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Polarized XAS on vectorially oriented single monolayers of cytochrome c

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Polarized x-ray absorption measurements have been performed in fluorescence mode under total reflection conditions on a frozen hydrated monolayer of yeast cytochrome c (YCC). The protein molecules were oriented by tethering their naturally occurring and unique surface cysteine residues to the isolated sulfhydryl endgroups of an organic self-assembled monolayer, itself covalently attached to an ultra-pure silicon wafer. The experimental spectra will be compared with theoretically generated output from FEFF7 using both crystallographic data and results from molecular dynamics simulations as input parameters. Accurate structural information on the prosthetic groups of such fully-functional protein monolayers is important in understanding the role of the protein-membrane interaction in the structure-function relationship of membrane proteins.

Keywords: polarized XAS, oriented, protein, monolayer

1. Introduction

Previously, we have shown the ability to vectorially orient a protein monolayer by tethering the protein molecules to the endgroups of an organic self-assembled monolayer (SAM) chemisorbed onto the surface of a solid substrate by employing designed specific interactions between particular residues on the protein's surface and the SAM's endgroups (Chupa et al., 1994). While other research groups have focused on measurements of the functional aspects of such (or closely related e.g. Langmuir-Blodgett) monolayer systems that were not structurally characterized (Song et al., 1993; Cullinson et al., 1994; Owaku et al., 1995; Jiang et al., 1996; Guo et al., 1996), we have focused instead on developing the physical techniques essential to determining the key structural features of the proteins within such vectorially oriented single monolayers. To date, this work has included resonance x-ray diffraction (Pachence et al., 1989), optical linear dichroism (Pachence et al., 1990), and x-ray interferometry/holography (Blasie et al., 1992; Chupa et al., 1994; Edwards et al., 1997 and Edwards et al., 1998). We have further demonstrated the potential of applying polarized XAFS to such systems in order to obtain information on both the orientation of the protein with respect to the substrate and the detailed structure around the metal site(s) of the protein (Zhang et al., 1997).

In this work, we have refined both our sample preparation techniques and our experimental configuration to overcome some of the problems encountered previously, yielding XAFS spectra for the cytochrome c protein that show significant differences depending on the direction of polarization. This data has been compared with theoretically generated output from FEFF7 to indicate the average orientation of the heme plane with respect to the monolayer (Zabinsky et al., 1995).

2. Sample Preparation and Experimental

We have performed XAFS total reflection experiments at the Fe K-edge on a vectorially oriented single monolayer sample of the protein yeast cytochrome c (from Saccharomyces cerevisiae). The silicon substrate used was an approximately 1x3 inch piece cleaved from a 100 mm diameter, 0.4 mm thick hyperpure polished Si(100) wafer. The substrate was soaked in 0.5 M EDTA for 100 minutes in order to reduce the iron content of the silicon. The substrate was then cleaned and coated with a 6:1 ratio of the organic compounds dodecyltrichlorosilane (methyl-surface)and 11-trichlorosilylundecyl thioacetate (protected sulfhydryl-surface) using the modified method of Sagiv (1980) as described by Xu (1993) with further modifications. This resulted in a SAM with a nonpolar surface on which the isolated sulfhydryl functional groups could be activated by immersing the coated substrate in a 50:50 mixture of methanol and concentrated hydrochloric acid for 1.5 hours to cleave the protecting group by acid hydrolysis. After rinsing well with ultrapure water, the activated surface was ready for immersion in a 10 μM solution of the protein, yeast cytochrome c in 1 mM TRIS, pH 8.0. This protein exhibits a naturally occurring and unique surface cysteine residue that would, therefore, be available for covalent disulfide bonding with the activated sulfhydryl endgroups of the SAM. The specimen was incubated in the protein solution for approximately 24 hours, then removed, rinsed several times in 1 mM TRIS buffer, then placed in buffer for 45 minutes. This had previously been determined by optical spectroscopy to be the optimal rinsing procedure to remove nonspecifically bound protein.

XAFS experiments were performed at beamline X9 of the NSLS in focusing mode. Focusing in the vertical direction was achieved by bending a Ni coated mirror down stream and focusing in the horizontal direction by sagital focusing of the second crystal of the monochromator. The sagital focusing was adjusted during the scan. Other experimental details have been submitted on the Experimental Details form as requested.

The total reflection experiments were carried out using a small chamber with polyvinylidene windows at the center of a Huber 4-circle diffractometer. The sample was mounted on a copper plate in the chamber by using a small amount of thermally conducting grease on the back of the sample. The temperature of the copper plate could be controlled by thermo-electric cooling elements and was held constant at 263 K throughout the experiment to keep the sample hydrated and help prevent radiation damage. Dry nitrogen was flowed through the chamber to prevent condensation. The measurements were performed below the critical angle, with the incident angle determined by the distance between the direct and reflected beams one to two meters away from the sample. Data were collected for approximately 48 hours with the x-ray polarization parallel and 39 hours with it perpendicular to the substrate surface.

3. Results and Discussion

The data were summed then background subtracted and normalized using a PC-based version of the AT&T Bell Labs EXAFS package. There appeared to be no change with time in the shape or quality of each set of polarized spectra, indicating that radiation damage had been minimized. More details of the data analysis have been submitted on the Experimental Details form as requested. FEFF7 was used to generate theoretical spectra for comparison with the experimental data. Input coordinates for FEFF7 were obtained either from the crystallographic data (Louie, 1990) available on the Brookhaven Protein Database (Abola et al., 1987; Abola et al., 1997) or from Molecular Dynamics (MD) simulations of a single hydrated YCC molecule covalently tethered by its surface cysteine to one sulfhydryl-terminated organic chain surrounded by 89 methyl-terminated organic chain molecules comprising a self-assembled monolayer-like structure. Appropriate periodic boundary conditions were used to model an extended monolayer system. The MD simulations were performed at 300 K using the CHARMM program version 23 (Brooks et al., 1983), with an all-atom model for the SAM and a polar hydrogen model for the protein. The simulations were performed for 900 ps, but only results from the final 300 ps of the trajectory were used in the calculations of the average orientation of the heme plane with respect to the plane of the SAM. For both the crystallographic and MD simulated data, only atoms within a 5 Å radius from the heme iron atom were used as input for FEFF7. For the crystallographic data, polarized spectra were calculated for various tilt angles between the heme plane and the substrate/SAM plane until the best match with the experimental data was obtained. This occurred at 48°. Since experimentally, the protein molecules would also be azimuthally averaged, this effect on the spectra was also investigated and found to be minor. From the MD simulations, the average heme angle after 900 ps was 54 ± 3° and averaging over several time slices of the trajectory produced an even better match to the experimental data in terms of peak position and shape than was achieved with the crystallographic input (as shown in Figure 1).

Fourier transforms of the experimental polarized spectra taken over the same k ranges reveal a larger first shell peak for the case with the x-ray polarization parallel to the substrate surface than for the perpendicular case, also indicative of the heme plane being at an angle greater than 45° with respect to the substrate/SAM plane due to the larger backscattering amplitude of the axial sulfur ligand versus the heme plane nitrogens. Optical linear dichroism (LD) measurements on this system using quartz substrates have consistently indicated heme tilt angles above 45° in the range 55 - 61°. The experimental spectra and the FEFF7 generated theoretical spectra using both the crystallographic coordinates and the MD time-averaged results are shown in Figure 1.

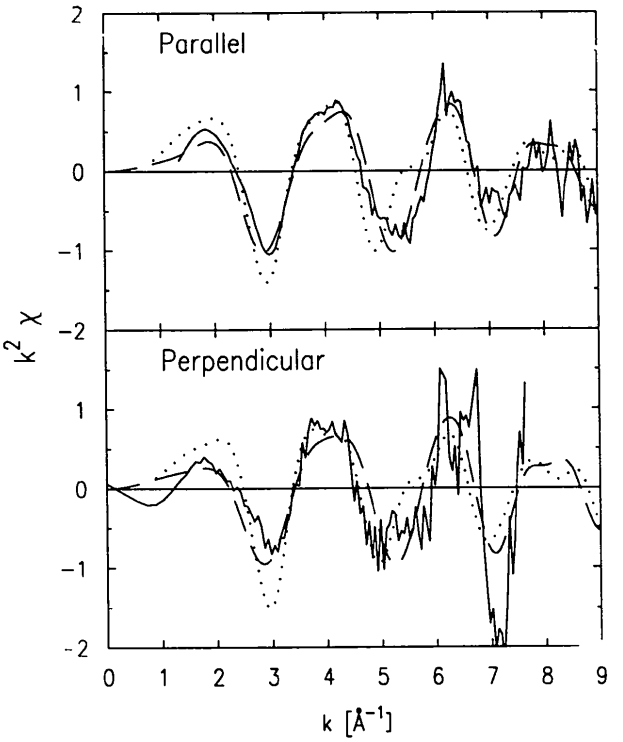

Figure 1. Experimental $k^2\chi$ data with x-ray polarization parallel and perpendicular to the monolayer (solid lines) vs. FEFF7 generated theoretical plots using the crystal structure of yeast cytochrome c (dashed lines) or MD simulations (dotted lines) as input parameters.

4. Summary

Polarized XAFS measurements have been performed on a frozen hydrated monolayer of YCC tethered to a non-polar SAM on a solid substrate. Cooling of the sample allowed data to be collected for the long time scales necessary in order to obtain reasonable statistics on such dilute systems without noticeable radiation damage. The sample system of a YCC monolayer tethered to a non-polar SAM surface produces an average tilt angle between the heme plane and substrate plane of around 48° according to comparison of the experimental data with FEFF7 simulations using crystallographic coordinates. However, a closer match to the experimental data in terms of peak position and shape was achieved using MD simulations of the system which predicted an average heme tilt angle of 54 ± 3°. Heme orientations in the range 55 - 61° were also indicated by experimental optical LD. The fitting of such experimental polarized XAFS spectra could now be further improved by refinement of the interatomic distances.

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