Real Time Observation of Low Frequency Heme Protein Vibrations Using Femtosecond Coherence Spectroscopy

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(Received 13 August 1993)

Femtosecond laser pulses, resonant with the Soret bands of myoglobin (Mb) and cytochrome *c*, are used to probe coherent low frequency nuclear motion of the heme group. The time domain analysis is in good agreement with frequencies obtained independently using spontaneous resonance Raman spectroscopy. The deoxyMb data reveal a strong oscillation near 300 fs ($\sim$100 cm^{-1}) and a persistent feature also appears near 50 cm^{-1}. This is near the frequency expected for heme doming motion, which has been associated with the ligand binding reaction coordinate of Mb.

PACS numbers: 87.15.He, 36.20.Ey, 42.65.Re

Heme proteins are ubiquitous biomolecules involved in a wide variety of fundamental chemical reactions that support the life process. Two prototypical examples are cytochrome *c* (cyt *c*), a protein that is crucial to biological electron transport, and the oxygen storage protein myoglobin (Mb), which has served as an example for numerous biophysical studies. Both Mb and cyt *c* contain the planar heme prosthetic group (Fe-protoporphyrin), which gives this class of proteins their rich spectroscopic properties. A strong π-π^* electronic excitation of the prophyrin ring near 400 nm, known as the Soret band, couples to the nuclear motion of the heme and its axial ligands. This coupling leads to spectral broadening of the optical transition [1] and strong resonance Raman scattering [2]. Ultrafast nonradiative decay to lower electronic states (probably involving iron orbital excitations) also broadens the transition and is indicated by the lack of observable fluorescence or hot luminesence from the Soret excited state [3].

In this Letter we demonstrate that femtosecond coherence spectroscopy [4] can be applied to heme proteins and present real time observations of low frequency coherent nuclear motion in solution at room temperature. We also present evidence that these low frequency modes are coupled to a blueshifted charge transfer resonance underlying the Soret band and suggest that they involve both fundamental and overtone activity of the heme doming motion. Previous ultrafast laser studies of other biomolecular systems have detected high frequency ($\gtrsim$800 cm^{-1}) modes at room temperature [5] or low frequency modes ($\lesssim$200 cm^{-1}) in frozen solutions that disappear at 290 K [6]. The present work demonstrates that impulsively excited low frequency ($\lesssim$200 cm^{-1}) modes in biomolecules are not overdamped in solution and can be observed at room temperature.

The experimental arrangement consists of a self-mode-locked Ti:sapphire laser (Coherent, Inc.) pumped by an argon ion laser, which produces 85 fs laser pulses centered between 800 and 900 nm. These pulses are doubled and compressed to 45 fs using a thin BBO crystal and SF10 prisms. The time-bandwidth product of the blue pulses is found to be 0.39, which is close to the theoretical transform limit of 0.32 assuming a sech^2 time profile. The pulse train is separated by a beam splitter and the polarization of the probe beam is rotated by 90° and delayed with respect to the pump beam by using a stepping motor system. The two beams are focused in a near parallel configuration into a thin quartz sample cell containing the biological solution. The sample cell is translated perpendicular to the beam with a 30 Hz frequency and 1 mm amplitude to minimize the background signal due to thermal blooming. The recollimated probe beam is separated from the pump beam by spatial filtering and polarization selection. The probe beam is then either monitored directly with a photodiode or passed through a double momochromator and detected with a photomultiplier. The output from the photodiode or photomultiplier tube is detected with a lock-in amplifier. The lock-in reference frequency is obtained from a chopper in the pump beam operating at 2.8 kHz. The resulting signal is filtered so that only changes in the probe beam intensity, induced by the pump beam excitation, are ultimately recorded. Control experiments, using nonresonant samples, demonstrate that cross-phase modulation processes are negligible when probing in the spectral wing of the pulse.

Lyophilized sperm whale metMb and horse heart cytochrome *c* were obtained from Sigma Chemical Co. (St. Louis). A 0.1*M* sodium phosphate buffer, adjusted to *p*H 6, was used for both samples. The myoglobin sample was reduced with sodium dithionite and the cytochrome *c* sample was reduced with ascorbic acid. The typical sample optical density is 0.3–0.5 in a 1 mm path length optical cell. However, the crossed beam geometry involves a shorter interaction path length within the cell.

The transient response obtained using broadened blue pulses of $\sim$120 fs duration, without spectral filtering of the probe signal, displays both fast (sub-ps) and slow ($\sim$5 ps) components of relaxation [7]. The signals, integrated over the bandwidth of the probe pulse, are dominated by the rapid Soret band bleach, followed by the creation of excited states that absorb in the vicinity of the Soret band [8]. The slower component of the signal is influenced by both electronic population decay and vibra-

0031-9007/94/72(2)/301(4)$06.00

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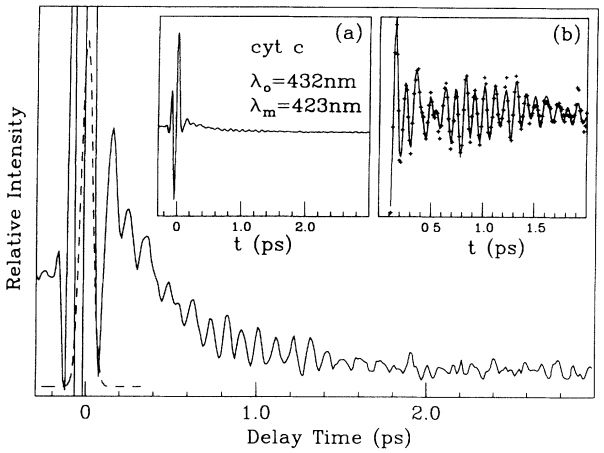

FIG. 1. The femtosecond coherence signal from ferrocytochrome *c*. Inset (b) shows the oscillatory component and the LPSVD fit.

tional cooling of the heme [9].

When the laser pulses are shorter than the vibrational frequencies of the material, the electric fields and frequencies contained in the pump and probe pulses lead to a third order polarization of the medium that is modulated by coherences involving the resonance Raman active ground vibrational states of the heme chromophore. The oscillation and decay of these coherences can be probed by suitably delaying the arrival of the probe fields. In resonance, there are several time-ordered histories that can contribute to the observed signal, and the reader is referred to the literature for a description of the various theoretical possibilites [10]. Here, we note that the rapid ($\lesssim$50 fs) electronic population dephasing of the resonant Soret state damps all oscillatory signals except those originating from the ground state vibrational coherences. Signals from excited electronic states appear only as population decay terms, without oscillation, because phase coherence is lost during the rapid nonradiative transitions from the Soret state [1,3] that lead to the creation of these states. Since the oscillatory part of the third order polarization signal probes the ground state vibronic manifold of the heme chromophore, the experiments described here are time domain analogs of resonance Raman scattering.

In Fig. 1 we present the results for cyt *c* excited and probed with 50 fs pulses centered at λ_0=432 nm. The monochromator used to analyze the transmitted radiation of the probe pulse is set to λ_m=423 nm. The full data set, including the coherent coupling signal that is present when the pump and probe light are temporally overlapping near Δt=0, is shown in panel (a). The autocorrelation trace, obtained when the sample is replaced by a thin BBO crystal, is shown by the dashed line in the lower portion of the figure. The oscillatory components of the signal are clearly apparent in the expanded plot and they correspond to coherent nuclear motion on the ground state potential surface. The oscillatory signal is analyzed by first fitting the decay due to electronic state population recovery and then using a linear predictive singular value decomposition (LPSVD) algorithm [11] to extract the vibrational frequencies and damping constants of the oscillatory residual. The rapid population decay is well fit with two, nearly equally weighted, exponentials having time constants of 300 fs and 1.7 ps. In addition, the electronic response has a long time component (~6 ps) with negative absorbance change [7], which shifts the baseline below the negative time data points in Fig. 1. The oscillatory part of the signal and its LPSVD fit are shown in panel (b). The fitting parameters and a comparison of the frequencies with those determined from the resonance Raman spectrum [12] are given in Table I.

In Fig. 2 we present data obtained using samples of deoxyMb. In the upper pair of panels we display the results obtained using 50 fs laser pulses and a monochromator tuned to the blue of the 435 nm maximum of the Soret band absorbance. The presence of a rich pattern of oscillations is clearly observed in the expanded data. In the lower pair of panels, we show the resulting signal when a broadened 75 fs pulse is used to excite and probe the sample. Because of the narrowed bandwidth, the

TABLE I. Low frequency nuclear vibration parameters of the heme. Raman frequency: $\tilde{\nu}_r$ (cm^{-1}); LPSVD frequency: $\tilde{\nu}_i$ (cm^{-1}); relative amplitude: A_i; lifetime: τ_i (ps); phase: ϕ_i (degree); fitting function: $\Sigma A_i e^{-t/\tau_i}\cos(2\pi\tilde{\nu}_i ct+\phi_i)$.

Deoxymyoglobin					Ferrocytochrome *c*				
$\tilde{\nu}_r$	$\tilde{\nu}_i$	A_i	τ_i	ϕ_i	$\tilde{\nu}_r$	$\tilde{\nu}_i$	A_i	τ_i	ϕ_i
···	52	1071	0.39	134	···	127	247	0.63	240
···	114	517	0.49	236	181	217	704	0.38	229
150	133[a]		0.34[a]		220	233	320	0.87	290
222	227	1340	0.68	180	264	284	399	0.79	226
242	252	664	0.81	245	304	313	800	0.51	179
291	293	2388	0.32	103	345-356	334	784	0.97	197
341	333	1630	0.54	81	371-379	368	357	0.61	262
372	367	1050	1.06	36	391-400	400	190	1.26	21
406	403	797	0.40	17					

[a]Obtained only with 75 fs pulses.

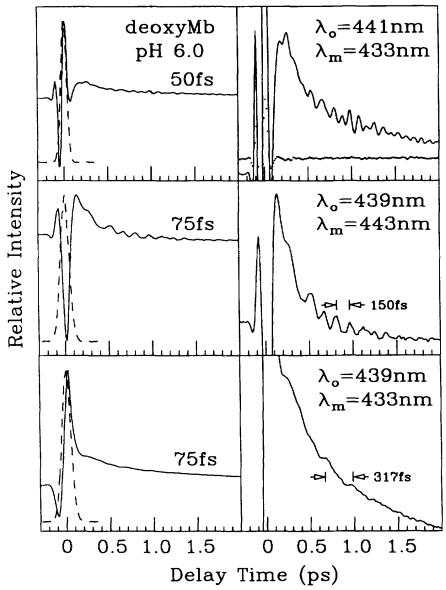

FIG. 2. The femtosecond coherence signals from deoxyMb as a function of pulse width and monochromator setting. The lower dotted line in the upper right-hand panel is the result of a control experiment using pure buffer as the sample.

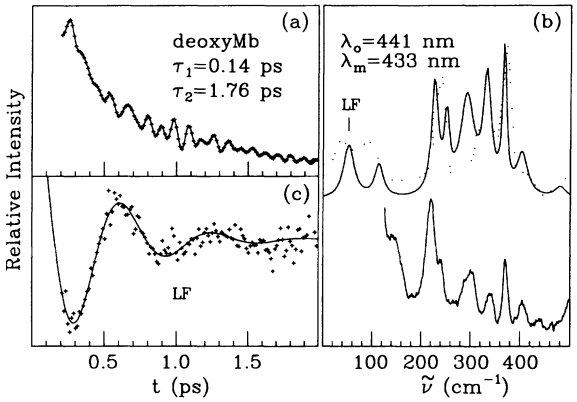

FIG. 3. (a) The 50 fs deoxyMb signal from Fig. 2 along with the LPSVD fit (solid line). The electronic decay constants, τ_1 and τ_2, obtained from an independent least squares fit, are in agreement with those found from the LPSVD analysis. (b) The discrete Fourier transform amplitudes (dotted line) and the power spectrum derived from the LPSVD analysis (upper solid line) are compared with the resonance Raman spectrum (lower solid line). (c) The low frequency residual and the LPSVD fit that describes its oscillation and decay.

high frequency oscillations are attenuated and the 317 fs (105 cm^{-1}) low frequency component can be observed directly. The middle pair of panels display the results when a different monochromator setting is used. Once again the low frequency oscillations are enhanced, but now the dominant component is found near 150 fs (220 cm^{-1}) and monitors the real time oscillation of the iron-histidine bond [2], which is the sole covalent link between the heme and the protein.

In analogy with resonance Raman excitation profiles, changes in the oscillatory pattern are expected as the monochromator is tuned through the vibronic resonances associated with different modes coupled to the resonant electronic state. However, the dramatic probe wavelength dependence, observed in the lower four panels of Fig. 2, involves modes (100 and 220 cm^{-1}) with vibronic resonances that differ by only $\sim$100 cm^{-1} and cannot be explained by coupling to a single electronic resonance. The results indicate that a separate iron-localized "charge transfer" excitation, underlying the Soret band, is coupling to the low frequency mode near 100 cm^{-1}. This suggestion is not unprecedented, since resonance Raman excitation profile measurements have revealed an unusual blueshift for a weak low frequency (150 cm^{-1}) Raman mode that appears in the spectrum of deoxyMb [13]. Evidently, tuning the monochromator to the blue has the effect of enhancing the contribution of the low frequency oscillations coupled to an underlying blueshifted charge transfer band.

It is important to realize that, if an iron-localized charge transfer band is responsible for the enhancement of the low frequency modes, it must have a significantly reduced (factor of $\sim$10) oscillator strength compared to the Soret band. In order to observe such modes, the electron-nuclear coupling to the charge transfer band must be substantially larger (factor of $\sim$100) than for the modes coupled to the delocalized π-π^* transition of the porphyrin. These issues have been discussed previously [13], where it was explicitly shown how such strong coupling leads to first and higher overtone scattering that is significantly enhanced relative to the fundamental. Since a persistent feature at 50 cm^{-1} also appears in both the LPSVD and Fourier transform analyses of all data sets shown in Fig. 2 (see below), we suggest that the low frequency modes at 100 and 150 cm^{-1} are potentially arising from overtone scattering associated with a 50 cm^{-1} fundamental that is strongly coupled to a blueshifted charge transfer band.

It should be noted that a recent normal mode study of the five coordinate heme [14] has placed the heme doming mode, γ_9, near 50 cm^{-1}. Independent studies have analyzed the low temperature kinetics of ligand binding to Mb, its kinetic hole burning, and the shifts in the optical transitions of the low temperature photoproduct, using a simple model that predicts [15] a heme doming frequency of 50 cm^{-1}. Recent investigations [16] of the low temperature ligand binding reaction in the presence of tunable far infrared laser radiation have also noted a kinetic enhancement near 50 cm^{-1} that could result from the selective excitation of such a mode.

In Fig. 3, we present a more revealing analysis of the data obtained using the 50 fs pulses. Panel (a) displays the expanded data, along with the fit obtained with the LPSVD analysis (Table I). The data were also analyzed by first fitting with a double exponential decay and ex-

tracting the oscillatory residuals, which were then subjected to a discrete Fourier transform analysis. In the upper portion of panel (b) we display as a solid line the sum of Lorentzian line shapes that arises from the frequencies and damping constants derived from the LPSVD analysis of the data. The dotted line is a plot of the amplitudes extracted from the independent Fourier analysis. The agreement is striking, and suggests that a low frequency mode near 50 cm^{-1} is a genuine feature of the data. However, the agreement is not perfect because of the possibility of compensation between the exponential decay terms and the low frequency oscillations (although the absolute frequency determination must be viewed with caution, the *existence* of modes below 150 cm^{-1} is unequivocally demonstrated by these data). The lower portion of this panel shows the resonance Raman spectrum of deoxyMb, which compares favorably with the "spectrum" extracted from the time domain analysis. Notice that the low frequency modes in the Raman spectrum are obscured by the Rayleigh wing. In order to display the 50 cm^{-1} mode (LF) more graphically, we plot in panel (c) the residuals of the data after subtraction of the LPSVD fitting function excluding the LF mode. The solid line shows the oscillatory behavior of the LF mode generated using its LPSVD parameters (Table I).

Finally, we note that the results obtained when the autocorrelation function is deconvolved from the data lead to the expected enhancement of the high frequency components. On the other hand, convolution of a broad smoothing function into the data attenuates the high frequency components and allows better visualization of the low frequency modes, which again appear at 50 and 100 cm^{-1}, but with higher intensity.

In summary, we have demonstrated that femtosecond coherence spectroscopy can be used to study low frequency modes of biological molecules in solution and at room temperature. The heme proteins cyt *c* and Mb display oscillatory signals that agree well with the vibrational modes observed using resonance Raman spectroscopy. In addition, we observe low frequency modes in the time domain signals that are not accessible using traditional frequency domain techniques, demonstrating that such modes are not necessarily overdamped in solution as is sometimes assumed [17]. In the case of deoxyMb, we find a robust oscillation near 300 fs ($\sim$100 cm^{-1}), particularly at blue settings of the monochromator. Such behavior is suggestive of strong coupling to a separate electronic transition underlying the Soret band. It is conceivable that this mode corresponds to the first overtone of another low frequency feature that persistently appears in the analysis quite close to the predicted (50 cm^{-1}) frequency of heme doming motion.

This work is supported by NSF 90-16860 and NIH AM-35090. We thank Glenn Millhauser for supplying us with the LPSVD algorithm and Y. Gu for the cytochrome *c* Raman spectrum. L. D Ziegler is thanked for numerous helpful conversations.

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