Communication

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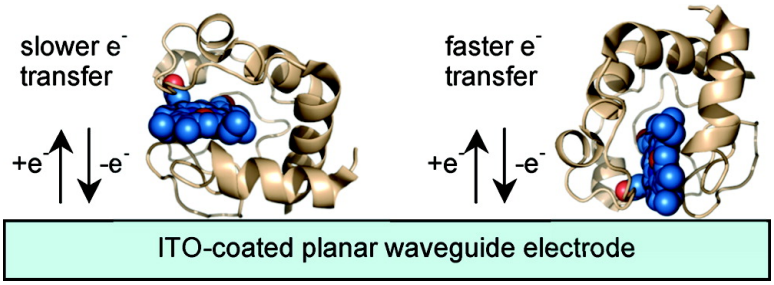

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Correlating Molecular Orientation Distributions and Electrochemical Kinetics in Subpopulations of an Immobilized Protein Film

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Immobilized metalloproteins have potential applications in several emerging areas such as bioelectronics and biomimetic energy conversion.[1−3] It is well recognized that immobilization of a metalloprotein may affect its electron transfer activity, because of an "incorrect" molecular orientation and/or altered conformation relative to the native state.[4−8] Horse heart cytochrome *c* (cyt *c*) has been frequently used as a model to study these effects.[5] Cyt *c* undergoes quasi-reversible electron transfer when immobilized on a variety of electrode surfaces, and current knowledge of the structural requirements for achieving protein-electrode communication are based primarily on studies of this protein.[6,7] Most of these studies have been performed on Au electrodes modified with self-assembled monolayers (SAMs),[8] although cyt *c* adsorbed directly to indium−tin oxide (ITO) is also electrochemically active.[9]

The influence of electrode−protein separation distance on the electron transfer rate constant (k^0) of immobilized cyt *c* has been thoroughly investigated.[6−8] Orientation is also predicted to play a significant role because the heme is not located at the center of the protein.[5c,10−12] Thus a distribution of molecular orientations will generate a distribution of heme-electrode separation distances and electron-tunneling pathways, producing a distribution of k^0 values. This concept has been invoked to explain the nonideal voltammetry of immobilized cyt *c* films,[10] but has not been experimentally verified. The difficulty lies in measuring the molecular orientation distribution of a cyt *c* film, measuring a distribution of k^0 values on that film, and establishing a correlation between the distributions. Dick et al.[5c] demonstrated that a more oriented cyt *c* film is more electrochemically reversible but their work did not address *distributions* of orientations and k^0 values.

Recently we used a combination of polarized internal reflection techniques to measure the tilt angle distribution of the heme molecular plane in a cyt *c* monolayer adsorbed to ITO (Figure 1).[11] The very broad orientation distribution should give rise to a broad distribution of heme-electrode separation distances and k^0 values. A complicating factor is that the total surface coverage, measured spectroscopically, is 22 pmol/cm^2, but the electroactive surface coverage, measured electrochemically, is only 9.5 pmol/cm^2.[13] Thus the orientation distribution and k^0 (measured electrochemically) cannot be correlated because the former is measured on the entire film whereas the latter is measured on only the electroactive portion (43%) of the film.

Herein we describe an electroreflectance (ER) technique that allows measurements of k^0 values for differently oriented subpopulations of the electroactive molecules in an immobilized cyt *c* film, without interference from the nonelectroactive subpopulation. To our knowledge, this is the first demonstration that distinct k^0 values correlated to differently oriented molecules can be recovered from *any* redox-active molecular film.

ER spectroscopy is an optical analog of electrochemical impedance spectroscopy in which changes in the reflectance of a redox-active molecular film deposited on an electrode are measured as a function of the frequency at which the potential at the electrode is modulated.[14,15] Most UV−vis ER studies reported to date have been performed using an external reflectance geometry at a Au electrode or in a transmission geometry using an optically transparent electrode. Attenuated total reflectance (ATR) is an alternative that provides a larger optical path length because the probe beam is internally reflected multiple times down the length of a waveguide electrode.[16] In addition, measurements can be made in both transverse electric (TE) and transverse magnetic (TM) polarizations, which can provide information about molecular orientation. This approach, termed potential modulated ATR (PM-ATR), has been used to examine electro-optical and charge transfer processes in conducting polymer and Prussian blue films deposited on ITO-coated planar waveguides.[17]

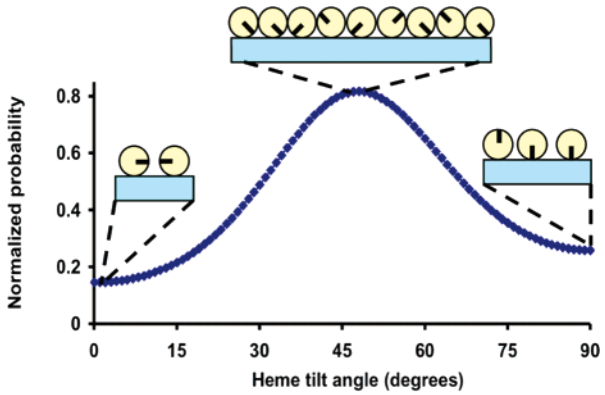

Figure 1. Distribution of heme plane tilt angles, relative to the electrode surface plane, in a cyt *c* monolayer adsorbed to ITO. Data are replotted from the polar coordinate data presented in Figure 3b2 in ref 11 and normalized to a total probability of unity. The insets show representations of molecules with heme tilt angles near 0°, 50°, and 90°.

Here PM-ATR was used to measure k^0 values for cyt *c* adsorbed to ITO when probed with either TE or TM polarized light. ITO-coated planar waveguides were fabricated by sputtering a ca. 100 nm thick layer of ITO on 150 um thick glass coverslips. Cyt *c* (Sigma) was prepared and adsorbed on ITO from pH 7 phosphate buffer, as described previously,[11] to produce films of near monolayer surface coverage. The electroactive surface coverage, measured by cyclic voltammetry, was 7.9 pmol/cm^2.[13] The double layer capacitance (C_{dl}) and uncompensated solution resistance (R_s) were determined on independently prepared films using electrochemical impedance spectroscopy and found to be 9.1 μF/cm^2 and 1.1 kΩ.cm^2, respectively. The time constant of the electrochemical cell with a bare ITO electrode was 4.9 ms.[13,17]

Complete descriptions of PM-ATR theory and instrumentation are given elsewhere.[17] Here a polarizer and a 417 nm bandpass filter (3 nm fwhm) were placed between the light source and ATR cell to control polarization and spectral bandwidth, respectively. Sinusoidally modulating the electrode potential over a small range near the midpoint between the oxidation and reduction potentials

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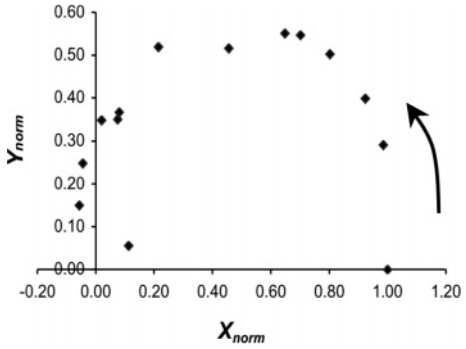

Figure 2. Complex plane plot composed of pairs of X_{norm},Y_{norm} values measured on a cyt *c* film adsorbed to ITO over a frequency range of 0.1−250 Hz using TM polarized light and $E_{dc} = 5$ mV. The arrow indicates the direction of increasing frequency.

of the cyt *c* film produces a modulated change in the electroreflectance (*R*, which is proportional to the intensity of light outcoupled from the waveguide) at the same frequency, due to the difference in molar absorptivities of ferro- and ferricyt *c*. Only electroactive molecules contribute to the modulated component of *R*. The midpoint potential (E_{dc}) that produced the largest change in *R* was determined from optically detected voltammograms measured on each film while applying a potential modulation of 8 mV at a frequency of 1 Hz (see Supporting Information (SI)). E_{dc} values were in the range of −12 to 12 mV versus a Ag/AgCl reference electrode. The modulation amplitude ($E_{ac} = \pm 22$ mV) was selected by monitoring *R* over a range of amplitudes (±4 to ±50 mV) centered at E_{dc} at a frequency of 1 Hz (see SI).

Figure 2 is a representative result from a PM-ATR experiment. The real (in-phase, *X*) and imaginary (out-of-phase, *Y*) portions of *R* were measured over a modulation frequency range of 0.1−250 Hz. To enable comparison of results from different films, normalized values of *X* and *Y* were calculated as described previously.[17] X_{norm},Y_{norm} curves were fit to a polynomial function to determine the frequency (ω) at which $X_{norm} = 0$. Electron transfer rate constants were then calculated from $k^0 = 0.5\omega^2 R_s C_{dl}$.

Data were acquired in both TM and TE polarizations on each cyt *c* film, yielding $k^0_{TM} = 4.0 \pm 0.8\ s^{-1}$ ($n = 3$) and $k^0_{TE} = 1.2 \pm 0.3\ s^{-1}$ ($n = 3$), respectively. On the same cyt *c* films, conventional cyclic voltammetry was performed, from which k^0_{CV} was calculated from the anodic and cathodic peak separation.[9] The result, $3.1 \pm 0.3\ s^{-1}$,[13] represents the mean rate constant for the entire electroactive portion of the cyt *c* film. In contrast, the k^0_{TM} and k^0_{TE} values correspond to subpopulations of the electroactive portion. These subpopulations are partially *overlapping*, which is due to several factors: (1) TM light is composed of both *z*- and *x*-polarized components, whereas TE light is *y*-polarized.[18] (2) The heme absorption is circularly (or elliptically) polarized in the molecular plane.[5,11] (2) The tilt-angle distribution should be uniaxial about the *z*-axis.[5,11]

Molecules at all tilt angles (from 0° to 90°) therefore contribute to *both* k^0_{TM} and k^0_{TE}, but their contributions are weighted by the extent to which their absorption dipoles project onto the electric fields of TE and TM polarized light. Molecules with large tilt angles contribute greater to TM absorption, and if most of these are adsorbed in a "face-down" orientation, that is, with the face of the protein that surrounds the heme crevice in contact with the ITO surface (see Figure 1), then their heme-electrode separation distances should be relatively small.[5a,9,11] TE light is more strongly absorbed by molecules with small tilt angles, for which heme-electrode separation distances should be larger, and this difference is reflected as $k^0_{TM} > k^0_{TE}$. Furthermore, because k^0_{CV} represents all electroactive molecules in the film, k^0_{CV} should be intermediate between k^0_{TM} and k^0_{TE}, as observed. This analysis assumes that adsorption of cyt *c* to ITO does not induce structural changes that alter the relationship between tilt angle, heme-electrode separation distance, and electrochemical activity.[5,11]

In summary, we have used polarized PM-ATR to obtain two k^0 values assigned to differently oriented subpopulations of cyt *c* molecules in an adsorbed film. The k^0_{TM}/k^0_{TE} ratio of 3.3 is consistent with a shorter tunneling distance for proteins adsorbed in a vertical orientation relative to a horizontal orientation. To our knowledge, these data are the first to correlate a distribution of molecular orientations with a distribution of electron transfer rate constants in a redox-active molecular film.

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Supporting Information Available: Examples of an optically detected voltammogram, a plot of E_{ac} versus *R*, and a TE polarized complex plane plot. This material is available free of charge via the Internet at http://pubs.acs.org.

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