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Large oxidation dependence observed in terahertz dielectric response for cytochrome c

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Far infrared dielectric response is used to characterize the collective mode density of states for cytochrome c as a function of oxidation state and hydration using terahertz time domain spectroscopy. A strong absorbance and refractive index increase was observed with the oxidation. A simple phenomenological fitting using a continuous distribution of oscillators reproduces the frequency dependence of the complex dielectric response as well as demonstrates quantitative agreement with a uniform increase in either mode density or polarizability with oxidation in the 5–80 cm^{-1} frequency range. Hydration dependence measurements find that a difference in the equilibrium water content for ferri and ferro cytochrome c is not sufficient to account for the large change in terahertz response. The large dielectric increase at terahertz frequencies with oxidation suggests either a significant global softening of the potential and/or a significant increase in polarizability with oxidation.

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Protein function relies on structural flexibility, the ability of the protein to access changes in conformation. The critical importance of flexibility has begun to be exploited in drug design [1], and will likely influence strategies for protein engineering towards therapeutic and technological applications. Standard methods to quantify flexibility are mainly limited to measures of variation in structure as measured by atomic mean square displacement $\langle u^2 \rangle$ through X-ray crystallographic measurements and structural fluctuations determined from nuclear magnetic resonance (NMR) measurements. While the relationship between the measured $\langle u^2 \rangle$ and protein dynamics has been established, multiple structural measurements must be performed in order to extract the collective vibrational mode contribution to $\langle u^2 \rangle$ [2]. Further these measurements cannot readily determine the frequency dependence of the motions. Inelastic neutron scattering and far infrared or terahertz spectroscopy can give direct access to the collective vibrational mode spectra. An interesting example of the role of protein flexibility in protein function is the electron transfer protein cytochrome c (CytC). CytC consists of 104 amino acids with a covalently bound heme group with two oxidation states, the oxidized (ferri) and the reduced (ferro). Various measurements have found that ferri-CytC is less thermally stable, has higher hydrogen exchange and higher proteolytic digestion rate than ferro-CytC. These large changes are not accompanied by a significant structural change [3–5]. The changes in reactivity with oxidation have been attributed to an apparent increase in flexibility as measured by X-ray *B*-factor measurements [4,5], NMR structural fluctuation measurements [6], and compressibility measurements [7], however, a spectral change in the collective vibrational mode density has never been demonstrated.

Collective vibrational modes lie in the far infrared (FIR) or terahertz (THz) frequency range (1–200 cm^{-1}, 30 GHz–6 THz, 0.1–25 meV) [8]. One can quantify flexibility through the frequency dependence of the density of vibrational modes, $g(\nu)$, in the range of biological temperatures, that is $\leqslant$25 meV, $\leqslant$200 cm^{-1}. Methods to measure this distribution include inelastic neutron scattering (INS), Brillioun scattering and FIR spectroscopy. With the advent of terahertz time domain spectroscopy (TTDS), FIR dielectric measurements are more accessible than INS and sample control more straightforward. TTDS measures the complex dielectric response reflecting the distribution of the modes, as well as the dipole coupling strength in this critical frequency region. We examine the complex terahertz dielectric response of CytC films in the ferri and ferro states using TTDS and find a substantial increase in the response with oxidation, indicating a strong increase in $g(\nu)$ and/or dipole coupling in the 5–80 cm^{-1} range. Hydration dependence of the dielectric response suggests that the large increase in low frequency modes may come in part from a difference in equilibrium water content for the two states, in addition to an overall redistribution of modes with oxidation. These measurements reveal a significant change in protein collective mode dielectric response can occur without large-scale structural change.

Thick films (nominally 45 μm) of oxidized ($A_{530} \sim 2.5$) and reduced CytC ($A_{520} \sim 3.5$) were formed on infrasil quartz substrates from starting solutions. Lyophilized powders of CytC (bovine heart, Sigma no. C-2037) were purchased from Sigma and used without further purification. Starting CytC solutions were made by dissolving 40 mg of CytC powder into 200 μl of Tris buffer (pH 7.0). A sodium dithionite solution (20 mg/ml) was prepared to reduce the sample. The oxidized CytC solution was made using 10 μl of Tris buffer and 20 μl of the starting CytC solution. The reduced CytC solution was made by adding 10 μl of the sodium dithionite solution into 20 μl of the starting CytC solution. The CytC films for THz characterization are formed by pipetting 10 μl of the protein solution on infrasil quartz substrates. In order to achieve sufficient optical density for THz characterization, the pipetting and drying procedure was repeated giving a net 20 μl. One-half of the substrate was left clean to be used as a reference. Substrates are mounted in a humidity-controlled cell behind 5 mm diameter metal apertures. The data shown here is for two sets of samples: Reduced-CytC 1, oxidized-CytC 1 and reduced-CytC 3, oxidized-CytC 3 where films 1 and 3 were made from dif-

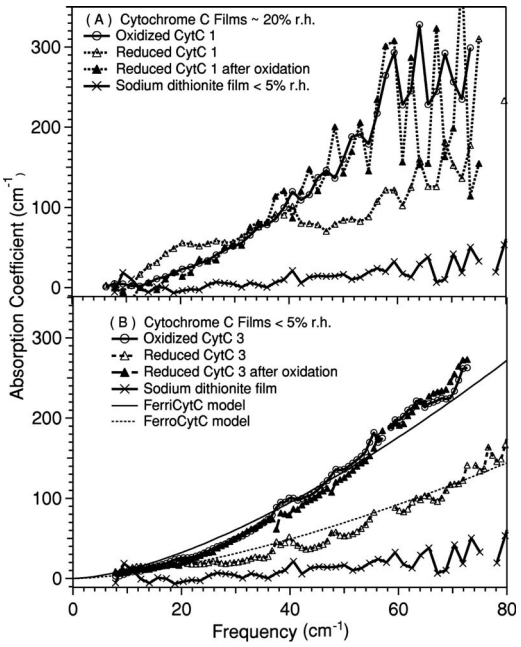

FIG. 1. Absorption coefficients of CytC films and sodium dithionite film (All measurements are done in room temperature, the humidity of CytC 1 films is ~20% r.h. (A), that of CytC 3 films and sodium dithionite is <5% r.h. (B). The broad absorbance is indicative of the high density of modes in this frequency range for proteins, as demonstrated by calculated normal modes for oxy-myoglobin shown in the inset. The absorbance modeled using a continuous distribution for the density of modes is shown in (B). See text for further discussion.

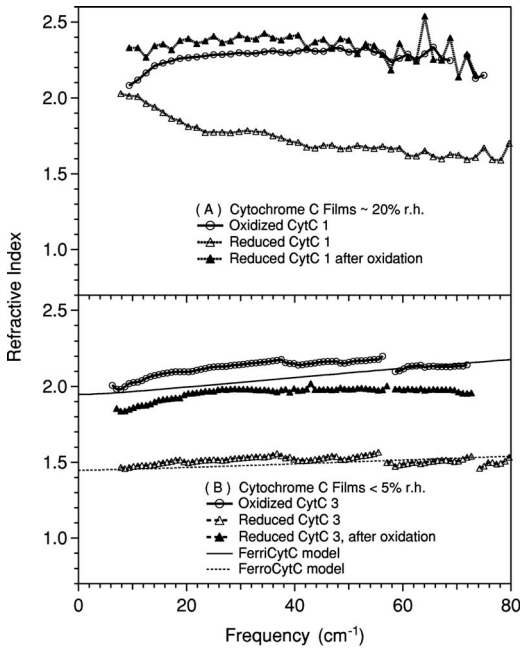

FIG. 2. Index of oxidized and reduced CytC films (All measurements are done in room temperature, the humidity of CytC 1 films is ~20% r.h. (A) and that of CytC 3 films is <5% r.h. (B). The absorbance modeled using a continuous distribution for the density of modes is shown in (B). See text for further discussion.

ferent starting CytC solutions. In addition to comparing films with different starting oxidation states, we also performed measurements in which the oxidation state is changed over time. After completing measurements on reduced films, we converted these same films to "oxy" by placing them in a cell with flowing oxygen and a highly hydrated environment maintained by wet sponges in the cell. This high humidity environment was essential to achieve conversion. The oxidized and reduced states of the films were verified by UV/Vis absorption measurements where the characteristic double peak at 520 and 550 indicates reduced CytC and a broad 530 nm absorbance indicates oxidized CytC [9]. The 695 nm peak is observed for all films, indicating the Fe-Met80 bond remains intact [10,11].

TTDS was used to monitor the change in absorbance and index with oxidation states of CytC. TTDS has been applied to a number of protein systems including short chain oligomers [12], short chain polypeptides, proteins [13–15] and protein complexes [15]. Our TTDS system consists of a dipole generator with electro-optic detection pumped with a 80 MHz Ti-Sapphire oscillator. The maximum THz power is <1 μW with no significant heating of the samples which are held at room temperature.

In Fig. 1 the absorption coefficient $\alpha(\nu)$ is shown for the oxidized and reduced CytC films as a function of frequency ν for CytC1 samples (relative humidity, r.h.=20%) and CytC3 samples (r.h.$<$5%). In all cases $\alpha(\nu)$ increases with frequency for the entire frequency range. This glass-like response has been observed for a number of proteins [13,16–20] and may arise from over-damping of modes or dielectric relaxation type behavior due to conformational polarizability [21–23]. An example normal mode calculation for myoglobin using CHARMm is shown in the inset, demonstrating the high density of modes for a similar size heme protein in this frequency range. As seen in Fig. 1, $\alpha(\nu)$ of the oxidized CytC is significantly larger than for the reduced CytC. The difference increases with frequency. Further as the reduced CytC films are oxidized, $\alpha(\nu)$ increases until it almost overlaps with that of the initially oxidized CytC films removing concerns about improper normalization of the films and contribution from the sodium dithionite used to reduce CytC. The negligible absorbance of a sodium dithionite film equivalent to the amount in the CytC films is also shown in the figure. The absorbance of the sodium dithionite film showed a slight *decrease* with time, confirming that it does not contribute to the *increase* in absorbance observed with oxidation of the reduced films with time.

The real part of the refractive index for the oxidized and reduced CytC films have a slow frequency dependence as shown in Fig. 2. The index of the reduced CytC films is smaller than that of the oxidized CytC films in both hydration conditions. While other measurements of dielectric response for CytC as a function of oxidation state are not available, the zero frequency dielectric response has been calculated using molecular dynamics with $\varepsilon=3.4\pm1.0$ for ferriCytC (oxidized CytC) and $\varepsilon=4.7\pm1.0$ for ferroCytC (reduced CytC) [24]. The trend is opposite to what we observe

(as well as opposite to what the B-factors would suggest), however, the values are similar to what we measure.

We consider a phenomenological description of the data. The dielectric response for a collection of oscillators is given by [25]:

$$\varepsilon(\nu) = \varepsilon_0 + \sum_j \frac{f_j}{(\nu_j^2 - \nu^2) + i\gamma_j \nu} \quad (1)$$

where ε_0 is the DC dielectric response and the sum is over all vibration modes j, with oscillator strength f_j and damping γ_j. The data does not suggest a discrete sum of oscillators. In general the calculated normal mode density of many proteins monotonically increases with frequency in the THz range [13]. An example is shown in the inset in Fig. 1(a) showing the normal mode distribution for oxy-myoglobin calculated with CHARMm. (Full molecular mechanics or molecular dynamics simulations of the dielectric response has not yet been done for ferri and ferro CytC. Currently, common databases such as CHARMm do not include the c heme group making these calculations relatively inaccessible to the nonspecialist.) Dielectric measurements strongly resemble these calculated mode densities, suggesting that all modes are dipole active and the oscillator strength is not a strong function of frequency [16,17,26]. We fit the data assuming a mode distribution with a power law frequency dependence and a frequency independent dipole coupling, setting $f_j = c\nu_j^m$ in Eq. (1). The parameter m determines the frequency dependence of the mode density, and c parameterizes both the dipole coupling and the mode density. Using a single damping term, γ, for all frequencies ν_j gives:

$$\varepsilon(\nu) = \varepsilon_0 + \sum_j \frac{c\nu_j^m}{(\nu_j^2 - \nu^2) + i\gamma\nu.} \quad (2)$$

Keeping the frequency dependence of $g(\nu)$ constant with $m = 1.6$, the fit has reasonable agreement with both the measured $\alpha(\nu)$ and $n(\nu)$ for $c = 12.5(50)$ and $\gamma = 2\ s^{-1}$ ($3\ s^{-1}$) for ferroCytC (ferriCytC), as shown in Figs. 1 and 2. This large increase in the parameter c with oxidation could indicate an increase in dipole coupling, normal mode density, or both. The oxidation dependence of CytC dipole moment has not yet been measured, however theoretical calculations [27] predict the change to be $\sim 6\%$.

If an increase in $g(\nu)$ is responsible for the observed results, such an increase in this frequency range may be due to an overall increase in the number of modes or a redistribution of the ferro state mode density due to a softening of internal couplings. An overall increase in $g(\nu)$ can only occur if the system size increases. While measurements of the ferro and ferri films were made at the same controlled r.h., the equilibrium water content for a given r.h. may not be the same. Only bound water needs to be considered as the first hydration shell of CytC is not fully occupied for r.h. $< 90\%$ and thus there is no bulk water contribution to the response measured [22]. If increased bound water content is the sole reason for the difference, a sufficiently hydrated ferro sample should reproduce the ferri response.

In Fig. 3 we show the data for CytC1 with increasing r.h. for ferroCytC. As seen in Fig. 3(a) ferro with r.h. 56%, the absorbance agrees at low frequencies with ferri at r.h. 21%. The difference corresponds to approximately 62 additional water molecules for ferri over ferro [22,23]. Even if such a large difference in equilibrium water content is correct, the increase in hydration cannot reproduce the frequency dependence of the ferri sample. The inability of equilibrium hydration differences to fully account for the difference in FIR dielectric response is particularly clear in the index measurements shown in Fig. 3(b). At 21% r.h. the index is 2.3 (1.7) for ferriCytC (ferroCytC). While the refractive index of the reduced CytC film increases with increasing relative hydration, it is still significantly smaller than ferriCytC, even at 83% r.h. More recent measurements show that in fact the equilibrium water content for ferro is *higher* than that of ferri for a given r.h. [28]. Thus while hydration plays a vital role in dielectric response and the hydration will certainly affect the dielectric response of ferro and ferri cytc, a net water content difference does not account for the change in the dielectric response for the two oxidation states. A large redistribution or red shift of $g(\nu)$ or significant change in polarizability must occur with oxidation.

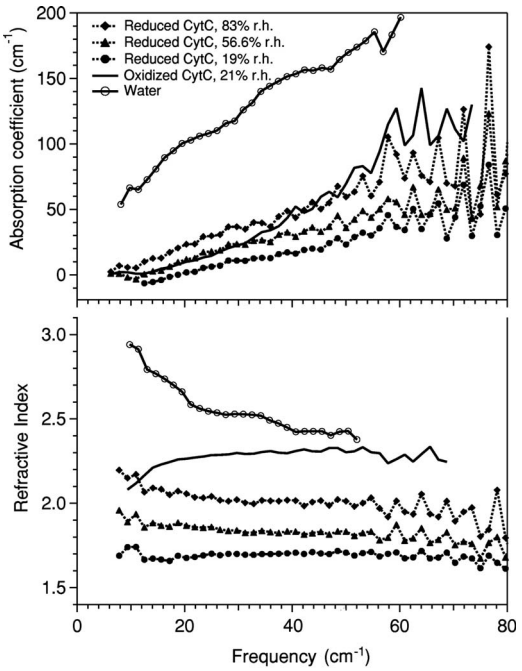

FIG. 3. Absorption coefficient and refractive index of bulk water, a ferriCytC1 film and a ferroCytC1 film as a function of hydration. The difference in the dielectric response with oxidation state cannot be entirely accounted for by different equilibrium water content.

It is interesting to compare these results to changes in the X-ray Debye-Waller factors. *B*-factor measurements of the total mean square atomic displacements do not reveal to what degree displacements are associated with a particular correlated motion. If one considers an average change in *B*-factors using Takano and Dickerson's [4,5] refined data for ferro (PDB 5CYT) and ferriCytC (PDB 3CYT) $\langle B \rangle$ increases from 18.7 in the ferro state, to 22.7 in the ferri state, suggesting a net increase in flexibility with oxidation consistent with our measurements [4,5]. Early INS measurements of CytC

could not conclusively show an increase in $g(\nu)$ with oxidation and it is hoped the results reported here will motivate new INS studies of this system [29]. The application of TTDS measurements of dielectric response is relatively straightforward and provides an immediate tool for determining the evolution of dielectric response with mutation, conformation, ligand binding or environmental changes in this important frequency range.

The measured dielectric response in the frequency region associated with conformational vibrational modes is strongly dependent on the oxidation state of CytC and the change is consistent with an increase in collective mode density with oxidation, however polarizability change with oxidation may also substantially contribute to the change in response. The data using terahertz dielectric spectroscopy strongly support the change in reactivity of CytC with oxidation arises in part from a change in the conformational fluctuations.

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