Ultrafast Vibrational Dynamics of the Myoglobin Amide I Band

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Mid-infrared transient absorption ("pump−probe") measurements on the amide I band of myoglobin in D_2O and in a glass-forming D_2O/glycer(ol-d_3) solvent mixture reveal very rapid vibrational energy relaxation. At 300 K, the exponential decay time is 1.3 ± 0.2 ps in D_2O. The temperature dependence of the vibrational relaxation in the solvent mixture is slight, changing from 1.9 ± 0.2 ps below 100 K to 1.2 ± 0.2 ps at 310 K. The lack of a strong temperature dependence is indicative of a low-order relaxation process where energy transfers into high-energy modes of the system rather than directly to low-energy solvent or protein "bath" modes. The pump−probe signal is also strongly wavelength-dependent. As the laser is tuned to the low-energy side of the absorption band, transient absorption contributions to the signal increase, indicating an anharmonicity of 15 ± 2 cm^{-1} for the amide I mode. The time-resolved polarization anisotropies at 300 and 100 K show a decay of about 10 ps, independent of temperature, which is attributed to energy transfer within the amide I band.

1. Introduction

Because of the sensitivity of the amide I mode to hydrogen bonding and conformation, this mode has been extensively studied both theoretically and experimentally. Until recently, infrared studies have been limited to frequency-domain measurements of band shapes and frequencies. Time-domain measurements of vibrational dynamics have demonstrated utility in investigations of intermolecular interactions and dynamics in condensed-phase systems[1,2] and often provide additional information about vibrational transitions than that obtainable from frequency-domain measurements alone.

We previously gave a preliminary report on amide I vibrational lifetimes in myoglobin[3]—a medium-sized protein containing ∼150 amino acids whose main structural component is the α-helix. More recently, Hamm et al. published a study of the amide I band in the smaller proteins apamin, scyllatoxin, and bovine pancreatic tripsin inhibitor (BPTI) using femtosecond pump−probe and hole-burning spectroscopy at room temperature.[4] The room-temperature myglobin amide I dynamics are the same as those observed for the peptides studied by Hamm et al. Here, we present a more detailed report of wavelength, temperature, and polarization-dependent picosecond infrared pump−probe measurements on the amide I mode of myoglobin. These measurements show that the amide I mode has a lifetime of 1.3 ± 0.2 ps that is only slightly dependent on temperature, a homogeneous line width of ≥4 cm^{-1}, and an anharmonicity of about 15 cm^{-1}.

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2. Experimental Methods

Vibrational relaxation was measured using a single-color pump−probe technique in which the transition is excited and probed at the same infrared wavelength. The experimental setup used was the mid-infrared pump−probe apparatus at the Stanford Picosecond Free Electron Laser Center. This technique, apparatus, and the characteristics of the Stanford mid-IR free electron laser (FEL) have been described in detail in a recent publication.[2]

The pump and probe pulses were nearly transform-limited Gaussian pulses of about 1 ps full width at half-maximum (fwhm). The average pulse autocorrelation and spectrum were continuously monitored during the measurements. The pump and probe beam polarizations were linear with their relative polarization angle controlled by two polarizers in the probe beam. The zero of time was determined by replacing the protein sample with an indium arsenide wafer in which the intense pump beam produces an instantaneous decrease in probe transmission.[5]

Samples were approximately 8 mM in protein, with a 15 μm path length. Sample absorbances, at the peak of the amide I band, were in the range 0.8−1.0. Horse heart or horse skeletal myoglobin (Mb) was purchased from Sigma and used without further purification. Deuterium-exchanged Mb was prepared by thrice dissolving and lyophilizing the Mb from 99.9 atom % D_2O (Aldrich). For temperature-dependent measurements between 6 and 310 K, a glass-forming solvent mixture of D_2O and 98 atom % glycer(ol-d_3) (Aldrich) in the ratio 20/80 by volume was used.

Figure 1 shows FTIR spectra (4 cm^{-1} resolution) in the amide I band region of Mb in the D_2O/glycer(ol-d_3) solvent mixture at 300 and 10 K. Infrared measurements on the amide I band of proteins are often performed in D_2O because the broad

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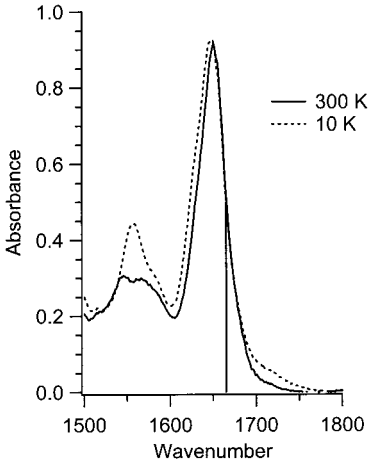

Figure 1. FTIR absorption spectra (4 cm^{-1} resolution) at 10 and 300 K for myoglobin in a 20/80 by volume D_2O/glycer(ol-d_3) glass-forming solvent mixture. The concentration is ~8 mM in protein, and the path length is 15 μm. The line at 1665 cm^{-1} indicates the frequency to which the laser was tuned for the temperature-dependent pump−probe measurements shown in Figure 2.

absorption band centered near 1650 cm^{-1} associated with the H_2O bending motion is shifted to 1215 cm^{-1}. The vertical line indicates the laser frequency (1665 cm^{-1}) at which temperature-dependent pump−probe measurements were made. The amide I band shape and position do not change significantly with temperature on the high-energy side of the band. For this reason, it was not necessary to tune the laser as the temperature was changed.

3. Results and Discussion

3.1. Temperature Dependence. Measurements were made at 10−20 K intervals from 6 to 310 K in the D_2O/glycer(ol-d_3) solvent mixture. Figure 2A shows the change in probe transmission vs time delay at 6 and 300 K with the laser tuned to 1665 cm^{-1}. Also shown is a typical experimentally measured pulse autocorrelation. The amplitudes of the signals have been normalized to 1.0 to facilitate comparison of the decay times. The change in transmission was typically about 1.0%. No differences in results were observed between heart and skeletal Mb.

Decays were fit, using an unweighted nonlinear least-squares method, to a convolution of a Gaussian representing the instrument response with the sum of a single-exponential decay and a δ-function. This function represents an exponential population relaxation plus a coherent scattering contribution as discussed below in section 3.2. Use of a biexponential decay did not improve the fits. The Gaussian width was allowed to vary or was held constant at the experimentally measured autocorrrelation width, with no significant difference in results. The laser pulse widths resulting from the fits agreed with those measured experimentally to within 20%. Experimental knowledge of time zero and pulse widths along with good a signal-to-noise ratio (S/N) allows for excellent time resolution even though the pulse widths and decay times are similar. For clarity, fits are not shown in Figure 2; see Figure 3 for examples of fitted decays.

Decays were obtained at each temperature for probe polarization parallel to and 55° to the pump polarization and, at a few temperatures, for perpendicular probe polarization. Although the relative amplitude of the coherent scattering contribution changes with polarization, the exponential lifetimes obtained from the fits had no systematic dependence on polarization. The

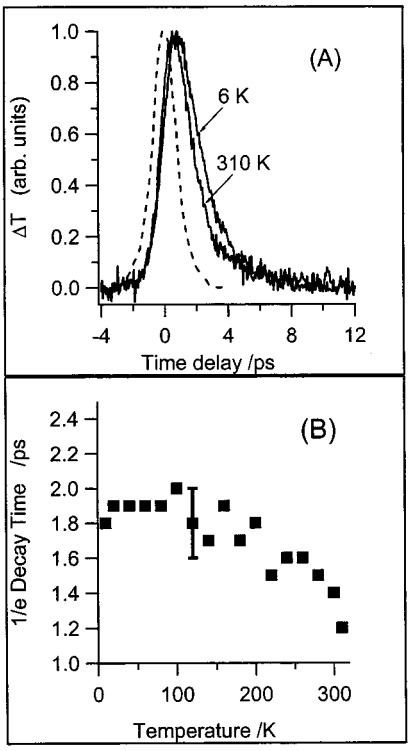

Figure 2. (A) Pump−probe signals at 1665 cm^{-1} showing the change in probe pulse transmission with optical delay for the amide I band of myoglobin at 6 and 310 K. The dotted curve is a typical FEL pulse autocorrelation (pulse fwhm = 1.3 ps). (B) Temperature dependence of the exponential decay times at 1665 cm^{-1}.

reported error of ±0.2 ps is the standard deviation in lifetimes obtained for a given temperature during two separate experimental runs and for different probe polarizations.

Figure 2B summarizes the exponential decay times obtained from the pump−probe data as a function of temperature. There is a slight increase in decay time as the temperature is decreased, changing from 1.2 ± 0.2 ps at 310 K to 1.9 ± 0.2 ps at temperatures below 100 K. The decay time of Mb in pure D_2O at 300 K is 1.3 ± 0.2 ps, the same as in the deuterated solvent mixture. The relaxation rate appears constant below 100 K. Above this temperature, the lifetime begins to decrease slightly. The glycerol/water mixture undergoes a liquid−glass phase transition near 180 K. This phase transition may affect the relaxation by changing the density of states and/or coupling via physical density and motional effects.[6,7]

The weak temperature dependence of the amide I relaxation strongly suggests it occurs via a low-order process, where relaxation is into high-frequency modes of the protein or solvent with frequency greater than 500 cm^{-1} rather than directly into several low-frequency solvent or protein "bath" modes. Tokmakoff et al.[6] and Kenkre et al.[7] have given detailed discussions of temperature dependencies in vibrational relaxation. In general, if two or more low-energy bath modes are involved, the vibrational relaxation will have a significant temperature dependence, since at higher temperatures, thermal population of the bath modes will increase the relaxation rate through stimulated processes.

Both the solvent mixture and the protein have high-frequency modes that, consistent with the temperature dependence, could be accepting modes for the amide I energy. Infrared spectra of proteins show that there are fundamental modes near in energy to the amide I mode, including the amide II N−D stretch (in this case the protein is deuterated) at ~200 cm^{-1} lower in

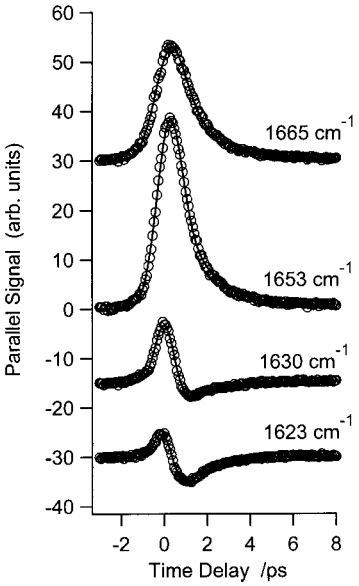

Figure 3. Effect of laser tuning within the amide I band on the pump−probe signals. The sample is myoglobin in D_2O at room temperature. The observation of a transient absorption as the laser is tuned to the red results from a small anharmonicity of about 15 cm^{-1}. Shown are data (open circles) and fits (solid lines) using either a positive or a negative amplitude exponential decay plus a positive δ-function. This fitting function was convolved with a Gaussian representing the pulse autocorrelation. The exponential lifetime is 1.3 ± 0.2 ps at all wavelengths.

energy. In addition, the entire region below about 1400 cm^{-1} has many overlapping bands, several of which are associated with motions of the backbone. Overtones and combination states of fundamental modes in the 500−1000 cm^{-1} region that are in resonance with the amide I mode can also act as accepting states. The density of states is high, and there are potentially many different pathways involving only one or no low-energy state.

3.2. Wavelength Dependence. As shown in Figure 3, the shape of the signal changes dramatically when the laser is tuned into the band maximum and then to the lower energy side of the band. The negative-going contribution, evident at 1630 and 1623 cm^{-1}, must arise from a transient absorption that is resonant with part of the probe pulse spectrum and has a different time constant than the remaining positive-going signal. In a preliminary report,[3] we attributed the wavelength dependence for the Mb amide I band to multilevel up-pumping, which has been observed in similar experiments on metal carbonyls.[8,9] However, signals such as those in Figure 3 can also arise from a combination of a transient increase or a transient decrease in transmission plus instrument response-limited coherent scattering that is in phase with the probe field. We now have polarization-dependent data that shows the pump−probe signals contain coherent scattering.[10]

Typically, coherent scattering is polarization-dependent and may be suppressed when the pump and probe beam polarizations are perpendicular. Figure 4 shows significant suppression of the positive-going signal at crossed polarization for a wavelength on the low-energy side of the band. As shown in Figure 3, the data are well fit (solid curves) by a single-exponential decay of positive or negative amplitude plus a positive amplitude δ-function convolved with a Gaussian representing the pulse autocorrelation. The δ-function represents the coherent scattering. A positive amplitude exponential decay is an increase in transmission of the ν_{10} transition after excitation. A negative amplitude exponential is a transient decrease in transmission

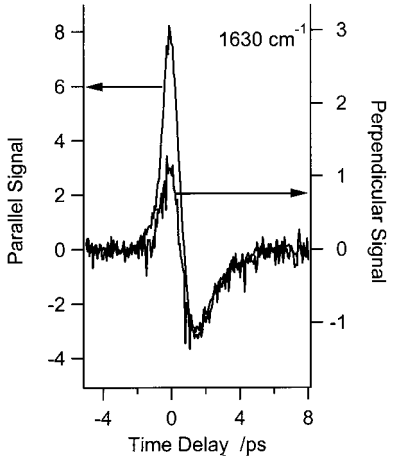

Figure 4. Effect of probe beam polarization on the signal at 1630 cm^{-1}. The positive component is suppressed relative to the amplitude of the transient absorption for crossed pump and probe polarizations, indicating the presence of a coherent scattering signal that is in phase with the probe field.

of the ν_{21} transition. Although both transitions are probed at each of the laser wavelengths, the bleach and absorption signals have the same time-constant but differing amplitudes at the different wavelengths. Thus, only one exponential is needed to fit the data and to obtain the lifetime of the first excited level.

The observation of transient absorption in the ν_{21} transition in single-color infrared pump−probe experiments depends on the relative values of the pulse spectral width, the mode anharmonicity, and the homogeneous and inhomogeneous line widths and requires overlap of the probe pulse spectrum with the ν_{21} transition. Thus, the signals in Figure 3 contain information on the anharmonicity. The anharmonicity of the amide I mode can be estimated from the data by comparison with a coupled rate equation model that includes a time-dependent excitation rate.[11] The same coupled rate equation model developed by Heilweil et al. to describe multilevel vibrational dynamics in metal carbonyls[9] was modified to include integration over an inhomogeneous band shape. The inhomogeneous band shape was defined so that, when convolved with a chosen Lorentzian homogeneous line width, the experimentally measured high-resolution (0.5 cm^{-1}) FTIR band shape results. Population was limited to the ground and first excited vibrational levels.

Experimentally determined laser parameters, a ν_{10} cross section of 2×10^{-22} m^2 estimated from myoglobin FTIR spectra, and an assumption that the upper transition lifetimes and cross sections scale linearly with vibrational quantum number[12,13] were used. The anharmonicity was estimated for reasonable values of the homogeneous line width by comparing the laser frequencies at which the calculated and experimental transients change sign. A vibrational lifetime of 1.3 ps means the homogeneous line width must be ≥4 cm^{-1}. FTIR deconvolution techniques show subbands within the myoglobin amide I band of 10−15 cm^{-1} fwhm,[14,15] and Hamm et al.[4] estimate an amide I homogeneous line width of 10 cm^{-1} from femtosecond hole-burning experiments on small proteins. Four cm^{-1} is the lower limit for the homogeneous line width, and 10 cm^{-1} is a reasonable upper limit. For a homogeneous line width of 4 cm^{-1} an anharmonicity of 13 cm^{-1} models the data, whereas for a homogeneous line width of 10 cm^{-1} an anharmonicity of 17 cm^{-1} is needed. The lower anharmonicity compares well with an anharmonicity of 12 cm^{-1} measured by infrared overtone spectroscopy of acetanilide crystals.[16] Acetanilide forms an α-helix type structure in the crystalline state and has

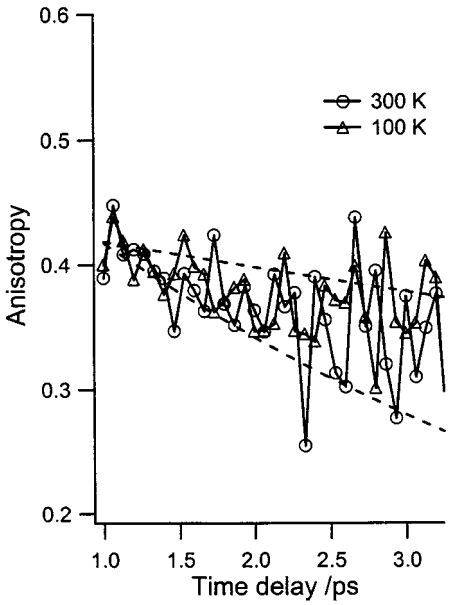

Figure 5. Anisotropy decays for the amide I mode at 100 and 300 K calculated from pump−probe transients taken with probe polarization parallel and perpendicular to the pump polarization. The laser was tuned to 1665 cm^{-1}. The decrease in anisotropy with time can be described by an exponential decay of several picoseconds as shown by the calculated 5 ps exponential curve (lower dashed line) and 20 ps exponential curve (upper dashed line). Within signal to noise, the anisotropy decays are the same at 100 and 300 K.

been used as a model for protein vibrations.[16] An anharmonicity of 17 cm^{-1} compares well with a value of 16 cm^{-1} estimated from time-resolved studies of another amide model compound, *N*-methylacetamide (NMA) in D_2O.[4]

The fits of the data in Figure 3, and at other laser frequencies not shown, provide room-temperature relaxation rates for the ν_{10} transition. It would be interesting to know if the relaxation rate is dependent on protein secondary structure. Since the transients measured on the low-energy side of the band are dominated by the ν_{21} transient absorption of amide modes whose ν_{10} transitions lie higher in energy, it is not easy to study the relaxation rate as a function of laser tuning in proteins. The case may be somewhat more clear-cut on the higher energy side of the band. Data at 1653 cm^{-1}, near the peak of the amide I band, should be mainly associated with the major structural component (α-helix). Myoglobin contains turn structures connecting the helices that have been assigned to frequencies between 1670 and 1680 cm^{-1}.[15] Pump−probe data taken at 1653 and 1673 cm^{-1} showed no difference in lifetimes.

The average room-temperature lifetime for all measurements across the amide I band is 1.3 ± 0.2 ps with no trend. However, at each laser frequency, the signal is an average over the 14 cm^{-1} laser bandwidth. Femtosecond pump−probe measurements on apamin, scyllatoxin, and BPTI, all of which contain both α-helix and β-sheet structures in differing proportions, show the same band average amide I vibrational lifetime of 1.2 ps.[4] In the femtosecond experiments, the laser bandwidth is >100 cm^{-1}; thus, the entire amide I band is excited by the pump pulse. Both those results and the data presented here suggest that the amide I vibrational lifetime is not significantly affected by secondary structure. However, a clearer demonstration would be to measure vibrational lifetimes of small polypeptides with different, well-defined secondary structures.

3.3. Polarization Anisotropy. Figure 5 shows anisotropy decays calculated from parallel and perpendicular pump−probe decays $((I_{||} - I_{\perp})/(I_{||} + 2I_{\perp})$ where I is the time-dependent probe intensity) taken at 300 and 100 K with the laser tuned to 1665 cm^{-1}. There is a decrease in anisotropy on a several picosecond time scale, as shown by the calculated exponential decay curves of 5 and 20 ps. A loss of polarization could arise from overall rotation of the protein, hindered low-energy motions of the peptide groups, or excitation transfer between amide modes within the amide band. Overall rotation of a protein the size of myoglobin would be on a much longer time scale, but the other processes can occur in picoseconds or femtoseconds. If the anisotropy decay were due to hindered internal motions, it should be temperature-dependent, with a significantly slower decay at 100 K. However, the anisotropy curves at the two temperatures are indistinguishable. This leaves energy transfer as the likely mechanism. Hamm et al.[4] observe a similar component of several picoseconds in the amide I anisotropy decays of smaller proteins at room temperature, also attributing it to energy transfer (equilibration) within the amide I band.

4. Summary

The important results of this study on myoglobin are the following: (1) the vibrational relaxation rate of the amide I band is 1.3 ± 0.2 ps at room temperature and appears to be independent of protein secondary structure; (2) the relaxation rate is only weakly dependent on temperature, indicating the relaxation is not directly into low-frequency solvent or protein bath modes but into high-frequency modes of the system; (3) the ν_{21} transient absorption was observed with laser tuning to the low-energy side of the band, consistent with an anharmonicity of 15 ± 2 cm^{-1}; (4) the polarization anisotropy, which decays over a several picosecond time scale, is the same at 300 and 100 K and is attributed to energy transfer within the amide I band.

All of these results are in agreement with femtosecond measurements reported for smaller proteins.[4] Despite differences in size and secondary structure, the amide I relaxation dynamics observed in both of these studies are essentially the same and appear to be fundamental features of the amide I mode in polypeptides and proteins.

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(10) If the transient transmission decay time were significantly longer than the pulse width, it would be clear whether coherent scattering was

present on the basis time constants alone. However, in nearly pulse-width-limited experiments, the case is not as clear.

(11) Since the pulse width and relaxation time are nearly the same, a coupled rate equation is not rigorously correct. However, it is sufficient for obtaining an estimate of the anharmonicity within the assumptions required for the upper-state parameters and the homogeneous line width.

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