG} = \alpha\left[\delta n(t)\right]^2 + \beta\left[\delta k(t)\right]^2, \qquad \text{(S1)}$$

where α and β are constants determined by the experimental conditions.

As the reaction progresses, the population of the intermediates or the products increase and that of the reactant decreases. The population change of chemical species, which induces the difference of the refractive index and the absorption, contributes to δn and δk, and this contribution is usually called the population grating (δn_p and δk_p, respectively). On the other hand, if the intermediates or the products have different molecular volumes from that of the reactant, such volume differences will contribute to δn and δk, and this contribution is called the volume grating (δn_v and δk_v, respectively). These contributions, the population grating and the volume grating, are usually called the species grating. In addition, δn also depends on the diffusion of the thermal energy and the contribution of the thermal energy is called thermal grating, δn_{th}. Hence, the TG signal can be expressed as follows

$$I_{TG} = \alpha\left[\delta n_{th}(t) + \sum \delta n_{spe}(t)\right]^2 + \beta\left[\sum \delta k_{spe}(t)\right]^2, \qquad \text{(S2)}$$

where δn_{spe} and δk_{spe} are the refractive index changes and absorption changes corresponding the species grating, respectively. When δk_{spe} is negligible at the probe wavelength, I_{TG} is proportional to the square of δn only as follows

$$I_{TG} = \alpha\left[\delta n_{th}(t) + \sum \delta n_{spe}(t)\right]^2 \qquad \text{(S3)}$$

In general, δn_{th} originates from a temperature change in the medium caused by the thermal relaxation from the excited states and the enthalpy change during the reaction. If the heating process is fast enough to release the thermal energy, δn_{th} is given by

$$\delta n_{th}(t) = \delta n_{th} \exp(-D_{th} q^2 t), \qquad \text{(S4)}$$

where D_{th} is the thermal diffusion coefficient and q is the grating wavenumber. The species grating, δn_{spe} and δk_{spe} disappear not only by the reaction but also by the diffusion of the molecule. Therefore, the species grating decays with the rate constant of the reaction, k and/or the rate constant of the molecular diffusion, $D_{spe}q^2$, where D_{spe} is the diffusion coefficient of the corresponding molecular species. When the molecular diffusion coefficient is time-independent and a reaction during the detection time window is negligible, the species grating signal can be expressed by the molecular diffusion equation.

$$R \xrightarrow{h\upsilon} P$$

In this situation, the reactant (R) undergoes the product (P) after the irradiation of pump light as shown above. Hence, the temporal profile of the species grating signal can be expressed by

$$\sum \delta n_{spe}(t) = \delta n_P(t) - \delta n_R(t) = \delta n_P \exp(-D_P q^2 t) - \delta n_R \exp(-D_R q^2 t), \qquad \text{(S5)}$$

where δn_P and δn_R are the refractive index change of the product and the reactant, respectively. The sign of δn_P is positive, whereas the sign of δn_R is negative, because the phase of the spatial

concentration modulation of the product is 180° shifted from that of the reactant. Considering the condition of $D_P \approx D_R$, eqn (S4) can be expressed as follows.

$$\sum \delta n_{spe}(t) = \delta n_P(t) - \delta n_R(t) = (\delta n_P - \delta n_R)\exp(-D_R q^2 t) \qquad \text{(S6)}$$

Therefore, in this case, the decay of TG signal is a single exponential decay and the rate constant is induced from the diffusion of the molecular species.

Reference

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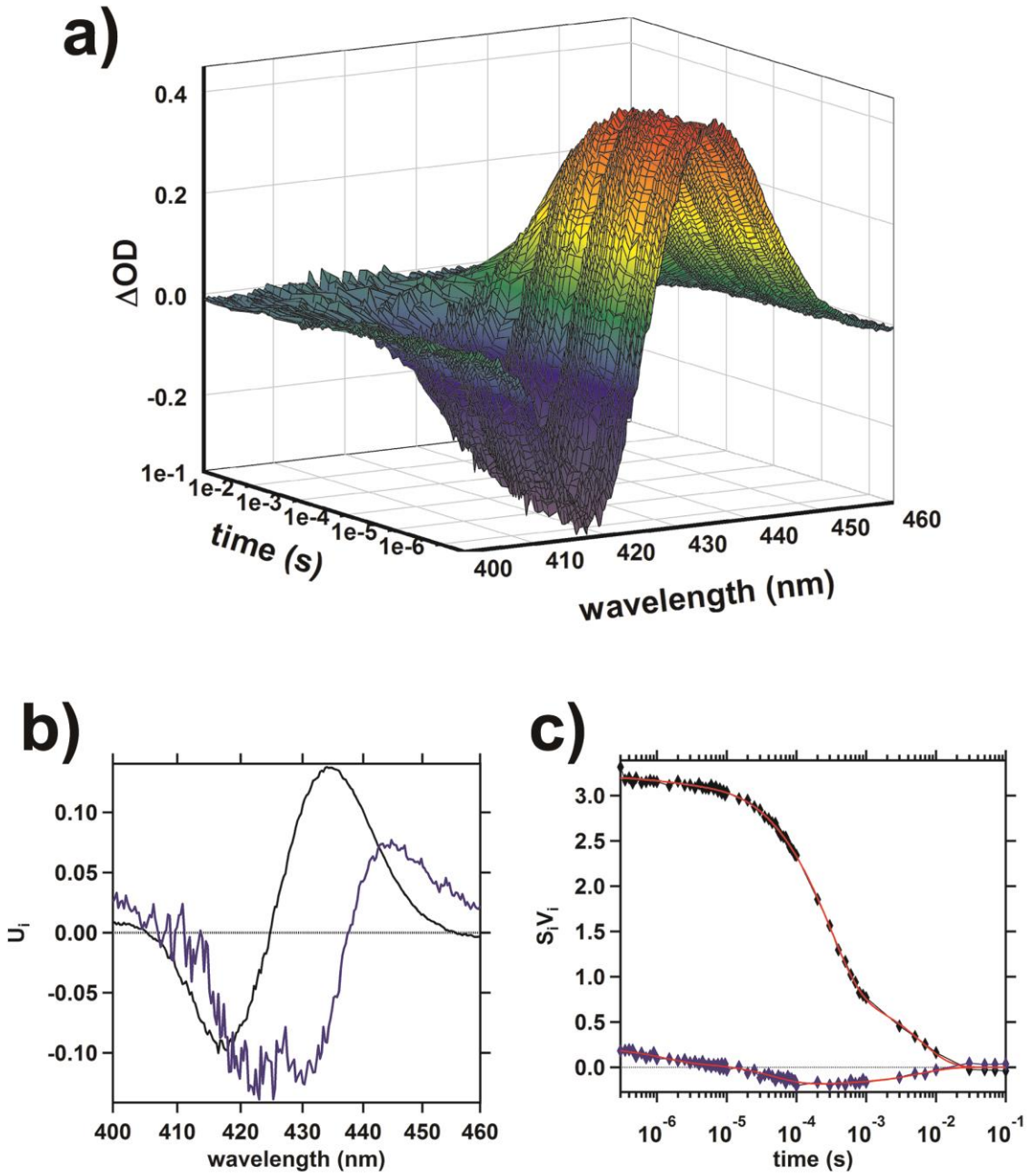

Fig S1. a) Transient Absorption (TA) spectra of 25 μM HbCO in 100 mM phosphate buffer (pH 7) excited at 532 nm. A spectral range of the measured wavelength was from 400 nm to 460 nm and time delays were from 300 ns to 100 ms. By using the singular value decomposition (SVD), TA spectra were analysed. As a result of SVD, only first two singular values are reliable. b) Time-independent left singular vectors (U_i). U_1 for the first singular value (black) and U_2 for the second singular value (blue). c) Time-dependent right singular vectors (V_i) multiplied with the singular values (S_i). S_1V_1 for the first singular value (black), S_2V_2 for second singular value (blue). Two *SV*s are fitted simultaneously by using the sum of four exponentials and the theoretical curves (red) are well-matched with the *SV*s. The decay time constants of 1.2 (±0.5) μs, 48 (±12) μs, 313 (±19) μs and 6.0 (±0.5) ms are determined by the fitting.