Evidence of protein collective motions on the picosecond time scale
Supplementary Material

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Sample Preparation and Characterization

Preparation of oxidized and reduced cytochrome c films measured in THz time domain spectroscopy is described in a previous study[1]. Solutions were pippetted onto clean infrasil quartz substrates with half the substrate left bare for referencing. The films were characterized by UV/Vis absorption to verify the oxidation state. The thickness of the dried films is measured to both verify uniformity and to determine absolute absorption coefficients and refractive indices. An optical microscope with a digital micrometer is used to measure the thicknesses of all samples. The film thickness is determined by the change in the microscope objective distance for focusing on the surface of the film and the surface of the substrate adjacent to the film. Typically seven measurements were made in this manner using different spots on the surface of the film and averaged the results for each film. Film thicknesses were typically ~ 100 μm with thickness variation < 5%.

UV/Vis spectroscopy was used to characterize the oxidation states of the CytC films. As seen in Figure 1 and according to the UV/Vis spectra of cytochrome c reported by Margoliash and Frohwirt [2], the response of films resembles that of solutions. A complete measurement for a given oxidation state sample consists of a series of THz TDS measurements at different hydrations. For each sample, a pair of films are made simultaneously from the prepared solution: a thick film for the THz TDS measurements and a companion thin film for the UV/Vis characterization. The thin film sample is used for the UV/Vis measurements to verify the native state absorbance and oxidation state. In figure 1(A), we show the absorbance of oxidized CytC and reduced CytC thin films made from the same solutions used to make the films for the THz measurements. The THz TDS measurements require thicker films to have sufficient absorbance at THz frequencies. Direct UV/Vis measurements on the THz TDS films suffer from insufficient dynamic range of the UV/Vis transmission measurement for such high optical densities, and contributions from scattering from thick samples. In figure 1(B), we show UV/Vis data for the thicker films. The thick reduced CytC film has the clear characteristic double peak at 520nm and 550 nm; however for the thick oxidized CytC film the expected absorption peak at 540 nm is broad with a flattened lineshape. We performed similar measurements on high OD solutions and found a similar result, demonstrating this lineshape distortion arises from the dynamic range limitations and/or scattering. The companion thin CytC film undergoes the same hydration cycling as the THz TDS film and is used to verify the oxidation state throughout the measurements. The UV/Vis setup consists of a light source, a Fiber-Lite PL-900 illuminator and a detector, the PC2000 PC plug-in spectrometer from Ocean Optics, Inc. with an UV/Vis range optical fiber. Complete hydration dependence measurements were achieved for at least two pairs of samples for each oxidation state with results consistent with those presented.

THz TDS

The system was purged with dry N_2 at least one hour prior to taking data to remove atmospheric water absorption. THz TDS, which measures the transmitted electric *field*, allows us to access both the real and imaginary part of the dielectric response in a single measurement; therefore, we can observe how both the refractive index and the absorbance vary as a function of frequency.

The real part of the refractive index and the absorption coefficient are extracted from the terahertz transmission data. The field transmission is determined by the measured transmitted field for the sample and reference using the following expression:

$$t = \frac{E_{sample}(v)e^{i\phi_{sample}(v)}}{E_{ref}(v)e^{i\phi_{ref}(v)}} = |t|e^{i\phi(v)} . \qquad (1)$$

The real part of the refractive index, n, is given by:

$$\phi = 2\pi(n-1)vd \qquad (2)$$

where $\phi(v)$ is the phase, v is the frequency in wavenumber, and d is the thickness of sample. The absorption coefficient is then given by:

$$\alpha(v) = -\frac{2\ln(|t|)}{d} . \qquad (3)$$

Isotherm Determination

To compare the picosecond dielectric response of ferro and ferri samples at equivalent water content we measured the isotherms for both oxidation states. The water content for a given relative humidity often follows the the B.E.T. thermal equation[3]:

$$h(x) = \frac{ah_m x}{(1+x)[1+(a-1)x]} \qquad (4)$$

where h(x) is the water content as % wt, that is, gm water of 100 gm protein; a is a parameter representing the potential water absorption capacity of the protein; h_m is a parameter representing the humidity for monolayer of water; and x is the relative pressure of the gas, namely, the relative humidity. Gascoyne and Pethig[3] determined the parameters for cytochrome c to be: a =9.8 and h_m=7.5, however Powell and Rosenberg[4] found a different set of parameters for CytC, a =14.1 and h_m=6.3, as shown in figure 2. The ability to apply previous determinations of the B.E.T isotherm fitting parameters is additionally complicated by the fact that neither group specified the cytochrome c oxidation state. Indeed as we will discuss, we find the hydration dynamics is slightly different for the two states. We measured the isotherms directly for both oxidized and reduced CytC films. A balance from Denver Instrument Company was used with precision up to 0.1 mg. The oxidized film is made by dissolving 100 mg of CytC powder into 500 μl Trizma buffer into a transparent petri dish and was dried by extra dry oxygen gas overnight. The reduced CytC film is reduced by adding an additional reducing agent, sodium dithionite and dried by extra dry nitrogen gas overnight. The oxidation state of the reduced CytC film is verified by UV/Vis spectrum at the beginning and at the end of the measurement. The hydration condition is controlled by putting samples and a specific saturated salt solution[5] in a closed environment for at least 8 hours to reach hydration equilibrium. The change in weight due to absorbed water at a specific humidity is measured by the balance right after the sample was moved from the closed hydration condition (within 10 seconds). The weight of 0% r.h. is

determined by weighing the sample dried by extra dry nitrogen/oxygen gas overnight. We fit the isotherm data to the B.E.T. isotherm equation and used the fitting to then calibrate the water content for the films measured using THz TDS.

Table 1. The parameters of the B.E.T. isotherm equation for oxidized and reduced CytC films

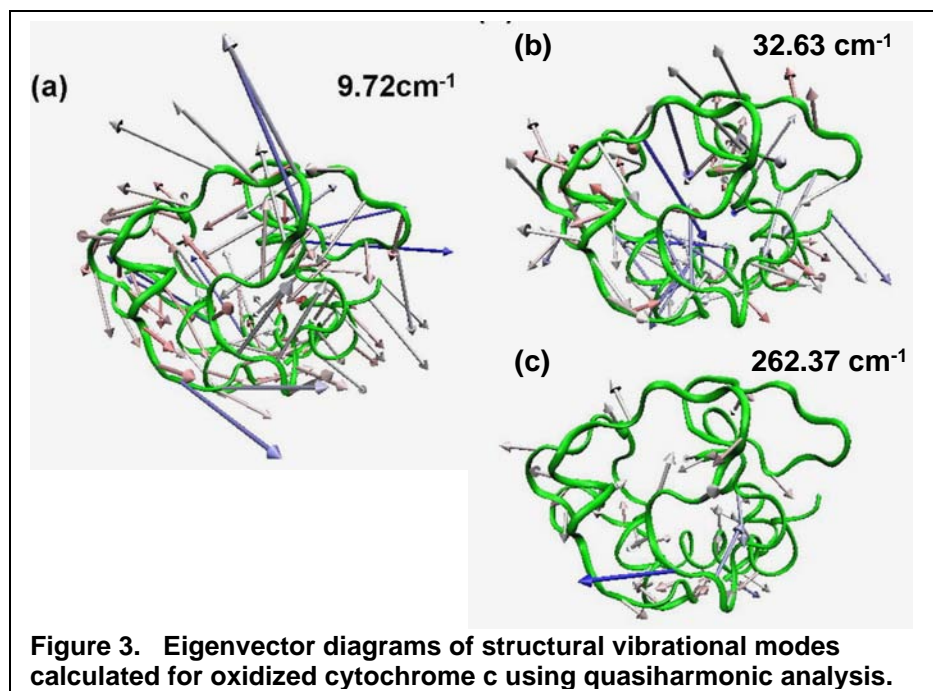| Status of Cytochrome c	A	h_m
Oxidized	19.95	5.64
Reduced	3.44	6.80
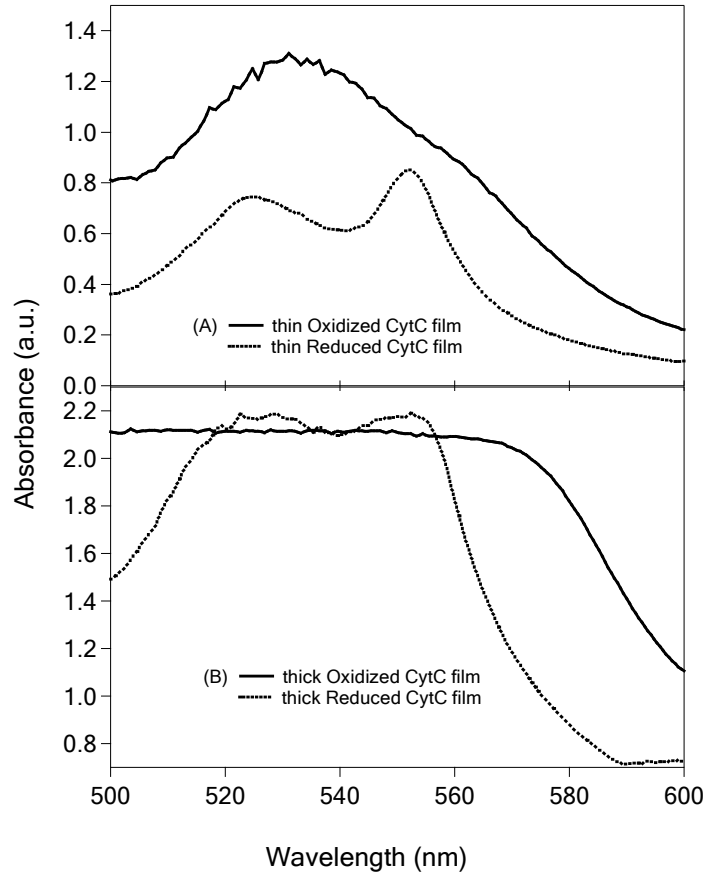
Figure 1. UV/Vis spectra of CytC films. Solid lines are the result of oxidized CytC films, and dotted lines are those of reduced CytC films. The upper panel (A) is the data of thin films used to characterize the oxidation states of CytC , and the bottom panel (B) is the data of thick films used in THz time domain spectroscopy.
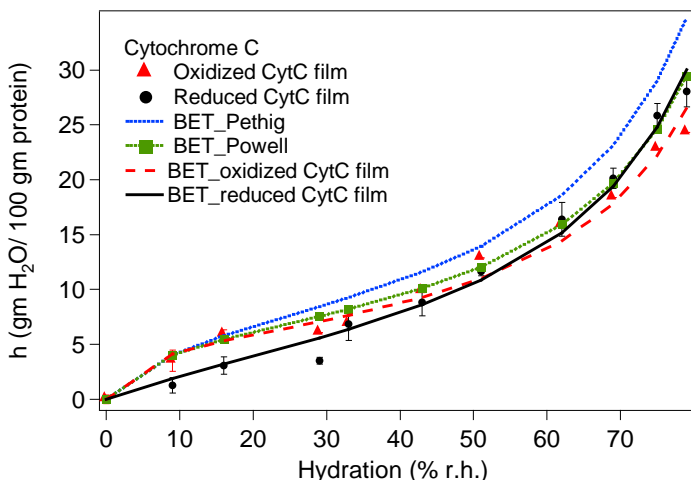
Figure 2. Water sorption isotherm of Cytochrome c. Triangles and dots are the data for oxidized and reduced CytC films respectively, and the error bars are the standard deviations among different trials. The dashed line and the solid line are fits to the B.E.T. isotherm equation. The dotted line is the B.E.T. equation using Pethig's fit parameters and the dotted line with squares uses the fit parameters from Powell.

Structural Extent of Calculated Quasiharmonic Modes

As discussed in the paper, normal modes and quasiharmonic modes refer to collective motions using a harmonic approximation. While quasiharmonic modes is based on the calculated trajectory, the motions extracted are only those harmonic motions and not diffusive or local rotations. To demonstrate these we show here several representative eigenvector diagrams in Figure 3. The lines originate from the alpha carbons on the polypeptide chain and their lengths are proportional to the atomic displacement for the net eigenvector of the mode. We note that the number of modes calculated per unit wave number is large, thus we denote each mode to the precision necessary to uniquely identify it, 0.01 cm^{-1}. precision. We do not suggest that any of these modes could actually be measured with such precision and others have shown that the modes are either critically damped or overdamped. The eigenfrequency of the modes is labeled in Figure 3 and all magnitudes of the displacement vectors are scaled in the same way (1000 times the calculated displacement). We see that the most extensive and largest amplitude modes are at the lowest frequencies, however even at 262.37 cm^{-1} mode, displacements are correlated over the full extend of the structure. This result demonstrates the fact that the quasiharmonic modes fail to capture the local rotational motions of the side chains, relaxational motions, that contribute to the dielectric response and only through direct calculation of the dipole-dipole correlation function can we capture all the dynamics contribution to the dielectric response.

Figure 3. **Eigenvector diagrams of structural vibrational modes calculated for oxidized cytochrome c using quasiharmonic analysis.**

Dependence of Oxidation Sensitivity to Initial velocities and trajectory length

In the main paper a number of 1 ns trajectories were calculated for structures with varying oxidation and hydration. The oxidation dependence observed in the THz measurements was reproduced by the power spectrum of the dipole-dipole correlation for these trajectories. To determine how robust the result is, the average power spectra of dipole-dipole correlation was calculated for multiple 2 ns trajectories and 8 ns trajectories, all at 4% hydration. The oxidation dependence is reproduced in the averages as shown in Fig. 4.
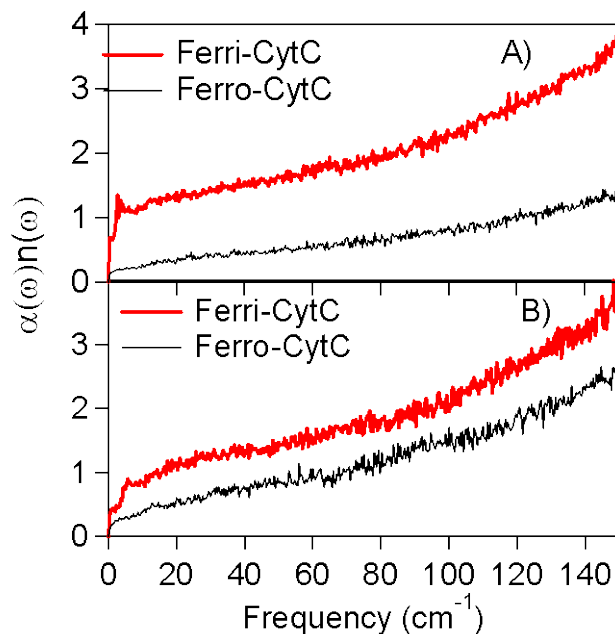

Figure 4. The average frequency dependent $\alpha(\omega)n(\omega)$ at 4% hydration calculated from the average power spectra of the dipole-dipole correlation from A) eight 2 ns trajectories and B) two 8 ns trajectories.

Supplemental Material References

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