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Multiply Excited Vibration of Carbon Monoxide in the Primary Docking Site of Hemoglobin Following Photolysis from the Heme

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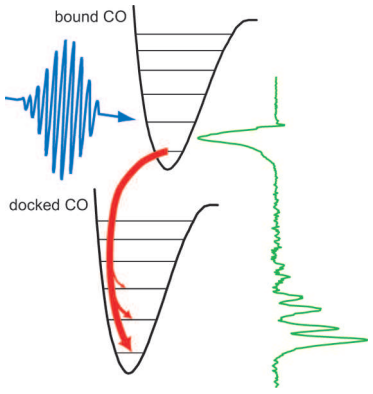
ABSTRACT We investigate ultrafast vibrational ligand dynamics in carboxyhemoglobin using chirped pulse upconversion and demonstrate the formation of vibrationally multiply excited carbon monoxide trapped in the primary docking site of hemoglobin after photolysis. The bleach signal due to ligand dissociation and the incipient docking-site absorption signal are about 200 cm^{-1} apart and differ by more than an order of magnitude in absorbance. In conventional approaches, these signals are monitored individually. Our method allows simultaneous observation of these signals with both high spectral resolution and high sensitivity. The large amount of vibrationally hot CO in the docking site as observed under Soret band excitation of the heme is discussed in the context of excess energy provided by the pump photon and is shown to be in quantitative agreement with predictions based on changes in the CO equilibrium distance upon instantaneous dissociation.

SECTION Biophysical Chemistry



The binding of diatomic ligands in heme proteins plays an important role, for example, for ligand transport, sensing, and storage. An ultrafast visible pump/mid-infrared (MIR) probe experiment can reveal the path of photolyzed ligands away from the heme, which may involve a docking site (or several) where the ligand is intermittently trapped. The heme proteins studied in most detail are myoglobin and hemoglobin, pioneered in ultrafast MIR studies by Anfinrud, Lim, and co-workers for the ligand CO. They observed the bleaching of carboxyhemoglobin (HbCO) after photolysis[1] and the CO absorption in the primary docking site of carboxymyoglobin (MbCO) and HbCO,[2] where the CO exists in two (presumably antiparallel) orientations parallel to the heme plane and hence almost perpendicular to the Fe-bound orientation.[3−5] Furthermore, a rise time for the docking-site absorption signal[6] and a spectral evolution due to interconversion of the two CO orientations[7] and to conformational changes of the protein[8] were reported, while the total amount of docked CO remained constant for hundreds of picoseconds. Our group further observed a progressive change of the absorption strength after CO photolysis[9] and demonstrated coherent MIR emission in MbCO[10] and coherent vibrational climbing in HbCO.[11]

Although MIR pulses can have sufficiently broad spectra, the photobleach of Fe-bound CO and the absorption of docked CO have never been recorded simultaneously with a single probe pulse. Instead, they are measured individually, usually even with MIR probe beams that are spectrally shifted between the experiments. There are two main reasons; first, to measure both signals, a 200 cm^{-1} spectral range has to be covered, with bands exhibiting a full width at half-maximum (fwhm) of less than 10 cm^{-1}. Common HgCdTe detectors for the MIR have 128 pixels or less; therefore, the best spectral resolution is achieved by covering one of the signals only. Second, the docking-site signal is more than 1 order of magnitude weaker than the bleach,[2] necessitating a detector with both a high sensitivity and a large dynamic range.

These issues can be circumvented with the chirped pulse upconversion (CPU) technique[12] advanced in this Letter. In CPU, the MIR probe spectrum is shifted into the visible spectral regime and phase-corrected;[13] therefore, very sensitive charge-coupled device (CCD) arrays can be used. The thousands of pixels and the 16 bit dynamic range of CCDs help to make ultrafast vibrational spectroscopy easier and more powerful. Using this method, we demonstrate the simultaneous observation of photobleach and docking-site trapping for HbCO and further reveal the existence of multiply excited vibrational states of docked CO after photolysis from the heme. The results are discussed in the framework of quantum mechanical calculations, taking into account the change in CO equilibrium distance due to photolysis.

To cover the bleach and the docking-site absorption simultaneously, we adjust the MIR probe spectrum so that its maximum is in the region of the docking-site absorption, while it is much weaker at the frequency of the bleach (Figure 1a). This

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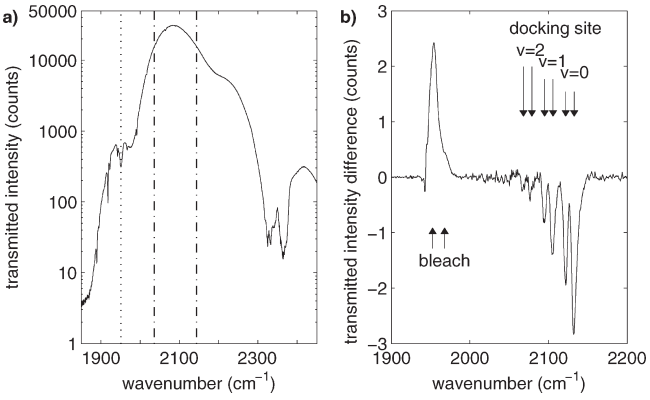

Figure 1. (a) MIR probe spectrum transmitted through the HbCO sample in the absence of a pump pulse. Its fwhm is indicated by dot−dashed lines, and the HbCO steady-state absorption is indicated by a dotted line. Further features are atmospheric lines of water vapor (narrow bands)[13] and CO_2 (antisymmetric stretching at around 2350 cm^{-1}). (b) The difference in transmitted intensity ΔI (after baseline correction) for a 4 ps delay between 400 nm pump and MIR probe pulses with parallel polarizations. The bleach and the docking-site absorption (with several vibrational states) exhibit similar signal levels.

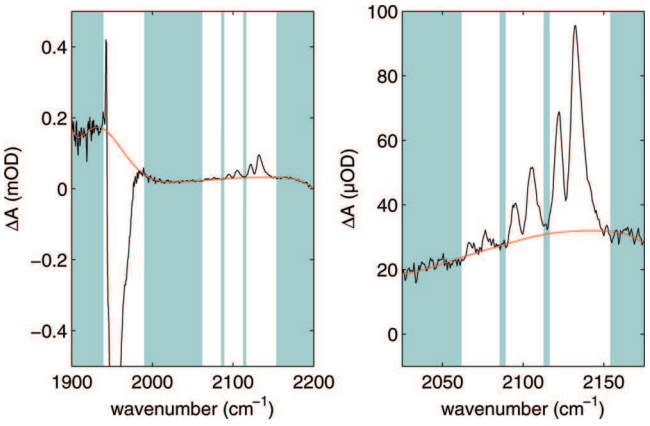

Figure 2. Raw transient absorption data of HbCO pumped at 400 nm and probed in the MIR with a delay of 4 ps. The present baseline is removed by a polynomial fit (red, 10th order) to regions without signal (gray shades). The right panel shows a magnification of the docking site region.

approach has the advantage that the difference in transmitted intensity ΔI for pumped and unpumped sample is of the same magnitude in absolute counts for both signals (Figure 1b). The bleach comprises the A_1 band at around 1951 cm^{-1} and the 1 order of magnitude weaker A_0 band at 1968 cm^{-1} (indicated by the arrows in Figure 1b). These two structural substates correspond to two orientations of the distal histidine in the heme pocket, causing two distinct absorption bands of the Fe-bound CO.[14] At the docking-site signal, almost 200 cm^{-1} to the blue, the two possible antiparallel CO orientations give rise to the higher-frequency B_1 band and the lower-frequency B_2 band. The copy of these B bands with lower amplitudes is attributed to vibrationally hot ($v = 1$) CO molecules,[2,15] with a red shift arising from the anharmonicity of the vibrational ladder of CO. This lowest hot band for docked CO decays with a population relaxation time T_1 of 600 ps[15] to the vibrational ground state of docked CO, while in the case of Fe-bound CO, T_1 for the first vibrationally excited state is as short as 25 ps.[11] In our data, a second copy of the B bands evidences the existence of even higher vibrational excitations ($v = 2$) of docked CO, as discussed in detail below.

Converted to transient absorption ΔA, the signals are very small, for example, the B_2 ($v = 2$) band is $< 4\ \mu$OD. A small baseline, yet of comparable magnitude to the low signals, is present in the data (Figure 2). There are several origins to this baseline; first, although the sample volume is exchanged for each laser shot, the pump pulse deposits energy in the sample, which gives rise to a varying thermal baseline due to heating of and diffusion in the solvent, which is very sensitive to alignment.[16,17] Note also that the transmission of a 100 μm film of H_2O in the spectral region used here is only a few percent.[18] Second, small contributions from nonstatistical fluctuations in the laser intensity also contribute to a varying baseline on a sub-mOD scale, as experiments with no sample have shown. Third, there is also a contribution depending on the studied protein system. Anfinrud and co-workers have also observed polynomial backgrounds for both A and B bands, where the latter was partially attributed to a broad absorption of unconstrained photolyzed CO molecules.[2]

To remove this baseline, a polynomial fit is subtracted from the signal, as illustrated in Figure 2 (here for ΔA; for ΔI, the results are quasi-identical). To ensure that no dynamics related to the system under study are removed by this procedure, those regions where transient absorption signals are expected are left out for the fit. Thus, an assumption has to be made about where one expects a signal, a disadvantage for measurements with completely unknown systems.

The simultaneously obtained transient absorption data of both bleach and docking-site absorption in HbCO after baseline correction are shown in Figure 3a, revealing the difference in absorptivity of the signals. Integrating the area under the A bands and comparing to the integral of all B bands, we find a ratio of 29 for the case of parallel polarizations (and ~53 if calculated to the magic angle). A direct reference for HbCO in H_2O is not available, but a value of 33 is given in ref 2, albeit for Hb^{13}CO in D_2O pumped with 527 nm pulses and probed under the magic angle at a delay of 100 ps. For MbCO, several values between 21 and 34 are reported.[2,9]

By magnifying the docking-site absorption (Figure 3b), the existence of CO molecules in hot vibrational states is further analyzed. A fit yields a splitting of 10.5 cm^{-1} between the maxima of the B_1 and B_2 bands and an anharmonicity of 27.5 cm^{-1}, agreeing well with other studies.[2] From this fit, we further deduce the populations in the vibrational bands to be 85% ($v = 0$), 13% ($v = 1$), and 2% ($v = 2$), taking into account that in the harmonic oscillator approximation, the absorbance scales with $v + 1$.

Two findings merit explicit notice; on the one hand, the docked CO is surprisingly hot compared to earlier studies, and on the other hand, no observation of $v = 2$ in the docking site has been reported at all so far.

While $v = 1$ populations of 15% for docked NO have been observed in MbNO a few picoseconds after Q-band excitation of the heme,[19] studies on MbCO and HbCO in various

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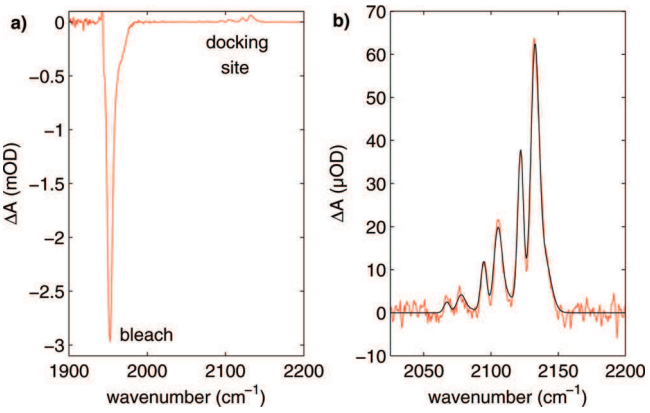

Figure 3. Transient absorption spectrum of HbCO pumped at 400 nm and probed in the MIR with a delay of 4 ps, after baseline correction. (a) Both the photobleach signal and the docking-site absorption are observed simultaneously. (b) A magnification of the docking-site absorption reveals the existence of the multiply excited CO vibration. The black line is a fit to the data.

solvents showed a $v = 1$ population of only ~4 %[2,15] (at a delay of 100 ps, which however means less than 5 % at a delay of 4 ps as used here). The Q-band absorption corresponds to an electronic transition to the heme's S_1 state which crosses repulsive states, leading to a CO ligand dissociation in less than 50 fs,[20] so that a preceding internal vibrational energy redistribution (IVR) is unlikely, as also concluded from time-dependent density functional theory studies.[21] The CO furthermore does not achieve a thermal equilibrium with the heme after photolysis, a conclusion drawn in a combined experimental and theoretical study,[15] where the docked CO also turned out to be much hotter than expected if the photon energy exceeding the one necessary for CO photolysis were distributed equally over all of the possible degrees of freedom before dissociation occurs. In other words, the photolyzed CO gets a larger share of vibrational energy than can be explained by pure thermalization processes.

In our experiments, 400 nm pump pulses excite the heme to the S_2 state, from where nonradiative decay leads to dissociative states and bond breaking probably occurs within 20 fs.[22] Being the only difference, we attribute the larger hot populations observed in our study to the excitation of the Soret band rather than the Q-band excited in previous studies. Two scenarios might provide an explanation; (i) since the CO gets a big share of the excess energy in the dissociation process, the vibrational energy will be even larger if this excess energy is larger. The initial nonradiative decay from S_2 to the repulsive states after Soret excitation gives rise to the population of hotter vibrational states of CO, which cannot cool before dissociation occurs. (ii) the share of excess energy is not the prevalent aspect, but rather the reaction speed from bound to unbound CO molecules. Assuming that this transition proceeds faster for Soret band than that for Q-band excitation, the former might be regarded as "more impulsive" and hence may lead to a different vibrational distribution in the photolyzed CO. A further scenario, a significant contribution from vibrational climbing[11] in the docking site induced by the probe pulse itself is ruled out since no such effect is observed for Fe-bound CO, despite the much larger absorptivity compared to that of docked CO.

Considering the limit of scenario (ii), an instantaneous transition from bound to unbound CO, we estimate the population distribution by using the change in the CO equilibrium distance x_e upon photolysis. Franzen[23] numerically determined $x_e^f = 1.141$ Å for free CO and $x_e^b = 1.169$ Å for Fe-bound CO in an iron−imidazole−carboxyporphine complex with H_2O, considering additional interactions of the CO with an imidazole. Starting with a rough estimate, we take a quantum harmonic oscillator with 1951 cm^{-1} ($\omega_e = 367.5$ rad/ps) transitions and calculate the square of the vibrational wave function overlap integral, that is, the equivalent of Franck−Condon factors, for the Fe-bound CO ground state and the nth state of unbound CO. This results in $\Delta\xi^{2n}e^{-\Delta\xi^2/2}/(2^n n!)$ for the case of a pure displacement, with $\Delta\xi^2 = \mu\omega_e(x_e^f - x_e^b)^2/\hbar$ and the reduced mass μ. This approach yields populations of 86, 13, and 1 %, which agrees remarkably well with our data.

Performing a more profound comparison, we evaluate these factors again, this time using the exact wave functions[24] of the Morse potential $V(x) = D_e[e^{-2\beta(x-x_e)} - 2e^{-\beta(x-x_e)}]$. Devereux and Meuwly recently showed that Morse potentials are essential to describe CO vibrations in MbCO.[25] Taking their Morse constants for Fe-bound CO ($D_e = 230.0$ kcal/mol, $\beta = 2.1$/Å), values for free CO ($D_e = 249.65$ kcal/mol, $\beta = 2.34$/Å, reported in ref 26), and Franzen's x_e values, one finds 85, 14, and 1 %, which is very close to the above calculation. Thus, a derivation of vibrational populations from Morse potentials of Fe-bound and unbound CO molecules can reproduce our experimental data.

For a further demonstration of the benefits from CPU, we simultaneously record a spectrotemporal map while keeping the measurement time low (Figure 4a, for which the pump−probe delay is scanned for 1 h around $\tau = 0$ and ΔI is plotted to have signals of comparable magnitude). The bleach at 1951 cm^{-1} is preceded by an oscillatory feature, the perturbed free induction decay (PFID),[27,28] leading to an exponential rise with the vibrational dephasing time T_2 for $\tau < 0$, while there is no PFID for the B bands. Signals of pump-created absorption bands exhibit a rise time which is determined by the instrument response function and true population rise times intrinsic to the system,[28] as becomes evident by comparing the transient absorption of the A_1 and the B_1 ($v = 0$) bands at 1951 and 2132 cm^{-1}, respectively (Figure 4b). The solid black curve models the data at 1951 cm^{-1} using $T_2 = 1.15$ ps,[29] while the dashed curve is identical except for a flipped sign. The dynamics of the B_1 ($v = 0$) state (red points, magnified by a factor of 71 to allow a comparison) set in around $\tau = 0$ and exhibit a rise time of 1.69 ps (see fit, green line), which agrees well with the 1.6 ps (shown for comparison as the blue line) reported in ref 6 for MbCO due to protein reorganization around the docked CO.

In conclusion, we have revealed the existence of multiply excited vibrational states of docked CO after photolysis in HbCO by making use of the benefits of CPU, namely, both high resolution and sensitivity over a broad spectral range. The observed hot populations are a consequence of the Soret band excitation of the heme and agree with quantum

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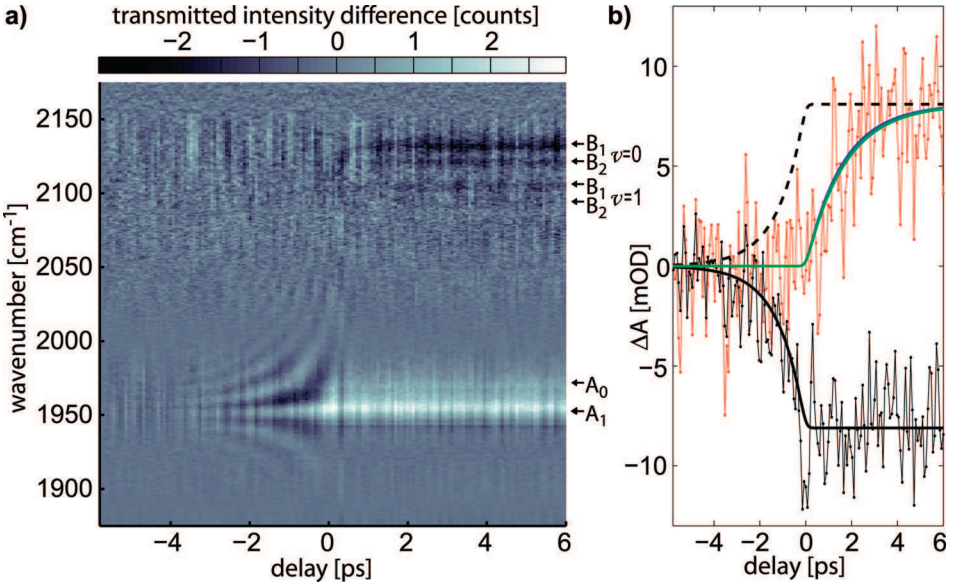

Figure 4. Pump−probe scans with 400 nm pump and MIR probe pulses with perpendicular polarizations. (a) Spectrotemporal map of ΔI. The bleach (bright, bands A_0 and A_1 indicated by arrows) with a preceding PFID is evident, as are the docking-site absorption signals (dark, bands B_1 and B_2 indicated by arrows for vibrational levels $v = 0$ and 1). (b) Transient absorption signal at 1951 (A_1, black data) and 2132 cm^{-1} (B_1, scaled red data). The black curve models the black data and is also shown multiplied by −1 (dashed black curve). The best fit to the red data is shown in green, while the behavior reported in ref 6 is shown in blue.

mechanical calculations taking into account the change in the CO equilibrium distance after photolysis. We further demonstrated the simultaneous observation of the bleach from ligand dissociation and the absorption from the docking-site, confirming effects which are usually studied separately in the different spectral regions and over a longer time, like the relative absorption strengths or the delayed appearance of docked CO. CPU has the potential for a more direct observation and concurrent analysis of ultrafast processes associated with weak absorptions in the MIR. Especially for proteins and mutants which are only available in small quantities or are just stable for short periods of time, CPU is a valuable alternative to more established techniques.

EXPERIMENTAL METHODS

The protein samples (protein concentration 5 mM) are prepared in a tris-HCl buffer made with H_2O (pH = 7.6) and an excess of sodium dithionite, kept under CO atmosphere and mounted as 100 μm films between calcium fluoride windows.

A part of the laser beam from a regenerative amplifier (800 nm, 1 kHz) is split off before the compressor, so that positively chirped pulses (120 ps fwhm) are obtained. The other part is compressed and used to generate 400 nm pump pulses and to pump an optical parametric amplifier followed by a difference frequency stage to generate MIR probe pulses whose spectrum is adjusted to cover all of the expected signals at once (Figure 1a). Pump and probe pulses are noncollinearly overlapped in the sample (in a rotating holder), and the temporal overlap is determined in a GaAs wafer between identical windows, yielding a time resolution of about 200 fs. The pump pulse energy is set to 250 nJ (280 nJ), the angle between pump and probe polarization is set to 0° (90°), and every second (third) pulse is chopped for the experiments shown in Figures 1−3 (Figure 4), respectively. We then use CPU,[12] as recently introduced to transient absorption[30,29] and multidimensional[31−34] spectroscopy, where the MIR probe pulses are upconverted to the visible with the chirped 800 nm pulses in a MgO:$LiNbO_3$ crystal. The chirp is subsequently corrected,[13] and a straightforward frequency calibration by comparison to atmospheric absorption lines is performed.

For the scan of Figure 4, the motor was continuously moved back and forth, and the data were fractionally binned[33] to 75 fs steps. For each pump−probe delay value (except for the turning points), on average, 6300 spectra with a pumped volume were recorded. For the fit in Figure 3b, a model with three Gaussians for the vibrational ground state was used.[2] The hot bands are considered as shifted and scaled replicas of the $v = 0$ band, resulting in a total of 12 fit parameters.

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