Ultrafast Dynamics of Carboxy-Hemoglobin: Two-Dimensional Infrared Spectroscopy Experiments and Simulations

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Supporting Information

ABSTRACT: This Letter presents a comparison between experimental and simulated 2D mid-infrared spectra of carboxy-hemoglobin in the spectral region of the carbon monoxide stretching mode. The simulations rely on a fluctuating potential energy surface that includes both the effect of heme and the protein surroundings computed from molecular dynamics simulations. A very good agreement between theory and experiment is obtained with no adjustable parameters. The simulations show that the effect of the distal histidine through the hydrogen bond is strong and is directly responsible for the slow decay of the frequency–frequency correlation function on a 10 ps time scale. This study confirms that fluctuations in carboxy-hemoglobin are more inhomogeneous than those in the more frequently studied carboxy-myoglobin. The comparison between simulations and experiments brings valuable information on the complex relation between protein structure and spectral diffusion.

Understanding the structure and dynamics of proteins is an important challenge to elucidate their physiological function. In this respect, the development during the past two decades of time-resolved coherent nonlinear spectroscopy, in particular, in the mid-IR spectral range, has allowed to study the real-time dynamics of proteins on times scales ranging from femtoseconds up to picoseconds. In this context, two-dimensional IR spectroscopy (2D-IR) is a powerful tool to analyze the vibrational couplings, fluctuations, and structural changes occurring in biological systems.

Hemoproteins play numerous important physiological roles including transport and storage of oxygen. Hemoproteins complexed with CO have been widely studied in the past, both experimentally7–12 and theoretically,13–28 In particular, carboxy-myoglobin (MbCO) shows a complex dynamics with interconversion between different substates. These substates are identified spectroscopically through three subbands of the CO stretching vibration denoted A1, A3, and A2 located, respectively, at 1965, 1945, and 1932 cm−1. The origin of these subbands has been attributed to different positions of the distal histidine His64 strongly interacting with the CO molecule. It is generally believed that the A1 band corresponds to an imidazole being rotated out of the heme pocket, while the A2 and A3 bands correspond to an imidazole in the pocket but with two different orientations.28 Far less spectroscopic studies have been dedicated to resolve the dynamics of carboxy-hemoglobin (HbCO).8,29–32 Compared with MbCO, similar subbands have been observed in HbCO,7,33 but despite the higher complexity of the protein, the absorption spectrum is dominated by one band denoted CIII located at 1951 cm−1. The second most intense band denoted CIV located at 1969 cm−1 has an intensity varying from 1 to 10% of the CIII band intensity depending on the sample used.7,29,33 Dynamical differences between aqueous MbCO and HbCO have been observed through the frequency–frequency correlation function (FFCF) deduced from vibrational echo spectroscopy.32 The origin of these differences remains to be elucidated.

Most theoretical studies simulating the nonlinear optical response of molecular systems in liquid phase rely on a semiclassical model, which takes into account explicitly the coupling between a vibrational system and the environment through frequency fluctuations. These fluctuations are usually extracted from an equilibrium molecular dynamics (MD) simulation using a simple relation between the vibrational frequency and the electrostatic field generated from the environment.28,34–37 This relation is either parametrized from experimental data38 or extracted from electronic structure calculation.28 This approach is efficient to describe systems dominated by long-range electrostatic interactions. For MbCO, several studies have successfully used this approach to both describe the fast fluctuations and state interconversion of the A1 and A3 states1,12,28 by disregarding the coupling between the CO and the heme group. For HbCO, we have recently introduced a new model that describes directly the fluctuations

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The potential energy surface (PES) originating from the electrostatic environment and the heme group was used to simulate vibrational ladder climbing and coherent control. A similar approach has been used by Lee et al. to simulate the 2D-IR spectroscopy of cyanide in water. We present high-resolution 2D-IR measurements of HbCO, complemented with detailed simulations based on a similar model, as presented in ref 39. Our approach is based on a 1D parametrized PES $V(r_0, \{r_i\})$ and dipole moment $\mu(r_0, \{r_i\})$ as a function of the Cartesian coordinates of all protein atoms $\{r_i\}$ and the CO stretch coordinate $r_0$ (Figure 1). While the model from ref 39 relied on a normal-mode representation of the CO stretch in the heme group, the present model is based on a gas-phase representation of the CO stretch. This representation of the CO stretch was found to yield a more precise description than the previous parametrization. The exact forms of the parametrized potential and the dipole moment are given in the Supporting Information. An equilibrium MD simulation using the CHARMM22 force field of a single alpha-subunit of HbCO was performed. By analogy to the A$_1$ state of MbCO, an $N_r$ protonation state was chosen for the distal histidine. Using this simulation, one can then evaluate the time-dependent PES $V(r_0, t) = V(r_0, \{r_i(t)\})$ and dipole moment $\mu(r_0, t) = \mu(r_0, \{r_i(t)\})$ that fluctuate in time due to the protein environment. By solving at each time step the 1D Schrödinger equation for the PES, one can obtain the time-dependent frequency $\omega_{01}(t)$, anharmonicity $\chi(t) = \omega_{01}(t) - \omega_{12}(t)$ and transition dipole $\mu_{01}(t)$, which are then used to calculate the linear absorption and 2D-IR spectra, as well as correlation functions, as detailed in the Supporting Information.

In a similar way, as done by Fayer and coworkers for MbCO, different contributions from the fluctuating PES are separated to identify the main sources of fluctuations, in particular, from the distal His58 residue side chain (Figure 1) or from the remaining protein. The fluctuating PES is thus divided accordingly into

$$V(r_0, t) = V_{\text{intra}}(r_0, t) + V_{\text{His58}}(r_0, t) + V_{\text{other}}(r_0, t)$$

where $V_{\text{intra}}$ is the interaction stemming from intramolecular heme, $V_{\text{His58}}$ from the distal His58, and $V_{\text{other}}$ is from the rest of the protein. On the basis of this separation, three models are considered: M1, where all contributions are taken into account, M2, where $V_{\text{His58}}$ is suppressed, and M3, where both $V_{\text{His58}}$ and $V_{\text{other}}$ are suppressed. The normalized experimental and simulated absorption spectra of HbCO are presented in Figure 2. To directly compare the experiment and the simulation, the position of the maximum of the simulated absorption was aligned to the experiment. The experimental spectrum shows a peak centered at 1951 cm$^{-1}$ and a bandwidth of 8.5 cm$^{-1}$, a value slightly larger than the value of 8.1 cm$^{-1}$ measured by Zhao and coworkers. The simulated spectrum using model M1 shows a very good agreement with the experiment with a 8.2 cm$^{-1}$ bandwidth. The simulated absorption spectrum for model M2 has a bandwidth of 5.5 cm$^{-1}$, while for model M3 the bandwidth is 2.7 cm$^{-1}$. This shows that the contribution of the His58 is strong in the overall bandwidth. Moreover, it shows that the contribution of the heme vibrations to the overall bandwidth is small but non-negligible.

The normalized experimental and simulated 2D-IR spectra of HbCO for different waiting times $T$ ranging from 0.5 to 20 ps are presented in Figure 3. The simulations are performed using the full model (M1). The experimental 2D-IR spectra are composed of two main peaks corresponding, respectively, to the bleaching and stimulated emission (positive peak) and the excited-state absorption (negative peak). The frequency interval between the two peaks gives a direct measurement of the intramolecular anharmonicity, which we found to be 25 cm$^{-1}$, in very good agreement with previous measurements. At $T = 0.5$ ps, the two peaks in the 2D-IR spectrum have an elongated shape. As the waiting time $T$ increases, their shapes are modified toward a more circular shape. This is a well-known phenomenon that has been observed in many molecular systems and is a signature of the spectral diffusion due to fluctuations. As can be seen in Figure 3, the simulated spectra are in very good agreement with the experimental spectra. In particular, the simulated anharmonicity is found to be 25 cm$^{-1}$, in perfect agreement with the experiment, and the simulated line widths are very close to the experimental line widths. The main difference appears in the non-Gaussian shape of the spectra. The simulated spectra appear narrower on the red side, while the experimental spectra appear narrower on the blue side.

To quantify the agreement between theory and experiment, we have calculated the diagonal ($\Delta_d$) and anti-diagonal ($\Delta_s$) widths (fwhm) for the positive peak of the spectra. With this definition, $\Delta_d$ of a homogeneously broadened transition is related to the absorption bandwidth (fwhm) $\Delta$ by $\Delta_d = \Delta_s = \Delta$, and $\Delta_d$ of a homogeneous broadening transition is related to the absorption bandwidth (fwhm) $\Delta$ by $\Delta_d = \Delta_s = \Delta$. The main difference appears in the non-Gaussian shape of the spectra. The simulated spectra appear narrower on the red side, while the experimental spectra appear narrower on the blue side.
whereas for an inhomogeneously broadened transition, one finds $\Delta_d = \sqrt{2}\Delta$. On the basis of $\Delta_d$ and $\Delta_a$, one can define the ellipticity $\epsilon(T)$ by

$$\epsilon(T) = \frac{\Delta^2_d(T) - \Delta^2_a(T)}{\Delta^2_d(T) + \Delta^2_a(T)}$$  \hspace{1cm} (2)

which, similarly as other quantities such as the center line slope,\textsuperscript{46,49} gives directly the FFCF within the short-time approximation and if the system do not experience fast fluctuations in the motional narrowing limit.

From the 2D-IR spectra measured at different waiting times $T$ and calculated for all three models considered, $\Delta_d$ and $\Delta_a$ are extracted and shown in Figure 4, together with the ellipticity $\epsilon(T)$. At waiting time $T = 0.5$ ps, the experimental diagonal and anti-diagonal widths are 12.3 and 4.7 cm$^{-1}$, respectively. As the waiting time increases, the experimental diagonal width decreases to 10.9 cm$^{-1}$, and the anti-diagonal width increases to 7.2 cm$^{-1}$ at $T = 20$ ps. Consequently, the experimental ellipticity decreases from 0.74 at $T = 0.5$ ps down to 0.40 at $T = 20$ ps. This is a direct consequence of the spectral diffusion and characterizes the fluctuations experienced by the CO stretch. It also indicates that the system experiences slow structural fluctuations longer than 20 ps. The simulation using model M1 predicts diagonal and anti-diagonal widths slightly smaller than the experimental values, in particular, along the diagonal. For $T = 0.5$ ps, the simulated diagonal and anti-diagonal widths are, respectively, 10.0 and 4.0 cm$^{-1}$, while the widths at $T = 20$ ps are, respectively, 9.1 and 5.3 cm$^{-1}$. The simulated ellipticity computed from model M1 shows a good match with the measured ellipticity for a time $T < 10$ ps with a similar decrease rate; however, for a delay time $T > 10$ ps, the slope of the simulated ellipticity decreases while the slope of the experimental ellipticity remains strong. Note that for the larger delay time, $T$, the experimental signal-to-noise ratio has

strongly decreased due to population relaxation. Considering the different contributions, we find that for M2 the bandwidths are approximately reduced by a factor 0.67 with respect to
model M1, and for M3 they are reduced by a factor \(\sim 0.35\). These values are consistent with the results for the linear absorption. The simulated ellipticity computed from model M3 is significantly smaller compared with models M1 and M2. This is a clear indication that the fluctuations caused by the heme occur on shorter time scales.

The comparison between experiment and theory shows that including all contributions (model M1) not only correctly reproduces the fluctuation amplitude in the surrounding of the CO molecule but also reproduces the time scale on which these fluctuations occur. Many experimental and theoretical studies relied on a Gaussian stochastic description of the transition frequency \(\omega(t)\) to quantify these fluctuations. Such a process is fully described by the average frequency, \(\bar{\omega}\), and the FFCF defined by \(C(t) = \langle \delta \omega(t) \delta \omega(0) \rangle\), where \(\delta \omega(t) = \omega(t) - \bar{\omega}\). For each model, the FFCF was calculated from 0 to 40 ps and reported in Figure 5. All FFCFs show a very fast decay, followed by a slow exponential decay behavior with an oscillation with a period of 2.5 ps corresponding to a frequency followed by a slow exponential decay behavior with an

\[
C(t) = \Delta_1^2 + \Delta_2^2 \exp(-t/\tau_1) + \Delta_3^2 \exp(-t/\tau_2) \tag{3}
\]

where a constant term was added to describe slow fluctuations processes occurring on time scale longer than 40 ps. The results of the fitting are summarized in Table 1.

For all models, the fast decay is in the motional narrowing limit \(\Delta_1\tau_1 < 1\) inducing a strong homogeneous contribution to the spectra; therefore, we cannot directly compare the ellipticity decay with the FFCF. For the full model M1, the FFCF shows a very rapid decay on the 100 fs time scale followed by a slow decay on the 9.5 ps time scale. This FFCF computed here is a clear indication that the interaction between the CO vibration and the distal His58 is strong and responsible for a large portion of the fluctuations and that the fluctuations induced by the motion of the His58 side chain occur on longer time scales as compared with the other fluctuations. This is similar to what has been observed in the case of MbCO with the distal His64. Removing completely all of the contributions from the protein and only keeping the heme vibration (model M3), the amplitude is reduced by \(\sim 35\%\) with respect to the full model M1, and the slow decay is reduced to \(\sim 1.2\) ps. Note also that the fast decay below 20 fs cannot be evaluated due to the temporal resolution used in the calculation of the correlation functions. Note that in the case of model M3, both exponential decay terms are in the motional narrowing limit \(\Delta_1\tau_1 < 1\) and \(\Delta_2\tau_2 < 1\).

As for His64 in MbCO, the interaction between the distal His58 side chain and the CO vibration in HbCO plays a key role due to a hydrogen bond between the H_\text{原子} atom of the distal Histidine and the CO molecule. To extract structural and dynamical information on this side chain and, in particular, on the hydrogen bond, we have quantified the correlation between the position of distal His58 with respect to the CO molecule and the CO vibrational frequency. We have used the distance, \(d_{O-H_\text{distal}}\), between the H_\text{原子} atom of the His58 side-chain and the O atom of the CO molecule as a tracer of the position of the distal His58 side chain and the hydrogen-bond strength (Figure 1). Figure 6a show the 2D density \(p(\omega, d_{O-H_\text{distal}})\) computed from the MD simulation. This density was obtained by a 2D histogram based on \(6 \times 10^6\) snapshots, with bins of \(\Delta \omega = 6\) cm\(^{-1}\) and \(\Delta d = 0.025\) Å. The density shows a clear correlation between the fundamental vibrational frequency \(\omega\) and the distance \(d_{O-H_\text{distal}}\), showing that the proximity of His58 to CO and a shorted hydrogen bond strongly modify the vibrational frequency. Figure 6a we have also indicated the maximum of the density along the frequency axis, \(\omega_1\), as a function of \(d_{O-H_\text{distal}}\), and the maximum of the density along the distance axis, \(d_{O-H_\text{distal}}\), as a function of the frequency, \(\omega\). Both curves meet at the maximum of the distribution. The average of the tangents of these two lines at the maximum of the density was used as an estimate for a linear correlation fit according to \(\Delta \omega = \alpha(d_{O-H_\text{distal}} - d_0)\), with \(\alpha = 47\) cm\(^{-1}\)/Å and \(d_0 = 2.7\) Å. The effect of the hydrogen bond on the frequency of CO stretch vibration is known, in particular, in the context of amide-I vibration in peptides. For the amide-I vibration a similar linear shift as a function of the distance has been found with \(\alpha = 30\) cm\(^{-1}\)/Å. Using perturbation theory, one can show that the frequency shift of an anharmonic oscillator coupled to an external force depends linearly on the force and is proportional to the square-root of the anharmonicity of the oscillator. For the amide-I vibration, the anharmonicity has been measured to be 16 cm\(^{-1}\), a value smaller than the 25 cm\(^{-1}\) measured for HbCO. Therefore, the effect of the hydrogen bond on the CO vibrational frequency is stronger in HbCO.

Figure 6b shows the distance-distance correlation function, which directly characterizes the dynamics of the hydrogen bond, defined by \(D(t) = \langle \delta d_{O-H_\text{distal}}(t)\delta d_{O-H_\text{distal}}(0) \rangle\), with \(\delta d_{O-H_\text{distal}}(t) = d_{O-H_\text{distal}}(t) - d_{O-H_\text{distal}}\) where \(d_{O-H_\text{distal}}\) is the average distance. As for the FFCF, this correlation function shows also a clear
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Figure 6. (a) Correlation plot between \(d_{O-H_e}\) distance and the fundamental frequency \(\omega\). Maximum of the density along the frequency axis \(\omega\) as a function of \(d_{O-H_e}\) and the maximum of the density along the distance axis \(d_{O-H_e}\) as a function of the frequency \(\omega\) are reported as dashed lines. The average of the tangents of these two lines at the maximum is reported as a solid line. (b) Distance \(d_{O-H_e}\) correlation function (red line) with the corresponding fit (blue line).

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The human carboxy-hemoglobin samples (heme concentration 10 mM) are prepared in a Tris-HCl buffer made with D2O (pH 7.6) and an excess of sodium dithionite, kept under CO atmosphere, and mounted as 100 \(\mu\)m films between calcium fluoride windows. Femtosecond mid-infrared pulses of spectral width 150 cm\(^{-1}\) fwhm are generated by difference frequency mixing of the signal and idler pulses produced by a titanium-sapphire pumped optical parametric amplifier. 2D-IR spectra are measured with a home-built 2D spectrometer in the pump–probe geometry. The transmitted probe spectra are measured with a silicon CCD by chirped-pulse upconversion, and corrected for cross-phase modulation as described elsewhere. The time delay \(\tau\) between the pump pulses is fastscanned over a total range of 30 ps, and data are averaged over 20 consecutive scans for each waiting time, allowing the retrieval of the 2DIR spectrum with a spectral resolution along the \(\omega\) axis of \(\sim 1.1\) cm\(^{-1}\).

# EXPERIMENTAL METHODS

Detailed description of the computational methods. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.5b00811.

# ASSOCIATED CONTENT

Supporting Information

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Notes

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