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Supporting Information

Protein Conformational Dynamics of Homodimeric Hemoglobin Revealed by Combined Time-Resolved Spectroscopic Probes

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Experimental Setup

Transient grating spectroscopy. The second harmonic (532 nm) of a Nd:YAG laser (Brilliant B, pulse width 6-7 ns) is used as an excitation beam and a photodiode beam (780 nm, Thorlabs) is used as a probe beam. The diffracted probe beam (TG signal) is isolated from the excitation laser beam with a glass filter (Thorlabs FGL715S) and a pinhole. The isolated TG signal is detected by a photosensor module (Hamamatzu H7732-10) with a photomultiplier tube (Hamamatzu R-928) and is digitized with a digital oscilloscope (Tektronix TDS 3052B). The repetition rate of the photoexcitation is set to 2 Hz. The energy of the excitation beam is kept below about 15 μJ to prevent multiphoton absorption. The TG signal is averaged 32 times to obtain a sufficient signal-to-noise ratio. The size of the excitation beam at the sample position is focused to be about 1 mm diameter. The laser-irradiated volume is small compared to the total volume of the sample solution. The sample cells are kept inside a temperature-controlled sample holder. All experiments are carried out at room temperature.

The value of the grating wavenumber (q) in the TG measurement is determined from the decay rate of the thermal grating signal of the reference sample, $CoCl_2$, because the energy of the photon absorbed by $CoCl_2$ is released as heat into the solvent with a quantum yield of unity.

Transient absorption spectroscopy. Transient absorption (TA) experiments are performed in 100 μM of HbICO in a phosphate buffer solution (pH 7) using the second harmonic (532 nm) of a Nd:YAG laser (Brilliant B, pulse width 6-7 ns) as an excitation source and a 250 W Xe lamp as the probing light source. The probing light, oriented perpendicular to the excitation laser beam, is passed through a grating monochromator, and is detected with a photomultiplier (Hamamatsu R-928) and a digital oscilloscope (Tektronix TDS 3052B).

Photoacoustic spectroscopy. The photoacoustic apparatus setup is similar to that reported earlier [23-27]. The second harmonic emission (532 nm) of a Q-switched Nd:YAG laser (Brilliant B, pulse width 6 - 7 ns) is used as the excitation source. A $CoCl_2$ solution is used as a reference sample for photoacoustic colorimetric measurements. The reference sample and HbICO sample absorptions are adjusted to be the same at 532-nm excitation. The

photoacoustic signals are monitored at the various temperatures ranges (275 K to 278 K). The photoacoustic signal is detected by a piezoelectric transducer (Panametrics V125-RM 2.25MHz), and then the signal is monitored with a digital oscilloscope (Tektronix TDS 3052B).

Principles

TG method. The TG intensity (I_{TG}) is proportional to the sum of the square of the refractive index (*dn*: phase grating) and/or the absorbance (*dk*: amplitude grating) differences between the peak-null of the grating pattern;

$$I_{TG}(t) = a\left[d\,n(t)\right]^2 + b\left[d\,k(t)\right]^2 \tag{S1}$$

where α and *b* are constants determined by the experimental conditions. The *dn* mainly comes from (i) the thermal releasing process ($dn_{th}(t)$) and (ii) the sum of refractive index changes of each chemical species ($\sum dn_{spe}(t)$):

$$d\,n(t) = d\,n_{th}(t) + \sum d\,n_{spe}(t) \tag{S2}$$

Therefore, all TG signals can be expressed as follows

$$I_{TG}(t) = a\left[d\,n_{th}(t) + \sum d\,n_{spe}(t)\right]^2 + b\left[\sum d\,k(t)\right]^2 \tag{S3}$$

Generally, the thermal grating is due to a temperature change in the medium induced by the liberated energy from the decay of excited states and by the enthalpy change of the reaction. If the heating process is fast enough to release the thermal energy, the temporal profile is given by

$$d\,n_{th}(t) = d\,n_{th}\exp(-k_{th}t) = d\,n_{th}\exp(-D_{th}q^2t) \tag{S4}$$

where D_{th} is the thermal diffusivity. A rate constant, equal to $D_{th}q^2$, is an indicator that assigns the signal to the thermal grating.

The species grating (dn_{spe}) consists of the following two contributions; (i) the population grating (dn_{pop}) and (ii) volume grating (dn_{vol}), where dn_{pop} and dn_{vol} are the refractive changes due to the change in the absorption spectrum and the change in the molecular volume, respectively ($dn_{spe} = dn_{pop} + dn_{vol}$). In the case of the HbICO dissociation reaction, the HbICO is dissociated to yield HbI and CO, and then HbI and CO recombine to recover HbICO. Hence, three chemical species, HbICO, HbI and CO, are involved in the species grating. Since the *D* of HbICO is very similar to that of HbI, the time profile of the species phase

grating is expressed by a bi-exponential function given by

$$d n_{spe}(t) = d n_{CO} \exp(-k_{CO} t) + d n_{HbI} \exp(-k_{HbI} t) \quad \text{(S5)}$$

where dn_{co} is the refractive index change induced by the presence of CO and dn_{HbI} is proportional to the difference between the refractive index changes due to the presence of HbI and of HbICO. The species grating signal disappears by the combination of a rate constant of Dq^2 (D; diffusion constant of the species) and a time constant of the recombination reaction (k_{rec}). Thus, the decay rate constants are given by

$$\begin{aligned} k_{CO} &= D_{CO} q^2 + k_{rec} \\ k_{HbI} &= D_{HbI} q^2 + k_{rec} \end{aligned} \quad \text{(S6)}$$

where D_{CO} and D_{HbI} are the diffusion coefficients of CO and HbI, respectively. On the other hand, the amplitude grating (dk) consists of a periodic grating pattern of a chemical species that absorbs the probe light. The contribution of CO vanishes in this case, because CO possesses no absorption at the probe wavelength. The time profile of the amplitude grating is given by

$$d k(t) = d k_{HbI} \exp(-k_{CO} t) = d k_{HbI} \exp(-(D_{CO} q^2 + k_{rec}) t) \quad \text{(S7)}$$

where dk_{HbI} is proportional to the difference between the absorption changes by HbI and HbICO.

Photoacoustic (PA) spectroscopy. In PA experiments, a pressure wave generated by the photoexcitation of a sample is detected by a piezoelectric transducer. The pressure wave is induced by the thermal expansion of the matrix and the molecular volume change. If a chemical reaction takes place (with a quantum yield of Φ), a reaction process can contribute to the overall volume change (? V_{total}).[1,2].

$$\begin{aligned} &? V_{\text{total}} = ? V_{\text{th}} + ? V_{\text{struct}} \quad \text{(S8)} \\ &? V_{\text{th}} = Q(\beta / C_p \rho) \end{aligned}$$

where ? V_{th} and ? V_{struct} are the solution volume changes due to thermal expansion and salvation/molecular structural changes respectively, Q is the amount of heat released to the solvent, β is the coefficient of thermal expansion, C_p is the heat capacity and ρ is the density. In this case, if the chemical reaction is completed within the instrumental response time, the PA signal intensity is expressed as follows.

$$S = E_a K ? V_{\text{total}} = E_a K(? V_{\text{th}} + ? V_{\text{struct}}) = E_a K[Q(\beta / C_p \rho) + ? V_{\text{struct}}] \quad \text{(S9)}$$

where E_a is the number of Einsteins absorbed by the sample and K is the instrument's

response constant . Since a calorimetric reference molecule such as $CoCl_2$ releases all the absorbed photon energy to the medium as a heat quickly without salvation/molecular structural changes, the PA signal intensity of the reference molecule is expressed by

$$R = E_a K E_{h?} (\beta / C_p \rho) \quad \text{(S10)}$$

where $E_{h?}$ is the photon energy at 532 nm. Thus, the ratio of the PA signal intensity of the sample to the reference molecule can be given as follows

$$f = S / R = [Q + ? V_{struct} (C_p \rho / \beta)] / E_{h?}$$

$$f \cdot E_{h?} = (S / R) \cdot E_{h?} = [Q + ? V_{struct} (C_p \rho / \beta)] \quad \text{(S11)}$$

As shown in Eq. (S11), a plot of $f \cdot E_{h?}$ versus $C_p\rho/\beta$ leads to a linear relationship with a slope corresponding to $? V_{struct}$ and an intercept equal to Q. For processes with a quantum yield (F) less than one, the reaction enthalpy and volume changes are obtained using Eq. S12 and S13, respectively.

$$? H = (E_{h?} - Q) / F \quad \text{(S12)}$$

$$? V = ? V_{struct} / F \quad \text{(S13)}$$

Diffusion coefficient of HbI calculated from the molecular weight. Young et al[3] reported that the size and volume of a protein can be determined from its molecular weight because the partial specific volume of a protein has a mean value of 0.73 cm^3/g, and they proposed an empirical equation to calculate the D of a protein.

$$D = 8.34 \times 10^{-8} \cdot \frac{T}{\left(h \cdot M^{1/3}\right)} \quad \text{(S14)}$$

where T is temperature, η is the solvent viscosity and M is the molecular weight of a protein. From Eq. S14, the D of HbI was calculated to be ~ 0.75×10^{-10} m^2s^{-1}.

References

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