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Dynamics in globular proteins: vibrational echo experiments

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Abstract

The temperature-dependent vibrational pure dephasing of the CO stretching mode of carbonmonoxyhemoglobin (HbCO) in an ethylene glycol:water mixture is reported and compared to previously measured dephasing of carbonmonoxymyoglobin (MbCO). HbCO displays a $T^{1.3}$-dependent pure dephasing rate between 15 and $\sim$150 K, suggesting glass-like behavior. Above 150 K, the temperature dependence becomes steeper. The functional form of the HbCO and MbCO pure dephasing temperature dependences are identical within error. However, the hemoglobin pure dephasing is 27% smaller at all temperatures studied, suggesting that the fast dynamics or the protein electric field–CO coupling is smaller in hemoglobin than in myoglobin.

1. Introduction

Protein motions on atomic distance scales and on all time scales are a critical link between equilibrium protein structure and protein function. Dynamical measurements on various times scales can yield insights into these processes. One common technique employed is to use ligands or other reporter molecules to probe protein motions [1–3]. On ultrafast time scales, nonlinear optical techniques have been extensively employed at very low temperatures [4–7]. The vibrational echo technique provides a new approach for the study of fast protein dynamics using an ultrafast, nonlinear experiment. The measurements, from low temperatures to biologically relevant temperatures, can provide insights into protein dynamics and interactions between the protein and a ligand bound at the active site [8–11].

The vibrational echo measures the pure dephasing of a vibration. Pure dephasing is a measure of a vibrational mode's energy level fluctuations. The fluctuations are induced by the interactions of the vibrational oscillator with its dynamic environment. For a solute oscillator in a solvent, pure dephasing reflects the solvent dynamics and nature of the solute–solvent intermolecular interactions. For protein oscillator (a vibrational mode of a particular group of the protein) pure dephasing is related to the dynamics of the protein and the manner in which the protein dynamics are communicated to the oscillator. Recently, vibrational echo experiments have been presented that provide information on the temperature-dependent dynamics of myoglobin. The experiments investigated the pure dephasing of CO bound at the active site of myoglobin (MbCO) [8–12]. Here, the experiments are extended to the study of CO bound at the active sites of human hemoglobin (HbCO).

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Human hemoglobin A (Hb) is a tetrameric protein composed of two α and two β subunits. The intrinsic differences in the subunits' ligand affinities and the interactions between them are necessary for the proper functioning of Hb [13]. When isolated, the subunits exhibit different behavior from that of the $\alpha_2\beta_2$ tetramer [3].

The CO stretch of carbonmonoxy hemoglobin (HbCO) at room temperature displays a dominant peak, C_{III} at ~ 1951 with a FWHM of ~ 8 cm^{-1}. From difference absorption spectroscopy, it is known that the subunits have slightly different absorbances; the α subunit's maximum absorbance is at 1950.5 cm^{-1}, and the β subunit's maximum absorbance is at 1951.6 cm^{-1} [3]. This difference indicates that the CO ligand's environments are not identical in the two subunits. The Mb protein has three distinct CO stretching modes, which have relative amplitudes that are sensitive to changes in pH, temperature, and amino acid sequence [2]. In MbCO, the dominant line, A_1, is about 75% of the integrated band area while in Hb, the C_{III} line is $> 96\%$ [3,14].

The secondary and tertiary structures of Mb are similar to those of the α and β subunits of intact Hb [15]. The similarities in structures are noteworthy as the primary sequences of the three chains are the same at only 24 of 141 positions [16]. Most of these differences are subtle changes of size or polarity, such as a leucine in Hb_α and Mb at F1 where a phenylalanine is found in Hb_β. Some differences are more dramatic, such as F7 where a serine occurs in Mb but an alanine occurs in Hb_α and Hb_β [16]. These changes in amino acid polarity do not significantly change the secondary or tertiary structure of Hb. However, changes in the polarity will change the electrostatic nature of the protein.

In this letter, IR vibrational echo experiments and IR pump–probe experiments on HbCO, are presented. The experiments were conducted as a function of temperature from 15 K to 298 K. HbCO's pure dephasing rate displays a $T^{1.3}$ power law temperature dependence between 15 and ~ 150 K, suggesting glass-like behavior. Above 150 K, the temperature dependence becomes much steeper. The functional forms of the HbCO and the MbCO temperature-dependent pure dephasing rates are identical. However, the magnitude of the pure dephasing is 27% smaller for HbCO at all temperatures, suggesting that the amplitude of fluctuating electric fields at the heme is smaller in hemoglobin than in myoglobin.

2. Experimental procedures

The experiments on HbCO were performed using a Spectra Physics regeneratively amplified Ti:Sapphire pumped optical parametric amplifier (OPA) system. The previous studies on MbCO were performed using the Stanford Free Electron Laser [11]. The advent of commercial equipment capable of performing detailed vibrational echo studies of proteins and other systems [17] should expand the application of vibrational echo methods to a wide variety of problems.

For the experiments described here, the bandwidth was limited to ~ 18 cm^{-1} to avoid pumping to higher vibrational levels of the CO mode and to have the bandwidth of the IR pulses on the same order as the linewidth. The bandwidth is limited by placing a slit in the stretcher. The output of the regen has wings generated by the sharp cut-off of the bandwidth caused by the slit. However, the bandwidth is narrowed slightly and wings are eliminated by the use of a grating and four consecutive nonlinear processes in the OPA used to generate the IR. The IR pulses are 900 fs in duration and have nearly Gaussian shape as determined by IR autocorrelation. The laser spectrum was recorded with a SPEX grating monochromator. The IR bandwidth was ~ 18 cm^{-1}. At 5 μm, the OPA typically produces 6–7 μJ/pulse at 1 kHz. Substantially more energy is obtained when fs pulses rather than ps pulses are used.

The IR output of the OPA is directed into the vibrational echo and vibrational pump–probe experimental set up. The entire IR part of the experiment is enclosed in a dry-air purged compartment to eliminate the substantial atmospheric water absorptions. 15% of the IR beam is split off with a ZnSe beam splitter and directed to the sample. The remaining 85% of the beam passes down a computer controlled 0.1 μm step stepper motor delay line and is then sent into the sample. For the echo experiments, the weak beam is chopped at 500 Hz; for the pump–probe experiments, the strong beam is chopped at the same

frequency. Two, 6″ focal length, 90° off-axis parabolic reflectors are used to focus to ~100 μm, and then recollimate the IR beams, including the vibrational echo beam. The sample is contained in a custom built copper cell with CaF_2 windows which is mounted in a continuous flow cryostat. After the focussed IR beams pass through the sample and are recollimated, either the probe (weak excitation beam) or echo beam is directed into a HgCdTe detector. The signal from the detector is sampled by a gated integrator, the output of which is measured using a lock in amplifier. The 500 Hz signal from the lock-in is digitized for storage by a computer. Pulse energies up to ~4 μJ/pulse were available at the sample. Power studies were performed at high and low temperatures to insure that there were no intensity or heating artifacts.

To determine the pure dephasing linewidths, $1/(\pi T_2^*)$, both a vibrational echo and a pump–probe experiment are performed. The pure dephasing time, T_2^* (pure dephasing rate, $1/T_2^*$), is calculated using

$$\frac{1}{\pi T_2} = \frac{1}{\pi T_2^*} + \frac{1}{2\pi T_1}, \tag{1}$$

where T_2 is the homogenous dephasing time (exponential vibrational echo decay, Lorentzian homogeneous line shape) measured by the vibrational echo decay, and T_1 is the vibrational lifetime measured by the pump–probe decay. In principle, orientational relaxation can also contribute to the homogeneous dephasing. However, because the protein is so large, orientational relaxation does not occur on the time scale of the experiments. T_2 is obtained from the vibrational echo decay, $S(\tau)$, using

$$S(\tau) = S_0 e^{-4\tau/T_2}, \tag{2}$$

where τ is the time delay between the excitation pulses.

The sample was prepared as follows. Approximately 1.4 g human Hb was slowly added to 5 mL of 50:50 (v:v) ethylene glycol (EgOH): pH 7 phosphate buffer mixture until all the protein was dissolved. The sample was purged with Ar for a few minutes and a 10 molar excess of sodium dithionite was added. CO gas was then bubbled through the solution for ~2 hours. The resulting concentration was ~18 mmol. Thermal stability of proteins is not greatly affected by relatively high concentrations of EgOH [18–20], and concentrations up to 22 mol percent are not thought to cause structural perturbations.

FTIR spectra were recorded as a function of temperature across the temperature range of the echo experiments. The room temperature peak is centered at 1951.8 cm^{-1}, has a width of 8.0 cm^{-1}, and an absorbance of 0.5 on a 1.2 background. The peak center and width change slightly and monotonically as the temperature is lowered. By 50 K, the peak center has shifted to 1949.3 cm^{-1} and the width has grown to 8.1 cm^{-1}. The OPA wavelength was tuned to the absorption center at each temperature, so the α and β CO modes were both driven. The absorption cross-section of the β subunit appears to be somewhat larger than that of the α subunit [3]. Therefore, the measured pure dephasing rates for Hb represents a weighted average pure dephasing rate for the α and β subunits. The difference between the subunits is enhanced by the nonlinear nature of the vibrational echo signal, which is dependent on the cross-section to the fourth power. The differences between the dephasing widths of hemoglobin subunits will be the subject of a future study.

At low temperatures, the vibrational echo data are well fit with a single exponential decay. At the highest temperatures, the vibrational echo decay time approaches the duration of the pulse. Therefore, the response is calculated by numerically evaluating all of the rephasing and non-rephasing terms for the third-order non-linear polarization that contribute to the signal in the vibrational echo geometry [21]. The intrinsic decays at high temperatures are taken to be exponential in accord with the decays at lower temperatures. Fitting the high temperature data with the calculation of the full polarization yields T_2.

3. Results and discussion

Fig. 1 displays a vibrational echo decay. These data are for HbCO in $EgOH/H_2O$, at 40 K. Also shown is an exponential fit to the decay. The decay time is 11.0 ps yielding a $T_2 = 44$ ps. These echo data were taken on a sample in which the protein has a very strong background absorption compared to the CO peak under study. In addition, the sample is

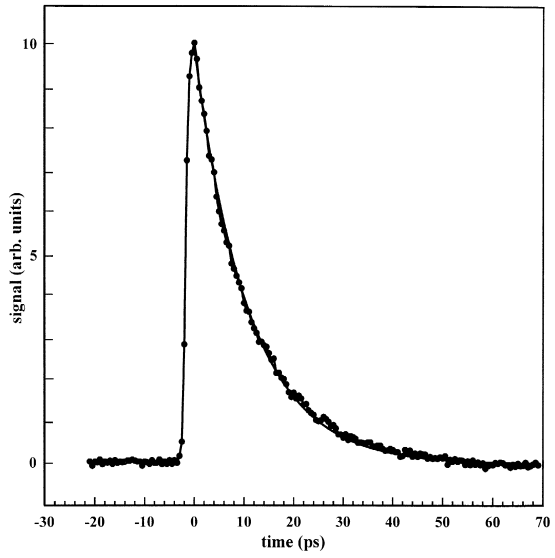

Fig. 1. An example of vibrational echo data taken on HbCO in ethylene glycol/water and a single exponential fit to the data. The data were taken at 40 K. The decay time is 11.0 ps giving a $T_2 = 44.0$ ps; the corresponding homogeneous linewidth is 0.24 cm^{-1}. The inhomogeneous linewidth is 8 cm^{-1}. The absorption line is inhomogeneous at all temperatures studied.

somewhat turbid to the eye. None-the-less, it is possible to obtain high quality vibrational echo data. The data took approximately 10 minutes to acquire.

Fig. 2 displays the temperature-dependent T_2 (triangles) and T_1 (circles, plotted as 2 T_1 for use in Eq. (1)) data and values of T_2^* (squares) obtained from Eq. (1). The lifetime has a mild temperature dependence, similar in slope to the lifetime data for Mb in the same solvent [22]. The lifetime of HbCO is approximately 20% slower than the MbCO lifetime at all temperatures. The room temperature lifetime is consistent with a previously reported value at room temperature [23]. As can be seen in Fig. 2, the pure dephasing is the dominant contribution to the dephasing time at high temperature, while the dephasing arises mainly from the lifetime at very low temperature.

Fig. 3 shows the pure dephasing rate versus temperature on a log plot. On a log plot, a power law appears as a straight line. The solid line through the data is a fit to a power law:

$$\frac{1}{T_2^*} = aT^{1.3\pm0.1} \qquad (3)$$

The dashed line is the continuation of the solid line to temperatures not involved in the fit. Vibrational echo measurements of MbCO show the identical $T^{1.3}$ power law in three solvents [11]. The fact that changing the solvent does not change the power law and a variety of other observations on MbCO [1,11], demonstrate that the MbCO dephasing arises from protein dynamics. It is reasonable to assume that the dephasing in HbCO is also solely due to protein dynamics.

The $T^{1.3}$ temperature dependence observed here is the same as the power law temperature dependences found in electronic excited state dephasing experiments [4–7] and other observables [24] in low temperature glasses. In low temperature glasses the power law exponent typically ranges from 1.0 to 1.5 with the most common values being 1.3 ± 0.1. The power law temperature dependences observed in many electronic excited state dephasing experiments

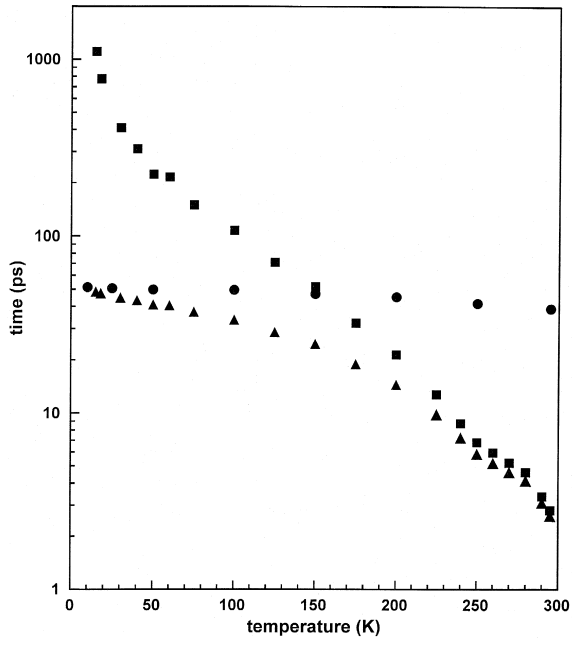

Fig. 2. Vibrational data for HbCO. The measured $v = 1$ lifetime (plotted as $2T_1$, in accordance with Eq. (1)) are shown as circles and have a very mild temperature dependence. The triangles are the homogeneous dephasing times measured with the vibrational echo experiments. The squares are the pure dephasing times, calculated from the lifetimes and homogeneous dephasing times using Eq. (1).

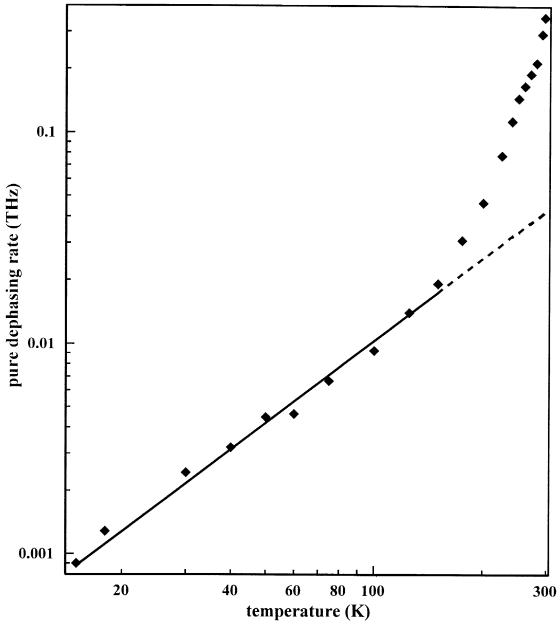

Fig. 3. Pure dephasing rates of HbCO as a function of temperature on a log plot. The solid line through the data is a $T^{1.3}$ power law. The data begin to deviate from the power law at approximately 150 K.

in glasses [4–7] as well as a recent vibrational dephasing experiment in a glass [8,25] have been analyzed theoretically [26–28] in terms of the two level system (TLS) model of glass dynamics [29,30]. The complex potential surface associated with the structural evolution of a glass is modeled as a distribution of double well potentials, i.e., TLS. The two wells represent different local glass structures. Phonon assisted tunneling from one side of the double well to the other produces a change in the local structure. A molecular oscillator is coupled to many TLS. When a TLS makes a transition from one local configuration to another, the structural change produces a change in the coupling of the solvent to the oscillator. The coupling of the TLS to the chromophore is modeled as a dipole interaction [7,28]. Many TLS undergoing transitions cause the oscillator energy to fluctuate, producing pure dephasing. If all TLS energy splittings, E (difference in energy of the two wells), are equally probable, theory predicts a T^1 temperature dependence [27]. Very broad, but not quite flat, distributions of E, produce power law temperature dependences, such as $T^{1.3}$ [27].

The observed $T^{1.3}$ HbCO pure dephasing, and the same power law found for MbCO, can be explained if proteins behave in a manner analogous to a glass below a protein glass transition temperature. There has been considerable discussion in the literature concerning a protein glass transition (See Ref. [11] and references contained therein). The protein glass transition is not a glass transition in the normal sense, since a transition from a liquid to a glass is a phenomenon associated with a bulk material. In contrast to a bulk material, a protein is a single molecule. However, its complexity is so great that it undergoes continual structural evolution among a vast number of configurations [1]. Below the 'protein glass transition temperature', T_g^p, sampling of protein configurations is greatly slowed. If the dynamics below T_g^p are dominated by a collection of protein TLS, then a power law temperature-dependent vibrational pure dephasing could arise in a manner analogous to that which occurs in a true glass. It is also possible to have a power law temperature dependence by mechanism that involves promotion over the barriers rather than tunneling through them [31]. In either case the power law arises from motion on a complex energy landscape.

The power law exponents are the same in myoglobin and hemoglobin, suggesting that the energy landscapes in the two proteins are similar in overall structure but perhaps different in the details [32]. Since the secondary and tertiary structures of the proteins are very similar, this may not be surprising. It remains to be seen if the $T^{1.3}$-dependence is universal in proteins or if proteins with very different secondary and tertiary structures have different low temperature dynamics.

The power law fits well up to ~ 150 K. Above this temperature, an additional broadening component becomes important. The break in the dynamics occurs somewhat above the glass transition temperature of the solvent mixture, 136 K [33]. Transitions from the power law to steeper temperature-dependent pure dephasing have been observed for MbCO in three solvents [8–11]. The transitions were attributed to a transition of the protein. A break in the MbCO temperature dependence at ~ 200 K was observed in the solvent trehalose, which is a glass to well above room temperature [11]. Thus, the transition does not depend on the solvent passing through

its glass transition. However, the solvent viscosity may influence the protein transition temperature in a manner akin to some observations on polymers [34]. Analogy to experiments on MbCO [11] indicate that above 150 K, the HbCO pure dephasing rate increases due to changes in both the temperature and the solvent viscosity.

Fig. 4 shows a comparison of pure dephasing rates versus temperature of MbCO and HbCO both in $EgOH/H_2O$. As can be seen from the figure, HbCO and MbCO dephasing data have the same functional form and only differ in the magnitude of the pure dephasing rate. The HbCO data is the same as shown in Fig. 3. The MbCO data and MbCO fit have been reported previously [11]. The line through the HbCO data is not a fit, but, rather, it is the MbCO fit multiplied by a constant, 0.73. These results show that the functional form of the temperature-dependent pure dephasing dynamics of HbCO is identical to MbCO, but the pure dephasing in HbCO is 27% slower across the entire temperature range.

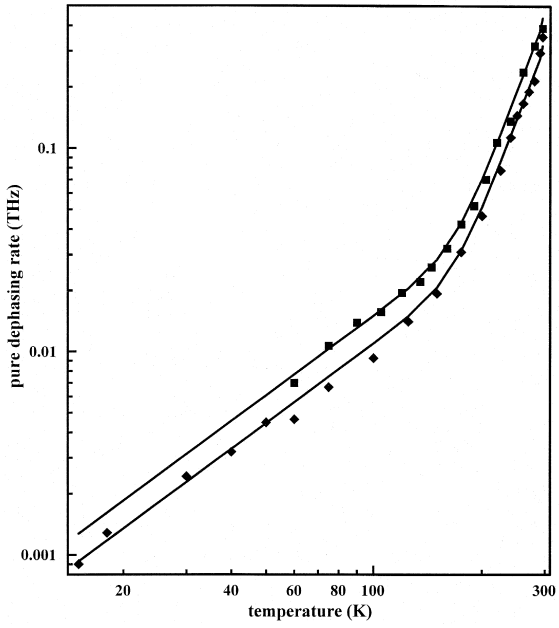

Fig. 4. Pure dephasing rates of MbCO and HbCO on a log plot. The data from HbCO is the same as Fig. 3. The line through the HbCO data is the line though the MbCO data multiplied the constant 0.73. The functional forms of the temperature dependences are identical.

Previously, it was proposed that the source of the pure dephasing in Mb involve motions of the polar groups in the protein that generate fluctuating electric fields [35] at the heme. The heme's delocalized π electron density is modulated by the fluctuating field, producing fluctuations in the back donation of electron density to the CO's anti-bonding π orbital. Time-dependent back bonding causes fluctuations of the CO oscillator's energy, and, hence, pure dephasing [9]. A vibrational echo study of the myoglobin mutant H64VCO (the distal histidine is replaced with a valine) was conducted [9]. The results show that the H64VCO has the same functional form of the temperature dependence as MbCO but has a 21% decrease in its pure dephasing rate. The distal histidine in Mb is responsible for a large part of the field felt at the active site [35], which is reasonable considering the proximity and polarity of the distal histidine to the CO. MD simulations of photolyzed Mb^*CO indicate that the reduced contribution to the fluctuating electric field from H64 agrees qualitatively with the difference between MbCO and H64V-CO vibrational echo measurements [35].

A possible explanation for the reduction in the rate of pure dephasing in HbCO compared to MbCO (Fig. 4) is that the magnitudes of the fluctuating electric field at the hemes in HbCO are less than in MbCO. The fluctuating electric field in HbCO may be reduced either because of the differences in the locations of polar groups or because the protein dynamics that fall within the vibrational echo measurement window ($\sim 0.1T_2$ to $\sim 10T_2$) are reduced in HbCO compared to MbCO. The fact that, within experimental error, the functional form of the pure dephasing temperature dependence in HbCO is identical to that in MbCO demonstrates that the fast global dynamics of the two proteins, as sensed by the CO ligand bound at the active sites of the proteins, are very similar. This suggests that the distribution of barriers controlling fast structural fluctuations have the same nature in the two proteins.

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