A Dynamical Transition in the Protein Myoglobin Observed by Infrared Vibrational Echo Experiments

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Ultrafast infrared vibrational echo measurements of the temperature-dependent pure dephasing of the A_1 CO stretching mode of myoglobin−CO (Mb-CO) were performed in the solvents trehalose and 50:50 ethylene glycol:water. The results are compared to previously reported data in 95:5 glycerol:water. The temperature dependence (11−300 K) of the pure dephasing in trehalose (a glass at all temperatures studied) is a power law, $T^{1.3}$, below $T \cong 200$ K, while at higher temperature it becomes dramatically steeper. The change in functional form occurs although the solvent does not go through its glass transition. In the other two solvents, the breaks in the temperature dependences occur at lower temperatures, and the temperature dependences are even steeper above the power law region. The results are discussed in terms of a combination of a temperature and viscosity dependence of protein dynamics.

I. Introduction

The dynamics of proteins on a wide variety of time scales are intimately related to protein function. Fast fluctuations (picosecond time scale) of protein structure enable a protein to sample the complex protein conformational energy landscape. Fast movement on the energy landscape gives rise to the slower processes associated with protein function. Molecular dynamics simulations have shown that a protein can sample thousands of conformations on a very short time scale.[1] Understanding such dynamics is key to determining the connection between protein structure, as measured by X-ray,[2,3] NMR,[4,5] or other experimental techniques,[6−10] or theory[1] and protein function.

Recently, the ultrafast infrared vibrational echo technique has been applied to the study of protein dynamics.[11−13] Unlike other ultrafast techniques,[14−18] which involve electronic excitation of chromophores, the vibrational echo experiments have directly examined the CO ligand bound to the active site of myoglobin (Mb-CO) in the ground electronic state. The vibrational echo is sensitive to ground state fluctuations of protein structure that are communicated to the ligand bound at the active site. Studies of the myoglobin mutant H64V suggest that the protein fluctuations communicated to the active site are global in nature. The protein fluctuations are coupled to the CO transition frequency by producing fluctuating electric fields which act on the heme.[12]

Myoglobin has been extensively studied by a wide variety of methods. Myoglobin is a 154 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 located in the protein's "pocket" and covalently bound at the proximal histidine of the globin. The Mb structure has no gaps for ligands to pass through. For ligands to move in and out of the pocket, they must traverse the intervening protein. Traversing the protein is made possible by the protein dynamics, which open paths for ligand diffusion through the protein.[19] Because of the ability of ligands to move through the protein, in some sense, the protein is considered to have liquidlike character.

A glass transition for a bulk material is recognized to be dynamical in nature.[20] Below the glass transition temperature, T_g, the system can no longer interconvert on a reasonable time scale among the full range of structural configurations accessed in the liquid. Previous temperature-dependent experiments on Mb-CO have revealed a type of dynamical transition in the protein at ∼200 K that has been referred to as a "protein glass transition".[21−23] This is not a glass transition in the normal sense, since a transition from a liquid to a glass as temperature is decreased 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. Below the "protein glass transition temperature", T_g^P, sampling of protein configurations is greatly slowed. Thus, like a true liquid/glass, a protein may undergo a type of dynamical transition. (When the term protein glass transition is used in discussing Mb, it is meant in the sense discussed in this paragraph.)

The myoglobin dynamics near ∼200 K, which suggest a protein glass transition, have been the subject of considerable investigation previously. For example, inelastic neutron scattering of hydrated Mb below 180 K observes only vibrational motion and, above 180 K, there is a dynamical transition, which is interpreted as the onset of torsional jumps between states.[24,25]

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MD simulations of the torsional transitions of the dihedral angles of Mb in water shows that the anharmonic mean square displacements change at 200 K, which is indicative of a glasslike transition.[26] In addition, IR[27−29] and visible[30] transition frequencies, dielectric relaxation,[27] specific heat of water in Mb crystals,[27] and Mössbauer spectra of ^{57}Fe−Mb[31] all show breaks near 200 K. Some experiments have suggested that this is a "slaved" glass transition; i.e., the protein undergoes a transition induced by the true glass transition of the solvent.[21−23]

In this paper, vibrational echo measurements on Mb-CO are presented as a function of temperature from temperatures well below the Mb protein glass transition to well above it. We begin with the idea that the many experimental and theoretical studies conducted previously (some of which have been cited above) have established that there is a dynamical transition in Mb in the vicinity of 200 K. The aim here is to use vibrational echo experiments to provide insights into the nature of fast protein dynamics at temperatures below and above Mb-CO's glass transition and how the solvent environment of the protein influences the protein glass transition. In the absence of the wealth of other data on the Mb glass transition, it would be possible to envision a variety of scenarios other than a protein glass transition that could give rise to the data presented below. However, given the many previous studies, it is reasonable to discuss the vibrational echo results in the context of a protein glass transition. There is evidence from experiments on $W(CO)_6$ in the glass-forming liquid, 2-methylpentane (2MP)[32] (discussed briefly below) that the homogeneous dephasing time, T_2, measured in a vibrational echo experiment, can be sensitive to a glass transition, but the general question of the fundamental relationship between vibrational dephasing and a dynamical transition, like a glass transition, is not addressed.

A previous vibrational echo study of Mb-CO in 95:5 mixture of glycerol:water displayed a change in the functional form of the temperature dependence of the pure dephasing at ∼180 K.[11] Because the break in temperature-dependent pure dephasing occurred near the solvent T_g, the change was discussed in terms of a slaved protein glass transition.[11] In this paper, new temperature-dependent vibrational echo experiments on Mb-CO in trehalose and 50:50 ethylene glycol:water are presented. Trehalose is a glass at room temperature, with $T_g = 353$ K. Nonetheless, in trehalose, a change in the functional form of the Mb-CO pure dephasing temperature dependence is observed at ∼200 K. Below 200 K, the pure dephasing rate has a $T^{1.3}$ temperature dependence. Above 200 K, the data can be fit as an exponentially activated process with $\Delta E \cong 650$ cm^{-1}, although other forms, discussed below, can also fit the data for $T > 200$ K. The results demonstrate that a change in the nature of the Mb-CO pure dephasing occurs although the solvent remains a glass; i.e., a change in the nature of the solvent dynamics does not occur. In the solvents 95:5 glycerol:water and 50:50 ethylene glycol:water, changes in the temperature dependence also occur but at progressively lower temperatures. In all three solvents at low temperatures, the pure dephasing rate temperature dependence is the same power law, $T^{1.3}$. The results are discussed in terms of a transition in the nature of the protein dynamics. Both the transition temperature and the temperature dependence above T_g^P are influenced by the solvent viscosity.

II. Experimental Procedures

A. Vibrational Echo Method. The infrared vibrational echo experiments were performed at the Stanford Free Electron Laser (FEL) Center. The FEL produces tunable, picosecond, mid-IR pulses. The experimental setup is discussed in detail elsewhere.[11,33] The IR pulses had an energy of ∼0.5 μJ and were nearly transform-limited Gaussians, 1.2 ps in duration. Both the autocorrelation of the IR pulse and the spectrum were monitored continuously during the experiments. The spot size (ω_0) was ∼100 μm. The energies in the two pulses used in the echo sequence were ∼150 and ∼50 nJ, respectively. Pump−probe experiments were also conducted to measure the vibrational lifetime, T_1.

A vibrational echo is a two-pulse time domain technique which measures the Fourier transform of the homogeneous vibrational spectrum. In these experiments, the Mb-CO absorption spectra (1945 cm^{-1}) are inhomogeneously broadened, even at room temperature. Therefore, the vibrational absorption spectrum alone cannot provide information on the protein dynamics.

In the vibrational echo experiment, two picosecond IR pulses, tuned to the Mb-CO transition frequency, are crossed in a sample at an angle θ with a variable time delay, τ, between them. The vibrational echo pulse, which is formed at 2τ, propagates along a path that makes an angle 2θ with that of the first pulse. As τ is increased, the protein dynamics cause increasingly large accumulated phase errors among the ensemble of CO oscillators, and the signal amplitude of the vibrational echo is reduced. A measurement of the vibrational echo intensity vs τ is an echo decay curve. The Fourier transform of the echo decay is the homogeneous line shape.[34,35] The vibrational echo makes the vibrational homogeneous line shape an experimental observable in spite of inhomogeneous broadening.

For an exponential decay of the off diagonal density matrix elements (Lorentzian homogeneous line shape), the echo decay is given by

$$S(\tau) = S(0)e^{-4\tau/T_2} \tag{1}$$

where T_2 is the ensemble-averaged homogeneous dephasing time. The homogeneous line width is $1/\pi T_2$.[34] Combining the vibrational echo measurement of T_2 with a pump−probe measurement of T_1 provides a determination of the ensemble average homogeneous pure dephasing time, T_2^*, through

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

T_2^* is determined by the fluctuations in the transition frequency caused by the protein dynamics.[11]

B. Sample Preparation. The experiments were performed on native horse heart myoglobin (Sigma, used without further purification). The trehalose sample was prepared by making a solution ∼10 wt % of trehalose in 0.1 M pH 7 phosphate buffer and dissolving lyophilized metmyoglobin in it to obtain a final protein concentration of 1−2 mM. The solution was saturated with CO, and a 10 molar excess of sodium dithionite was added. A few drops of the resulting solution were placed on a sapphire window and allowed to dry under CO atmosphere for a few days. The final protein concentration was ∼20 mM. To improve optical clarity, the sample was annealed at 90 °C for 1 h. IR absorption measurements verified that the annealing of the sample did not denature the protein. A second sapphire window was placed on the sample and mild pressure was applied to obtain good thermal contact. The final sample thickness was approximately 100 μm. The sample was inserted into a copper sample holder attached to the coldfinger of an optical cryostat.

The ethylene glycol sample was prepared by adding a 15 mM aqueous solution of lyophilized metMb to 50:50 (v:v) ethylene

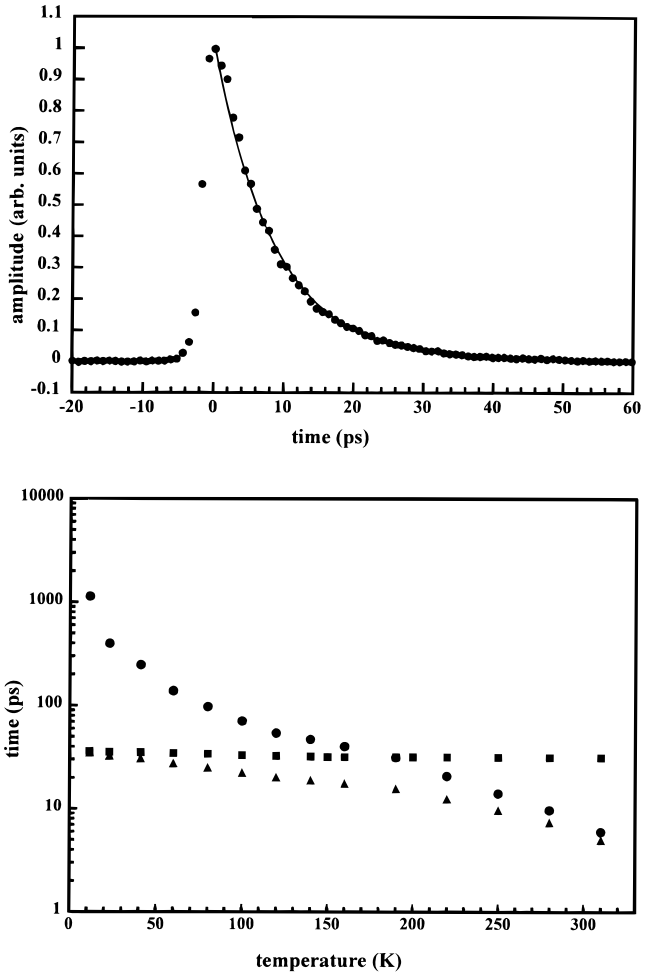

Figure 1. (a, top) An example of a vibrational echo decay of Mb-CO in trehalose glass at 11K. These data are fit well by an exponential curve as are all of the decays at all temperatures. The decay constant is 7.0 ps. This value gives a homogeneous dephasing time of 28 ps and a homogeneous width of ~0.3 cm^{-1} using eqs 1 and 2. (b, bottom) The temperature-dependent data for Mb-CO in trehalose. The triangles are the homogeneous dephasing times, T_2, measured with vibrational echo experiments. The squares are twice the vibrational lifetime, $2T_1$, measured with IR pump−probe experiments. The lifetime is plotted as $2T_1$ in accordance with eq 2. The circles are the pure dephasing times, as determined at each temperature from eq 2.

glycol/0.1M pH 7 phosphate buffer. The resulting solution was then stirred under CO atmosphere for 8 h before being reduced by a 10-fold molar excess of dithionite. A copper sample cell with CaF_2 windows was filled with the resulting solution. The path length was 200 μm.

III. Results and Discussion

Figure 1a shows a vibrational echo decay of Mb-CO in trehalose glass at 11 K. The signal-to-noise ratio is excellent in spite of the fact there is a large background absorption by the protein and the trehalose. The data are fit well by a 7.0 ps decay constant exponential curve, which corresponds to a homogeneous dephasing time of 28 ps and a homogeneous width of 0.38 cm^{-1}. At room temperature, the homogeneous width increases to ~2 cm^{-1}. The absorption line width is ~15 cm^{-1} fwhm at all temperatures. Therefore, the Mb-CO absorption line is inhomogeneously broadened at all temperatures studied.

Figure 1b is a semilog plot of the temperature dependence in trehalose of T_2 (triangles), $2T_1$ (squares), and T_2^* (circles). T_2^* was computed from the other two quantities using eq 2. T_1 has

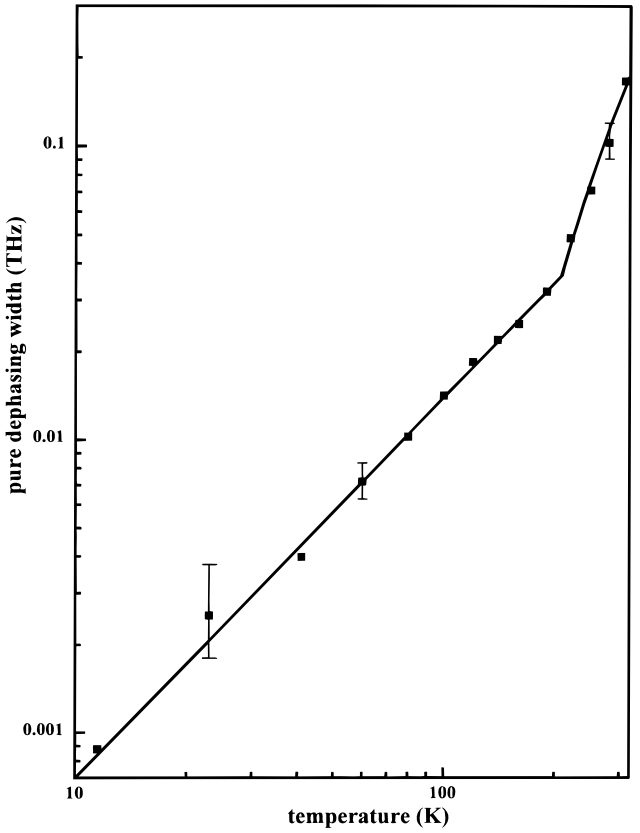

Figure 2. Temperature-dependent pure dephasing rate of Mb-CO in trehalose. The error bars on the data are larger for the $T < 50$ K data because of the potential error caused by subtracting two similar numbers (see Figure 1b). The data are fit with a power law, $T^{1.3}$, below ~200 K and with an exponentially activated process with $\Delta E = 650$ cm^{-1} above 200 K.

only a slight, essentially linear temperature dependence. It can be seen that at low temperatures the homogeneous line width is nearly lifetime limited. By room temperature, the homogeneous line width is dominated by pure dephasing.

The manner in which the global Mb protein fluctuations are communicated to the CO bound at the active site to produce pure dephasing has been discussed in detail previously.[12,13] Figure 2 shows the pure dephasing contribution to the line width, $1/\pi T_2^*$, vs temperature on a log plot for Mb-CO in trehalose. On a log plot, a power law graphs as a straight line. As can be seen in Figure 2, between 11 and ~200 K, the functional form of the data is a power law

$$\frac{1}{\pi T_2^*} = aT^{1.3} \tag{3}$$

where the prefactor $a = 3.5 \times 10^7 \pm 0.1 \times 10^7$ Hz/(deg K)$^{1.3}$. The error bar on the power law exponent is ±0.1. This power law has been reported previously for Mb-CO in glycerol:water.[11] However, the trehalose data are for a much broader range of temperatures and leave little doubt as to the functional form of the data. The same power law is observed in ethylene glycol:water (see below).

In Figure 2, there is a change in the functional form of the data at ~200 K. The points above ~200 K can be fit with

$$\frac{1}{\pi T_2^*} = 3.3 \times 10^{12}\, e^{-650/k_B T} \text{ Hz} \tag{4}$$

where k_B is Boltzmann's constant, $k_B T$ has units of cm^{-1}, and

the error bars on the prefactor and activation energy are $\pm 0.2 \times 10^{12}$ Hz and ± 25 cm^{-1}, respectively. It is clear that there is a change in the functional form of the temperature dependence at ~200 K. However, it is important to emphasize that the form of eq 4 is not unique given the small number of points. It is possible to describe the data with the power law plus another function without an abrupt switching of the functional form. If this is done with an exponentially activated process, the value of the exponent changes, but the power law is identical. A very good fit is obtained if the data are fit to a power law plus a Vogel−Tamman−Fulcher (VTF) type equation.[20,36,37] A VTF equation describes many processes, such as viscosity, in supercooled liquids as they approach the glass transition temperature. The VTF equation for the homogeneous line width is

$$\frac{1}{\pi T_2^*} = b e^{-E/k_B(T-T_0)} \tag{5}$$

For a true glass-forming liquid, T_0 is the "ideal" glass transition temperature. It typically has a value a few tens of degrees below the laboratory T_g.[20,36,37] A fit to the data with eq 3 plus eq 5 yields a T_0 of ~180 K and an E corresponding to a temperature of ~230 K. These parameters can vary somewhat about the given values because of the wide range of fits that can be achieved when fitting four points with three parameters. However, the power law is always identical, independent of the form used to fit the points above ~200 K. If the exponential fit and the VTF fit are extended to higher temperatures, they do not become distinguishable below 500 K. Therefore, experiments at temperatures below the Mb denaturation temperature cannot distinguish these two forms. Regardless of the form that is used to fit the data, it is clear that there is a sudden change in the nature of the temperature dependence of the pure dephasing.

Switching from a power law to an activated process or the power law plus the VTF function is appropriate if there is a transition in the fundamental nature of the dynamics. A power law plus an activated process does not require a change in nature of the dynamics, but only that the more rapidly increasing activated process overtakes the power law. In fact, this overtaking of a power law by an activated process has been demonstrated to occur in the electronic excited state dephasing of chromophores in low-temperature glasses very far below the glass transition temperature of the solvent.[38−42] In the absence of other information, these vibrational echo experiments alone cannot distinguish between a fundamental change in the nature of the protein dynamics or one process simply overwhelming another.

However, given the many other types of studies (discussed in the Introduction) that have observed changes in the Mb dynamics at ~200 K and that have been interpreted as evidence of a protein glass transition,[11] it is reasonable to assume that the vibrational echo data do, indeed, display a manifestation of a change in the basic nature of the protein dynamics; i.e., they reflect the protein glass transition. As has been discussed previously, Mb-CO pure vibrational dephasing arises from global fluctuations of the protein structure.[11−13] The CO dephasing is caused by electric field fluctuations produced by overall motions of the protein, rather than very local protein dynamics near the CO. A change in the nature of the protein dynamics, which influences the various observables that have been studied previously, can also produce a change in the nature of the temperature dependence of the vibrational pure dephasing.

A system that has been studied in detail with vibrational echo experiments is $W(CO)_6$ in the glass forming liquid, 2-methylpentane (2MP).[32] 2MP has $T_g = 80$ K. In this system, the temperature dependence of the pure dephasing contribution to the homogeneous line width is a power law, T^2, below the 2MP T_g. Above T_g, there is a very distinct break in the data; the temperature dependence becomes much steeper. As the temperature approaches room temperature, the slope of the temperature dependence decreases. The data are fit with the sum of the power law and a VTF equation (eqs 3 and 5). The data are measured up to 300 K, providing enough range to demonstrate that the VTF equation provides a very good fit to the data. However, in contrast to the 2MP viscosity, which has $T_0 = 59$ K, for the pure dephasing $T_0 = 80$ K, the laboratory T_g.[32] The $W(CO)_6$/2MP vibrational echo experiments demonstrate that vibrational pure dephasing can be sensitive to a true glass transition, adding credence to the proposal that the break in the Mb-CO pure dephasing data is associated with a protein dynamical transition.

In a previous vibrational echo study of Mb-CO in glycerol:water, a change in the temperature dependence was also observed at ~180 K.[11] In that system, the change is approximately at the solvent T_g. It was suggested that the change in solvent properties was responsible for the change in the dynamics of the protein.[11] However, the data in the solid trehalose sample demonstrates conclusively that the apparent break in the functional form can occur without the solvent going through its glass transition. The trehalose glass transition is at $T_g = 353$ K. The break in the data occurs ~150 K below the trehalose T_g, so there is no change in the viscosity or other properties of the trehalose solvent around 200 K. Thus, the data suggest that the protein itself is undergoing a transition in the nature of its dynamics that is not slaved to a change in the solvent dynamics.

Another issue is that of time scales. Vibrational echo measurements of the homogeneous dephasing time are sensitive to fluctuations on a time scale of approximately an order of magnitude faster than T_2 to an order of magnitude slower than T_2. In Mb-CO at ~200 K, this range spans ~1−100 ps. The picosecond time scale is in contrast to the time scale of some processes in supercooled liquids, such as long-range spatial diffusion, which become infinitely slow as T_g is approached from above. It has been observed in a wide variety of low-temperature glasses, far below T_g, that the temperature dependence of the optical dephasing of electronic transitions is independent of the time scale. Photon echo experiments on a 100 ps time scale and optical hole burning experiments on a 100 s time scale produce different pure dephasing widths.[38−42] The hole burning experiments yield broader pure dephasing line widths because of spectral diffusion caused by very slow processes that do not contribute to the photon echo experiments.[40,43,44] However, both types of experiments display the same $T^{\sim 1.3}$ temperature dependence of the pure dephasing contribution to the line width.[38−42] (The exponent varies somewhat depending on the glass.) In the $W(CO)_6$/2MP system, vibrational pure dephasing in the supercooled liquid exhibits VTF behavior, which has its onset at T_g. The dephasing's sensitivity to the glass transition has a shift to higher temperature than viscosity, possibly because dephasing is sensitive to short time scale fluctuations rather than the slow processes associated with viscosity. In the vibrational echo study of $W(CO)_6$/2MP, it was suggested that the VTF type dephasing turns on because of the onset of local solvent molecule orientational and translational motions above T_g.[32] Thus, dephasing can display

the same or similar temperature dependences as the long time scale process and can be sensitive to processes which are generally associated with long time scales.

The Mb-CO vibrational absorption line is inhomogeneously broadened both below and above the proposed protein dynamical transition temperature, T_g^P. At first glance, this may seem inconsistent with making a transition from a glassy like state to a liquidlike state. In a glass, the time scale for the system to sample all possible structural configurations is, essentially, infinitely long. In contrast, in a liquid, the time scale is relatively short. Vibrational echo experiments on metal carbonyls in true glass-forming liquids, e.g. $W(CO)_6$ in 2-methylpentane, demonstrate that vibrational lines can be inhomogeneously broadened well above the glass transition temperature, T_g.[32] The vibrational echo measures the homogeneous dephasing time. In a liquid, on some relatively short time scale, all structural configurations will be sampled. However, the time scale may be long compared to the homogeneous dephasing time, and, therefore, the vibrational line is inhomogeneously broadened. On a time scale longer than T_2, spectral diffusion will eventually result in the transition frequency sampling all possible values. The fact that spectroscopic lines can be inhomogeneously broadened above T_g is true for electronic transitions as well. Ultrafast photon echo experiments, even in room temperature liquids, have shown that the electronic transitions of chromophores in glass-forming liquids can be inhomogeneously broadened.[45−48] Thus, passing from a glassy state to a liquid state ensures that a chromophore will sample the full range of transition energies contained in the spectroscopic line, but the time scale for the sampling can be long compared to the homogeneous dephasing time measured in a vibrational echo experiment or a photon echo experiment, resulting in an inhomogeneously broadened absorption line.

Figure 3 shows pure dephasing line widths as a function of temperature for Mb-CO in three solvents. The diamonds are the trehalose data shown in Figure 2; the triangles are data for Mb-CO in the solvent 95:5 glycerol:water;[11,12] and the circles are data for Mb-CO in the solvent 50:50% ethylene glycol:water. The line through the trehalose data is the same as in Figure 2. The lines through the other data are guides to facilitate discussion.

In all three solvents, at temperatures below their respective break points, the data fall on the same $T^{1.3}$ power law line. The fact that the vibrational dephasing comes solely from protein fluctuations, and not from the solvent, has been discussed in detail previously.[11−13] The identical power law temperature dependences, which have the same slopes and the same values of the dephasing in three solvents, is another demonstration that the vibrational pure dephasing is a measure of protein dynamics. The power law temperature dependence is reminiscent of the temperature dependence of processes in low-temperature glasses (< a few K). Power laws have been seen in measurements of the heat capacities of glasses[49,50] and optical[43] and vibrational[51] dephasing of chromophores in glasses. Power law temperature dependences of heat capacities, optical and vibrational dephasing, as well as other observables have been explained in terms of the tunneling two-level system (TLS) model of glasses.[52,53] Generally, the TLS model is only invoked up to a few K. In optical dephasing experiments in organic glasses below a few K, T^α with values of α in the range of 1.1−1.5 are frequently observed with $\alpha = 1.3$ the most common value. A power law temperature dependence of the vibrational pure dephasing line width was observed for a solute in an organic glass up to ∼20 K.[51] TLS in glasses arise from slight differences in local structures. The complex structural energy landscape is modeled as a distribution of double wells having a broad distribution of energy differences between the two sides of the double well. The dynamics in glasses at low temperatures are caused by phonon-assisted tunneling among local structures modeled as transitions between the two sides of the double wells.

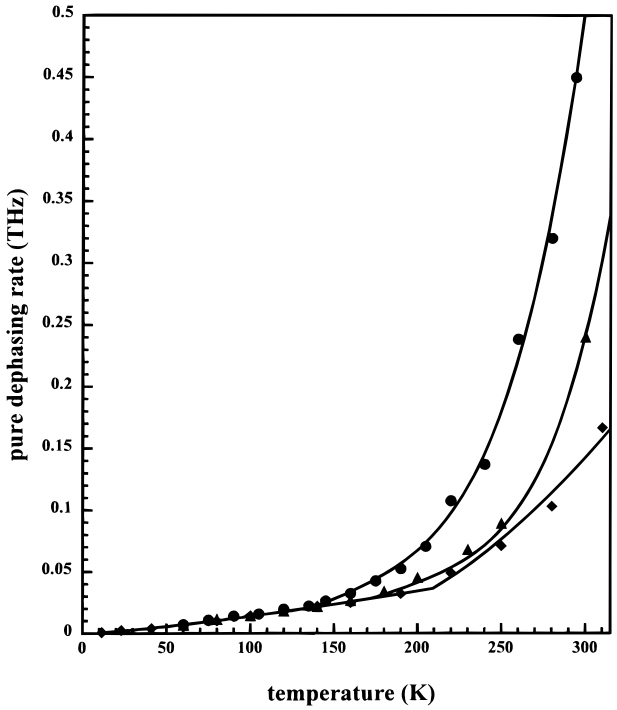

Figure 3. Pure dephasing of Mb-CO in three solvents. The squares are the trehalose data shown in Figure 2. The triangles are data in 95:5 glycerol:water.[11] The circles are data in 50:50 ethylene glycol:water. Below ∼150 K, the dynamics for all three solvents are fit with the identical $T^{1.3}$ power law. Above ∼200 K, the trehalose data is fit with an exponentially activated process. The glycerol:water and ethylene glycol:water data have both a temperature dependence and a viscosity dependence at the higher temperatures.

In the Mb-CO vibrational dephasing, the $T^{1.3}$ temperature dependence is observed to much higher temperatures than in true glasses. One possible explanation of the power law temperature dependence is thermally assisted tunneling among slightly different protein configurations. Small internal protein structural changes might be described in terms of protein two-level systems (PTLS).[11,12] The PTLS are akin to the two-level systems of very low temperature glasses except the protein energy landscape would have to be such that tunneling is the dominant process even at temperatures up to 200 K. If this is the case, the same statistical mechanics machinery used to describe the low temperature (∼1 K) optical dephasing of electronic transitions of chromophores in low-temperature glasses[43,54] can be used to describe the PTLS induced vibrational dephasing of Mb-CO at much higher temperatures (∼100 K). Alternatively, the power law temperature dependence could arise from activation over barriers rather than tunneling if there is the appropriate energy landscape to provide the necessary broad distribution of activation energies.[55,56] In either case, the $T^{1.3}$ temperature-dependent pure dephasing can occur because of motion on a broad protein energy landscape in a manner analogous to dynamics in true glasses. While an entirely different mechanism cannot be ruled out, the similarity of the vibrational echo data for Mb-CO to dephasing and other measurements on low-temperature glasses is suggestive of a type of glassy-like state of the protein.

Considering Figure 3, it can be seen that the breaks in the dephasing in the three solvents do not occur at the same temperature and that the temperature dependences are not the same in the three solvents at higher temperatures. At all temperatures studied in trehalose, the viscosity is essentially infinite. The observed temperature-dependent pure dephasing comes from protein fluctuations in a solid medium in which the topology of the protein/surface interface is fixed. In the two liquid solvents, the situation is quite different. As the temperature is increased, the viscosities of the solvents decrease. Very recent vibrational echo studies on Mb-CO conducted at room temperature as a function of solvent viscosity show that there is a strong viscosity dependence to the Mb-CO pure dephasing.[57] At constant temperature, as the viscosity of the solvent is decreased, the Mb-CO vibrational pure dephasing rate increases. Therefore, the temperature dependences observed in the glycerol:water and ethylene glycol:water solvents are actually combinations of a pure temperature dependence and a viscosity dependence. For this reason, the data in these solvents were not fit with the functions given in eq 4 or 5. The trehalose data characterizes the protein dynamics responsible for the pure dephasing with the protein/solvent boundary condition static.

The order of the break in the functional form of the temperature dependences displayed in Figure 3, from lowest temperature to highest temperature, is ethylene glycol:water (~150 K), glycerol:water (~180 K), and trehalose (~200 K). This is also the order of the solvents' glass transition temperatures. From Figure 2, it is clear that the dynamical transition displayed in the vibrational echo data does not depend on the solvent undergoing a glass transition. However, the data in Figure 3 show that if the solvent goes through its glass transition at a temperature below T_g^P, then T_g^P is reduced.

A way to visualize what might be happening is to consider a hypothetical phase-separated polymer blend in which the minority component, A, is contained in nanometer size domains,[58] which are essentially pure A, embedded in the host polymer, B. If $T_g^A < T_g^B$, then the nanodomains can undergo the glass to liquid transition while B is still solid. Above T_g^A, the liquidlike nanodomains will have a fixed nanodomain/host surface boundary. However, if $T_g^A > T_g^B$, then the host will become liquid at a temperature at which the nanodomains are still solidlike. This changes the boundary condition and could very well reduce the nanodomain glass transition temperature because, for a nanometer-sized object, the surface region has a substantial influence on the interior. There is some evidence to support this picture. A very thin film of a polymer (large surface to volume ratio) on a substrate with one surface unconstrained has a lower T_g than the bulk polymer.[59] The extra mobility afford by the free surface reduces T_g.

We suggest that the protein in a glassy solvent might be in some sense like the nanodomains in the polymer blend. In trehalose, $T_g^P < T_g^S$, where T_g^S is the glass transition temperature of the solvent. In this situation, the protein glass transition occurs with a fix protein/solvent interface, giving the highest T_g^P. In glycerol:water, $T_g^P > T_g^S$. The protein glass transition occurs in a very viscous liquid, but the interface is no longer fixed. This provides the protein with extra mobility, reducing T_g^P. In ethylene glycol:water, T_g^S is even lower. The liquid is less viscous as T_g^P is approached, and T_g^P is even lower. This is not a slaved protein glass transition, but, rather, a protein/solvent boundary condition influence on T_g^P.

While the vibrational echo experiments presented here and many other experiments[11] display a change in Mb dynamics at ~200 K, there have been other experiments that do not see a change near 200 K. The temperature dependence of the X-ray crystal structure has been determined, and the structure at 80 K does not seem to be different than that at room temperature.[60,61] In addition, the IR spectra of low-humidity samples are independent of temperature between 15 and 300 K and have a transition, depending on the degree of hydration, between 180 and 270 K.[27] Differential scanning calorimetry shows no unique glass transition of water associated with the protein but, rather, many transitions between 150 K and denaturation.[62,63]

The results presented here are consistent with the previous measurements, both those that show protein transitions and those that do not. The vibrational echo detects a change in the nature of the dynamics. Such a change does not require a change in the protein structure. The trehalose samples studied here contain significant amounts of water. It would be interesting to see if the degree of hydration changes the nature of the vibrational echo temperature dependence. The vibrational echo experiments detect the protein fluctuations that are communicated to the CO bound at the active site. Apparently, such fluctuations are not significantly influenced by changes in the state of the water bound at the protein surface that are detected by calorimetry.

IV. Concluding Remarks

Vibrational echo experiments can provide new insights into the nature of protein dynamics. In the experiments described above, Mb protein fluctuations that are communicated to the CO bound at the active site were examined. Because the experiments involve only the ground electronic state and involve time scales only out to a few tens of picoseconds, they should be amenable to simulations. The results show that, independent of the solvent, the low-temperature pure dephasing rate is a power law, $T^{1.3}$. This power law temperature dependence is suggestive of glasslike behavior, but at much higher temperatures than typically observed in true glasses. The results show that, even at low temperatures, fast protein structural fluctuations occur.

The observation of the change in the form of the temperature dependence of the pure dephasing rate at ~200 K in the solid solvent trehalose is consistent with the protein undergoing a transition in the nature of its dynamics that does not depend on a change in the solvent properties. If the power law pure vibrational dephasing temperature dependence below ~200 K is due to motion on a glasslike energy landscape, then the data above ~200 K could result from activation above the top of the landscape.[20] Another possibility is that the break in the temperature dependence indicates that the top of the landscape has been reached, and the ΔE in eq 4 arises from a barrier or a narrow ranges of barrier heights that are higher in energy than the top of the landscape. The fact that the temperature at which the break in the temperature dependence occurs depends on the solvent T_g and the temperature dependence above the break in liquid solvents depends on the solvent viscosity is indicative of the important role that the protein/solvent boundary condition plays in protein dynamics and, presumably, the nature of the energy landscape. The relationship of the vibrational dephasing to the solvent viscosity will be discussed in detail in a subsequent publication.

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