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Myoglobin's ultrafast dynamics measured with vibrational echo experiments

K.D. Rector *, M.D. Fayer

Department of Chemistry, Stanford University, Stanford, CA 94305, USA

Abstract

Ultrafast infrared (IR) vibrational echo experiments, which are used to examine the ultrafast dynamics of myoglobin and myoglobin mutants are described. Like the NMR spin echo and other NMR pulse sequences, the vibrational echo can extract dynamical and spectroscopic information that cannot be obtained from a vibrational absorption spectrum. The vibrational echo measures the homogenous vibrational linewidth even if the absorption line is massively inhomogeneously broadened. When combined with pump-probe (transient absorption) experiments, the homogeneous pure dephasing (energy level fluctuations) is obtained. Conducting these experiments as a function of temperature provides information on dynamics and intermolecular interactions. The nature of the method and the experimental procedures are outlined. The dynamics of the CO ligand bound at the active site of the protein myoglobin are examined and compared with that in myoglobin mutants. The results provide insights into protein dynamics and how protein structural fluctuations are communicated to a ligand bound at the active site.

1. Introduction

We present a vibrational echo study of myoglobin, a small 153 amino acid protein which has the primary biological function of the reversible binding and transport of O_2 in muscle tissues. Myoglobin's ability to bind O_2, and other biologically relevant ligands, such as CO or NO, is due to a non-peptide prosthetic group, heme, which is covalently bound at the proximal histidine of the globin. The interior of the protein consists almost entirely of non-polar amino acids while the exterior part of the protein contains both polar and non-polar residues. The only internal polar amino acids are two histidines [1]. The proximal histidine is covalently bonded to the Fe forming the fifth coordination site of the heme. The sixth coordinate site of the heme is the active site of the protein where the ligand bonds. The distal histidine is physically near the sixth coordinate site of the heme but not directly covalently bonded to it. When bound to Mb, the CO vibrational frequency is substantially red shifted from the gas phase frequency and separated into several distinct bands, labeled A_0, A_1, and A_3 in order of decreasing carbonyl frequency. These three bands, which occur around 1969, 1945, and 1930 cm^{-1}, reflect distinct conformational substates [2].

*Corresponding author. Tel.: 650 723 9847; fax: 650 723 8311; e-mail: rector@bjork.stanford.edu.

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The ultrafast infrared (IR) vibrational echo experiment eliminates the inhomogeneous broadening contribution to the line width and provides a direct measurement of homogeneous dephasing (homogeneous spectrum). Using vibrational echoes to measure the homogeneous dephasing time (T_2) and IR pump-probe experiments to measure the vibrational lifetime (T_1), the homogeneous pure dephasing time (T_2^*) can be obtained. The pure dephasing is the energy level fluctuations caused by the ultrafast intra and inter molecular perturbations. Thus, by using non-linear vibrational experiments in the time domain, it is possible to determine the homogeneous spectrum and all dynamical contributions to it.

2. The vibrational echo method

The echo method was originally developed as the spin echo in nuclear magnetic resonance in 1950 [3]. In 1964, the technique was extended into optical frequencies for electronic transitions as the photon echo [4,5]. Since then, photon echoes have been used extensively to study electronic excited state dynamics in many condensed matter systems [6–9].

The vibrational echo experiments permit the use of optical coherence methods to study the dynamics of the mechanical degrees of freedom of condensed phase systems from low (<3 K) to high (>300 K) temperatures. Because vibrational spectroscopic lines are relatively narrow, it is possible to perform vibrational echo experiments on well defined transitions and at temperatures which are physiologically relevant for biological studies. Further, vibrational echoes probe dynamics on the ground state potential surface only. Therefore, the excitation of the mode causes a minimal perturbation of the solvent. Recently, vibrational echoes have been used to examine vibrational dynamics in liquids, glasses, [10–12] and proteins [13–15].

Vibrational echo experiments in general yield decay curves of the form

$$I(\tau) = I_0 \exp(-4\tau/T_2) \tag{1}$$

where T_2 is the homogeneous dephasing time. The homogeneous linewidth is composed of both pure dephasing (T_2^*) and vibrational lifetime (T_1) components:

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

The experimental conditions as well as more details of the vibrational echo have been omitted due to spatial constraints. These details can be found elsewhere [10,11].

3. Myoglobin and myoglobin mutants results

Vibrational echoes and pump-probe experiments were performed on native horse heart myoglobin in the solvent 95:5% glycerol:water as a function of temperature between 60 and 300 K. Fig. 1 shows the results. The triangles are the

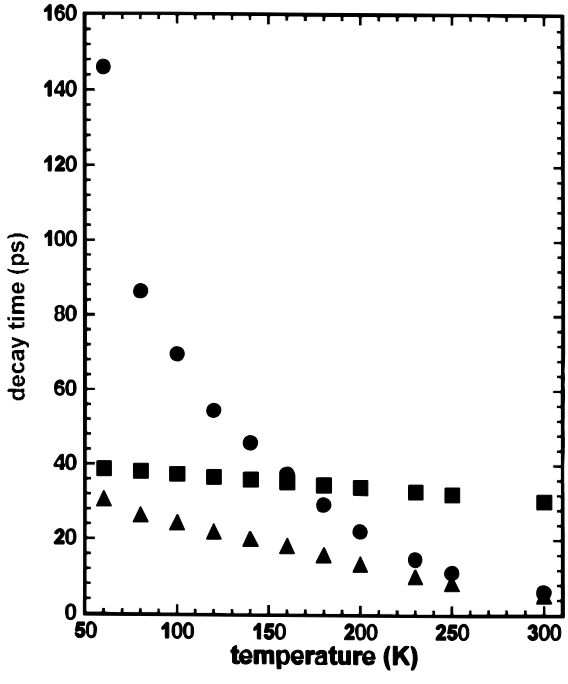

Fig. 1. Temperature dependent data for native Mb-CO. The triangles are the measured values of T_2 obtained from the vibrational echo decays using Eq. (1). The squares are $2T_1$ obtained from the decay constants measured in the pump-probe vibrational lifetime experiments. The circles are T_2^*, the pure dephasing times, obtained from T_2 and $2T_1$, using Eq. (2).

measured homogeneous dephasing time (T_2). The squares represent the vibrational lifetime, but are plotted as $2T_1$, in accordance with Eq. (2). The circles are the pure dephasing time (T_2^*) as determined from T_2 and $2T_1$, using Eq. (2).

Fig. 2 shows the pure dephasing rate, $1/\pi T_2^*$, versus temperature on a log plot. The line through the data is a fit to

$$\frac{1}{\pi T_2^*} = a_1 T^{\alpha} + a_2 \exp\left(\frac{-\Delta E}{kT}\right), \tag{3}$$

with $\alpha = 1.3 \pm 0.1$ and $\Delta E = 1000 \pm 250$ cm^{-1} [13]. The CO pure dephasing is caused by fluctuations of the protein and not by direct coupling of the CO to the solvent dynamics. This was verified by a variety of experiments [16–18] including vibrational echo studies of Mb-CO in the solvents ethylene glycol and trehalose [19]. In all three solvents, the low temperature dephasing is identical. Not only is the temperature dependence $T^{1.3}$ in all three solvents, but the actual values of T_2^* are identical at all temperatures below ~200 K. In addition, experiments discussed below on the mutant H64V, show that a change in the Mb amino acid sequence in the pocket of the protein does cause a change in the pure dephasing.

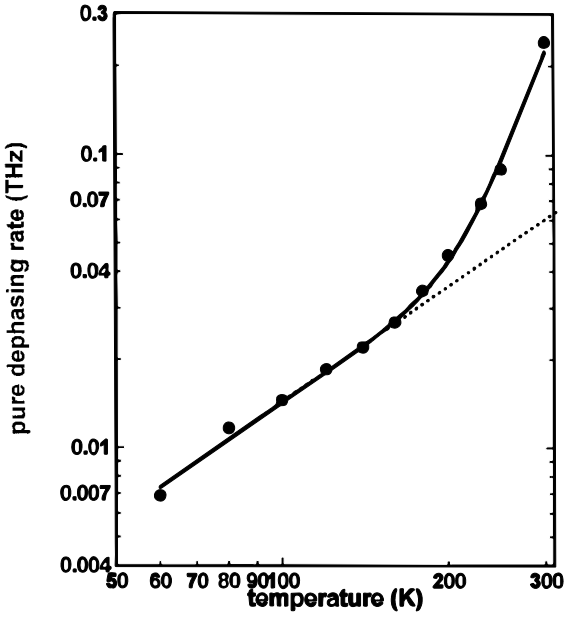

Fig. 2. Log plot of the pure dephasing rate, $1/\pi T_2^*$, versus temperature for native MbCO. Below ~200 K, the temperature dependence is dominated by a power law, $T^{1.3}$, which appears linear on the log plot. Above ~200 K, an exponentially activated process describes the data with $\Delta E = \sim 1000$ cm^{-1}.

The low temperature $T^{1.3}$ dependence of the CO pure dephasing on temperature is reminiscent of dephasing by the TLS dynamics observed for $Rh(CO)_2acac$ in dibutyl phthalate [12] and of many other observables in glasses [6,8,20,21]. However, in the protein, the power law temperature dependence extends to much higher temperature than in conventional glasses. The $T^{1.3}$ temperature dependence can arise from tunneling dynamics of a system of protein two level systems (PTLS) [13,14]. The PTLS are akin to the two level systems of very low temperature glasses. The same statistical mechanics machinery used to describe the low temperature (~1 K) heat capacities of glasses and the optical dephasing of electronic transitions of chromophores in low temperature glasses [8] can be used to describe the PTLS induced vibrational dephasing of Mb-CO at much higher temperatures (<200 K). A power law temperature dependence can also arise from activation over barriers rather than tunneling if there is the appropriate broad distribution of activation energies. In either case, the results suggest the existence of a complex protein energy landscape.

Near ~200 K, there is a break in dephasing temperature dependence. This reflects a change to an exponentially activated process. Initially, it was proposed [13,14] that the break arose due to the onset of the solvent's glass transition which changed the motions available to the surface of the protein. The glass transition for glycerol:water is ~180 K. However, recent experiments disprove this hypothesis. An unpublished vibrational echo study of Mb-CO in trehalose [19], which has a glass transition above room temperature, exhibits a break in the temperature dependence at ~200 K. Therefore, the change in the temperature dependence involves properties of the protein and is not triggered by the glass transition of the solvent. These results suggest that protein or the water molecules making the first hydration sphere of the protein makes a transition from a glassy state to a more fluid like state. In the fluid like state, the

protein can make transitions among a range of structural configurations that it could either not make or could only make at a very slow rate when in the glassy state. These transitions are activated processes with a narrow range of activation energies, giving the appearance of a single activation energy.

For vibrational dephasing of CO bound to the active site of Mb to occur, the fluctuations of the protein must be coupled to the vibrational states of the CO in a manner that causes fluctuations in the vibrational transition energy. Two models have been proposed to explain the dephasing [14,15]. One involves global electric field fluctuations and the other local mechanical coupling.

In the global electric field model, motions of polar groups throughout the protein produce a time dependent electric field. The fluctuating electric field causes modulation of the electron density of the heme's delocalized π-electron cloud. The heme π system is composed of the Fe d_π, N p_π, and C p_π atomic orbitals. The CO bonded to the Fe is a σ electron donor of electron density to the heme. To alleviate excess charge density at the heme, there is a substantial back donation (back bonding) of heme π electron density to the CO π^* antibonding orbital. The back bonding causes a red the shift of ν_{CO} because the electron density is donated to an antibonding orbital. It is well established from work on Mbs and other metal carbonyls that changes in the back donation of electron density into the CO π^* are responsible for static shifts of the CO vibrational frequency. Furthermore, experiments [2] suggest that in different Mb's, variations in electric fields resulting from different protein conformations are responsible for changes in back bonding, and, therefore, observed static shifts in vibrational frequency [22,23]. In the dephasing model, fluctuations of the heme π electron density modulate the magnitude of the back bonding to the CO π^*, causing time dependent shifts in ν_{CO}. These time dependent shifts are responsible for the vibrational pure dephasing. In essence, the protein acts as a fluctuating electric field transmitter. The heme is an antenna which receives the signal of protein fluctuations and communicates it to the CO ligand bound at the active site.

In the local mechanical fluctuation model, the local motions of the amino acids on the proximal side of the heme are coupled to the heme through the side group of the proximal histidine. The proximal histidine is covalently bonded to the Fe. This bond is the only covalent bond of the heme to the rest of the protein. Thus, motions of the α-helix that contains the proximal histidine are directly coupled the Fe. These motions can push and pull the Fe out of the plane of the heme. Since the CO is bound to the Fe, this motions may induce changes in the CO vibrational transition frequency causing pure dephasing.

To test these models, we have performed a temperature dependent vibrational echo and pump-probe study on two myoglobin mutants, H64V-CO and H93G(N-MeIm)-CO, both in 95:5% glycerol:water. These mutants were prepared using site-directed mutagenesis techniques [24,25]. To test the global electric field model, we studied H64V, a myoglobin mutant in which the polar distal histidine is replaced by a nonpolar valine. If the global electric field model of the dephasing is operative, then the decrease in the electric field in the mutant should reduce the magnitude of the frequency fluctuations, producing slower pure dephasing. To test the local mechanical model of pure dephasing, we studied H93G(N-MeIm), a myoglobin mutant in which the proximal histidine is replaced by a glycine. This severs the only covalent bond between the heme and the globin and leaves a large pocket on the proximal side of the heme. Inserted into this pocket and bound to the heme is an exogenous N-methylimidizole, which has similar chemical properties as the side group of the histidine. Effectively, the proximal bond had been broken without changing significantly the electrostatics properties of the protein. If dynamics of the f α-helix are causing the pure dephasing by producing motions of the Fe via the proximal histidine, then the dephasing of this mutant should be less than that of the native protein.

Fig. 3 shows the pure dephasing rates versus temperature on a log plot of the native protein and the two mutants studied. The circles represent the values for the native protein, which are the same as in Fig. 2. The triangles are the pure dephasing

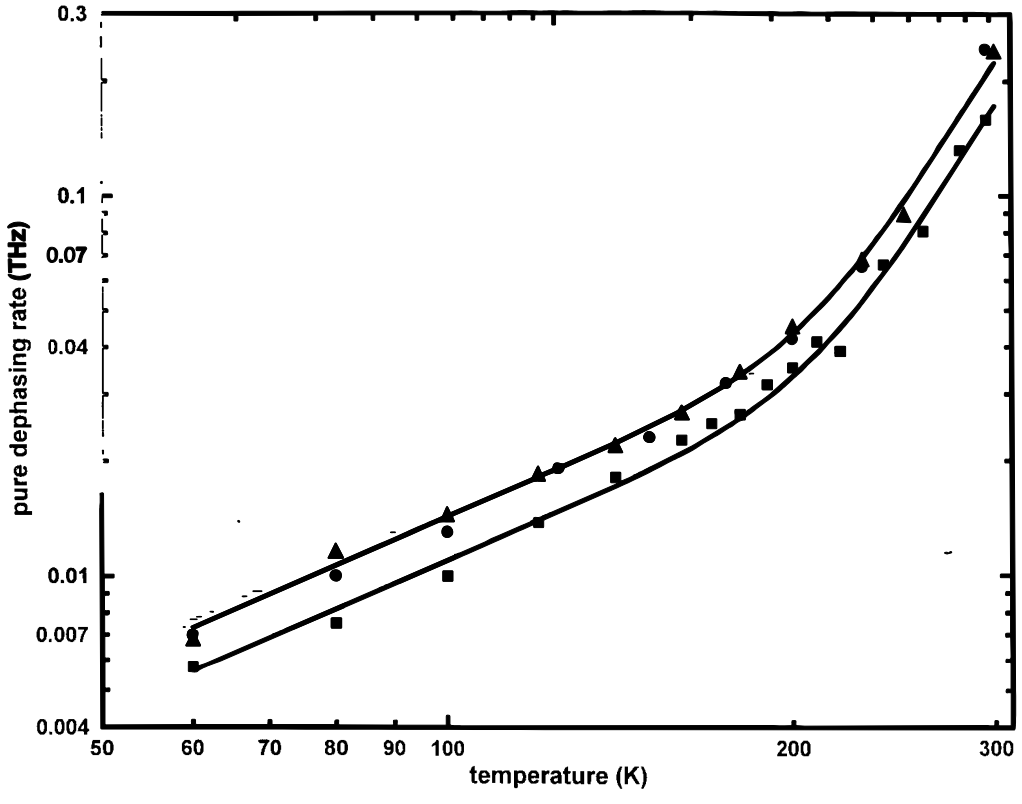

Fig. 3. Pure dephasing rate versus temperature for native MbCO (circles) (same as Fig. 2). Also plotted is pure dephasing for H64V-CO (squares) and H93G(N-MeIm)-CO (triangles). As is clear, the native MbCO and the mutant H93G(N-MeIm)-CO have identical pure dephasing temperature dependences. The H64V-CO has identical form of the pure dephasing but with a 21 ± 3% decrease in the pure dephasing rate at all temperatures studied.

rates for the mutant H93G(N-MeIm)-CO in 95:5% glycerol:water. Clearly, these values are identical to the native protein, indicating that the proposed local mechanical dephasing model is not active in myoglobin. The squares are the pure dephasing rates for the mutant H64V-CO in 95:5% glycerol:water. The data is fit well with using Eq. (3) using the same parameters as native; $\alpha = 1.3 \pm 0.1$ and $\Delta E = 1000 \pm 250$ cm^{-1}. However, the dephasing is 21 ± 3% slower than that of native at all temperatures. The functional form of the temperature dependence is unchanged because modification of one amino-acid does not significantly change the global dynamics of the protein. However, replacing the polar distal histidine with a non-polar valine removes one source of the fluctuating electric fields, reduces the coupling of the protein dynamics to the CO vibration, and slows dephasing. These results support the global electric field model of pure dephasing in myoglobin and suggest that the distal histidine contributes 21 ± 3% of the fluctuating electric fields felt at the heme. Recent molecular dynamics simulations [26] lend support to the ~20% electric field fluctuation produced by the distal histidine.

4. Concluding remarks

Vibrational echo experiments have also been applied to the CO stretching mode of myoglobin and mutant myoglobin proteins. Vibrational echo and lifetime measurements have been made on CO bound to the active site of native Mb, H64V, and

H93G(N-MeIm). The temperature dependences show that the pure dephasing of H64V is 21 ± 3% slower than native Mb with no change in the functional form of the temperature dependence. The temperature dependence of the pure dephasing of H93G(N-MeIm) is identical to the native Mb. The general mechanism proposed [13] to explain the coupling of conformational fluctuations of the protein to the vibrational transition energy of CO bound at the active site is supported by the H64V results. The model states that protein motions produce fluctuating electric fields which are responsible for the CO pure dephasing. Replacing the polar distal histidine with the non-polar valine removes one source of the fluctuating electric fields, thus reducing the coupling between the protein fluctuations and the measured pure dephasing. The picture that emerges is that the heme acts as an antenna that receives and then communicates protein fluctuations to the vibration of the CO ligand bound at the active site. Vibrational echo data obtained on H93G(N-MeIm) in which the covalent linkage between heme-CO and the protein is broken, show the temperature dependence of vibrational pure dephasing is identical to native Mb. The experiments demonstrate that local mechanical motions of the proximal histidine which directly couple the Fe are not responsible for vibrational pure dephasing of CO bound at the active site of myoglobin.

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