Long-Lived Amide I Vibrational Modes in Myoglobin

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Pump-probe experiments in the infrared measure vibrational relaxation rates. Myoglobin, which is almost entirely α helix in secondary structure, has an unusually long, nonexponential excited state relaxation generated by optically pumping at the blue side (5.85 μm) of the amide I band. The amino acid alanine and the predominantly β sheet protein photoactive yellow protein do not have such a long-lived state, suggesting that the α helix in proteins can support nonlinear states of 15 ps characteristic times.

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Proteins are remarkable nanoscale machines that efficiently convert chemical energy into mechanical energy or direct chemical energy to the breakage of a specific bond. Perhaps the most remarkable example of this nanoscale mechanochemical coupling is the reversible motor protein F1-ATPase, which can convert chemical energy to mechanical rotation, and vice versa [1]. Although stereochemistry can go a long way towards understanding how these specific molecular events occur, ultimately we need to understand at the picosecond time scale the progress of locally deposited vibrational energy from chemical events through the macromolecule.

High intensity picosecond IR pump/probe experiments can be used to deposit quanta of vibrational energy specifically into narrow spectral regions of a protein which are group specific and probe for intensity dependent vibrational lifetimes. The amide I transition in proteins is an exceptionally interesting level to pump since in the case of the α helix hydrogen bonding forms a chain which connects across the protein. The amide I band of a protein is heterogeneously broadened due to sensitivity of the amide I transition to internal hydrogen bonds which interact with the C═O group [2]. Simple amino acids such as alanine have a COO^- stretching mode which fits well to a single Lorentzian line shape of width 10 cm^{-1}, while proteins such as myoglobin and photoactive yellow protein have wider bands of approximately 40 cm^{-1} width with substructure which is a function of the secondary structure of the protein. A relatively simple protein such as myoglobin has a band predominantly due to the α helix, while a more complex protein such as photoactive yellow protein has a broader and more complex band presumably due to a greater variety of secondary structures. Figure 1 shows the amide I bands of these molecules in deuterium oxide and their x-ray structures [3,4].

Physical quantities in insulating solids which involve phonon energy transport such as heat conductivity are finite (and not infinite) because bond anharmonicity (nonlinear response) couples modes together and leads eventually

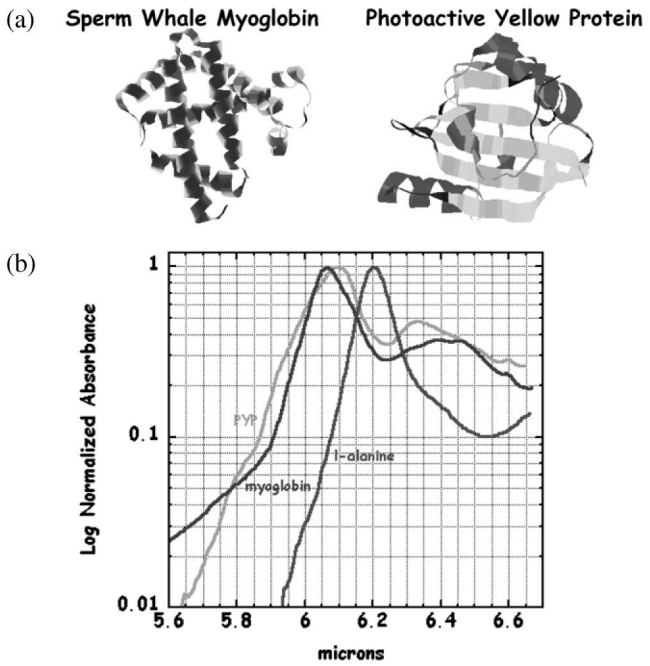

FIG. 1. The normalized amide I absorbance of sperm whale myoglobin (middle dark line), photoactive yellow protein (left gray line), and *L*-alanine (right dark line) in deuterium oxide. The x-ray structures of myoglobin (1) and photoactive yellow protein (2) are shown above the amide I spectra.

to equipartition of energy, and dispersive in time because of the finite lattice spacing. However, under the right combination of amplitude excitation anharmonicity can also lead to *cancellation* of nonlinear pulse sharpening and linear dispersion, resulting in the generation of a nondispersing "solitonic" excitation [5]. The α helix has been theoretically proposed as a conduit for coherent energy transfer in proteins, due to the linear hydrogen bonding network which stabilizes the structure and forms a path along the α helix backbone [6]. Pump/probe experiments are sensitive to the anharmonic response of a system since a completely harmonic (linear response) system cannot be saturated and thus has no saturation induced transparency and measured vibrational relaxation rates. We have used the infrared free electron laser facility FELIX [7] to provide continuously tunable, high peak power picosecond duration IR pulses capable of driving the system into the nonlinear response region.

Note carefully that the macropulse of FELIX, which consists of a string of 0.5 μJ picosecond duration micropulses separated by 40 ns (25 MHz), is 5 μsec in duration, so this is not a cw experiment. It is desirable to use as high an intensity of micropulses as possible in probes of nonlinearities, but the large absorption of even deuterium oxide at 6 μm limits sample thickness to 12 μm. The combination of thin sample, high energy micropulses, 50 μm focused spot size, and high micropulse repetition rates in the macropulse means that substantial sample heating can occur during the macropulse, even though each micropulse heats the sample only approximately 0.5 K. In order to ensure that our sample was at 20° our pump/probe signals are measured only within the first 0.1 μsec of the macropulse using gated boxcar integrators. Although our signals are small enough (1% transmission changes) that four-wave coherent mixing spikes are not visible, serious thermal grating coherent artifacts are observed at the rear of the macropulse as the sample heats and the coherent interference of the pump and probe pulses forms a temperature phased grating.

Vibrational systems should soften with high levels of excitation and exhibit a fundamental nonlinear response in an optical pump/probe experiment: bleaching (loss of absorbance) due to ground state depletion on the high frequency (blue) side of the band and an absorbance increase at the low frequency (red) side of the transition due to the anharmonic level shift $\Delta\nu$ which brings the 1-2 vibrational transition into resonance for a photon too low in energy for the 0-1 transition. A simple nonlinear potential surface which gives rise to mode softening as a function of high levels of excitation is the cosine function

$$V(x) = V_0[1 - \cos(2\pi x/a)] \sim V_0\left[\frac{1}{2}(2\pi x/a)^2 - \frac{1}{4!}(2\pi x/a)^4\right], \quad (1)$$

where x is the displacement from equilibrium of an atom of mass m, V_0 is the "ionization" energy of the atom (on the order of a few electron volts), a is a characteristic "ionization distance" (on the order of a few Å).

In first order perturbation theory [8,9] the nth energy level of the harmonic oscillator $E_n = (n + 1/2)\hbar\omega$ is shifted by an amount ΔE_n from E_n:

$$\Delta E_n = -\frac{3}{4}\epsilon(2n^2 + 2n + 1), \quad (2)$$

where $\epsilon = (V_0/4!)(2\pi/a)^4(\hbar/m\omega)^2$.

We have observed this signature signal both in simple amino acids (L-alanine) and in proteins, with similar size signals at a given pump energy for both simple amino acids and proteins. Figure 2(a) shows the amide I pump/probe signals on the red and blue side of the amide I peak for photoactive yellow protein. The anharmonic shift $\Delta E_2 - \Delta E_1$ between the 0-1 transition and the 1-2 transition can be calculated from the observed signal on the blue and red side of the amide I at a given absorbed flux of photons and extinction coefficient at the amide I and can be estimated to be approximately 5 cm^{-1} in the first vibrational excited state. This estimate comes from calculating that only

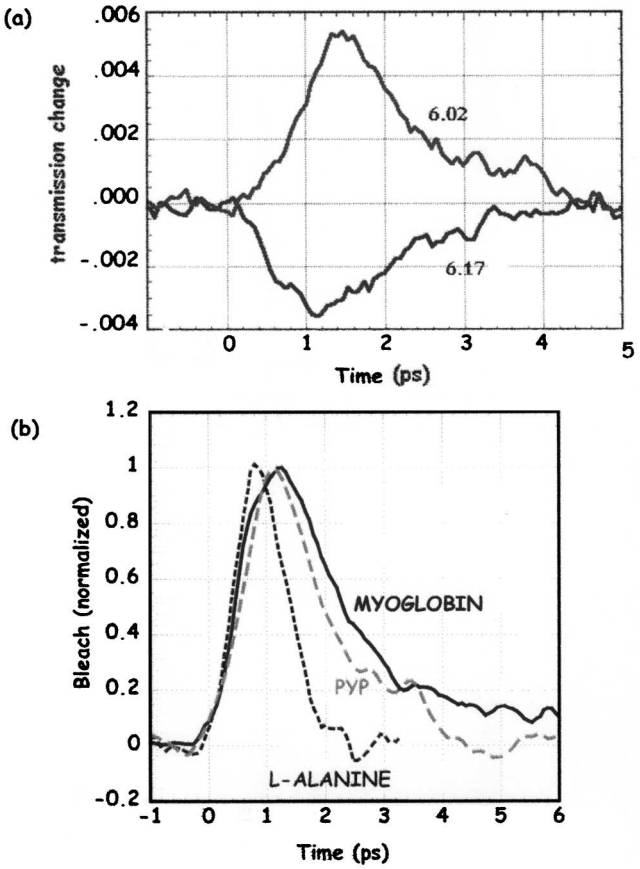

FIG. 2. (a) The pump/probe decay of PYP in D_2O at 20 °C at two different wavelengths, 6.02 μm (dark line) and 6.17 μm (grey line). A 20 nsec delayed reference pulse from the probe beam was used in a subtractive mode in the photoconductive detector to minimize common-mode micropulse amplitude variance. (b) A comparison of the normalized bleaching signal of L-alanine (dotted line), PYP (dashed line), and Mb (solid line).

approximately 1% of the amide I oscillators are excited at our measured flux at 6 μm of 0.1 μJ in a 50 μm diameter circle.

Unlike the simple *L*-alanine molecule, a biopolymer such as a folded protein with α helices can be viewed as a linear lattice of *coupled* anharmonic oscillators, and we can model the coupling as arising from springs of spring constant G representing the internal hydrogen bonds which hold the helix together. Although a linear lattice dispersively transports energy, and nonlinearities give rise to mode coupling (equipartition), a nonlinear lattice is capable of propagating energy without dispersion (solitons) at certain amplitudes. A simple nonlinear potential surface which gives rise to mode softening and kink soliton excitations as a function of high levels of excitation is the cosine function. Following the simple description of Remoissenet [10] in the continuum approximation we can write the wave equation for displacements of anharmonic oscillators in the $\cos(2\pi x/a)$ potential function as the sine-Gordon equation which supports kink solitons:

$$d^2\psi/dt^2 - c_0^2 d^2\psi/dx^2 + \omega^2 \sin(\psi) = 0, \qquad (3)$$

where $\psi(x) = 2\pi u(x)/a$ is the dimensionless displacement of the medium at position x, ω is small displacement harmonic angular frequency $\frac{2\pi}{a}(\frac{V_0}{m})^{1/2}$, and $c_0 = a(G/m)^{1/2}$ is the speed of sound down the lattice. We can roughly estimate this velocity from the mass of the oxygen atom (25.6×10^{-24} g), the lattice constant of the α helix (4.5 Å), and the effective spring constant of a hydrogen bond (10^4 dynes/cm), yielding $c_0 = 10^6$ cm/sec. Since the typical length of an α helix in a protein is only 40 Å, solitons will propagate down this lattice in only 0.4 ps. Thus, stable nonlinear excitations in these structures are more likely to be soliton/antisoliton pairs (breathers) rather than single solitons.

We have, in fact, observed an unusually long-lived bleaching signal only for myoglobin, and only with blue side amide I excitation at 5.85 μm. Neither photoactive yellow protein nor simple amino acids show any significant variance of the pump/probe relaxation rate with excitation wavelength, but myoglobin does. Note also that the amide I band relaxation rate is relatively insensitive to solvent conditions, since within the main band a simple amino acid in water has roughly the same short relaxation time as amino acids in a protein. Figure 3(a) compares the observed relaxation of the amide I mode of myoglobin at 5.85 μm, the extreme blue edge in a predominantly amide I protein, to the relaxation observed at 6.02 μm. This long-lived relaxation to the ground state population is indicative of the generation of a long-lived excited state lying to the red of the 5.85 μm pump wavelength. These are "deep statistics" runs on samples with inherently small pump/probe signals, and at present it is not realistic to obtain more spectral points without a substantial improvement in the signal-to-noise ratio on the FELIX pump/probe experimental beam line.

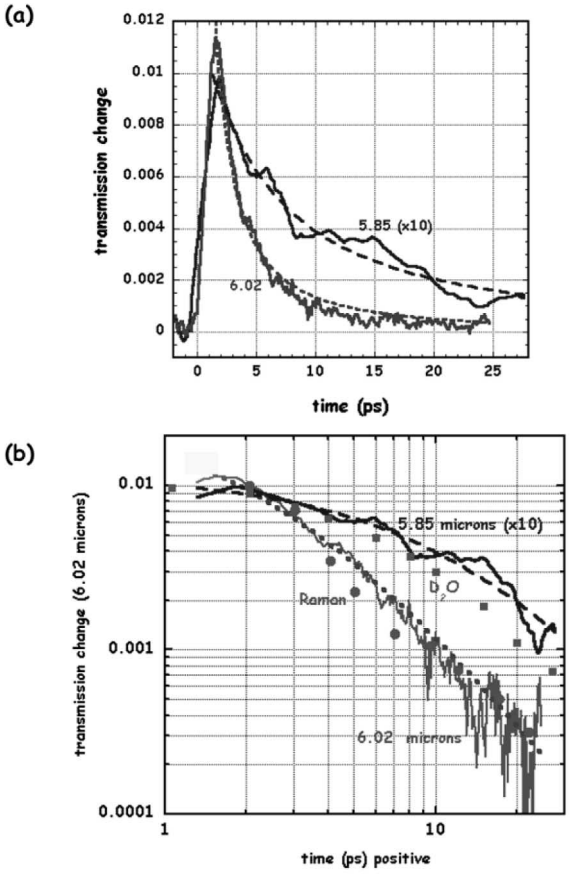

FIG. 3. (a) The pump/probe decay of Mb at 6.02 μm and 5.85 μm. The 5.85 μm decay amplitude has been multiplied by 10. The power law curve fits to the decay of the pump/probe signal are shown as dashed lines for the two wavelengths. The pulse width of FELIX was set to be as long as possible for these experiments, 1.5 ps or 10 cm^{-1} in linewidth. (b) Log amplitude vs log time plot of the data in (a). The heme Raman cooling data of Mizutani and Kitagawa are shown as gray circles, while the D_2O heating data of Lian *et al.* are shown as gray squares.

The general shape of the relaxation curves observed here can be due to a combination of T_1 anharmonic mode coupling and T_2 based spectral diffusion, both of which probe the distribution of excited state modes. Earlier recombination experiments which probe conformation distributions in proteins [11] and general principles of spin glass dynamics [12] predict that the relaxation decay due to a static mode distribution should fit a power law in time:

$$N(t) = N(0)(1 + t/\tau)^{-\alpha}. \qquad (4)$$

Figure 3(b) shows the results of fitting the data to such a power law decay. The normal amide I cooling rate fits well to a power law with exponent $\alpha = 2.1 \pm 0.2$ and $\tau = 2.7 \pm 0.5$ ps. The blue side peak is consistent with the same α and a much slower $\tau = 15 \pm 1$ ps. Thus the 5.85 μm relaxation rate is over 5 times slower than the relaxation rate within the core of the amide I band of photoactive yellow protein or myoglobin, and nearly 10 times slower than the relaxation rate of a solvent exposed amino

acid, and longer than any other relaxation we have observed in the 6 μm region for any molecule.

Anharmonic mode coupling leads to mode cooling. An independent measurement of the cooling time of vibrational modes confined to the heme group in myoglobin by time-resolved resonance Raman scattering by Mizutani and Kitagawa [13] also saw picosecond relaxation processes. We have coplotted the Raman results in Fig. 3(b), and remarkably their cooling times are identical with the main amide-I cooling rates and also fit to a power law of the same exponent and time constant, indicating that the heme cooling rates and the amide-I cooling rates in the center of the amide I band are very similar and distributed over many states. A pioneering measurement by Lian *et al.* [14] measured at 2 μm the rate of energy deposition in D_2O after optical excitation of the heme electronic states at 0.5 μm. We have coplotted Lian *et al.* data in Fig. 3 to show how the final rate of energy deposition into the solvent agrees with the power law cooling rate of the 5.85 μm amide I band, indicating that the proposed soliton-antisoliton breather mode may be the final bottleneck for vibrational energy flow into the solvent.

There have been other measurements of the cooling rate of energy deposited in a protein. The amide I pump/probe measurements of Peterson *et al.* [15] did not go farther to the blue side of the amide I than 6.0 μm, still well in the main band. The decay times in the center of the amide I are in agreement with our measurements there. Picosecond pump/probe measurements by Hamm *et al.* in the amide I of other proteins [16] did do multiwavelength measurements and did see a clean amide I bleach or absorb profile as a function of wavelength that is an indicator of anharmonic response but did not attempt narrow band far-blue side excitation.

The long-lived ground state population depletion produced by nonlinear generation of a long-lived state in the α helix of a protein that we report here would indicate that nonlinear excitations may play a significant and important role in energy transfer in biomolecules. It will be of interest to expand these pump/probe experiments to other proteins such as bacteriorhodopsin where the α helices span the membrane and may be directly involved in energy transduction. The indications from this experiment are that there may be some very clever aspects to energy transport in proteins that we have yet to explore.

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