Monolayers of proteins participating in biological electron-transfer reactions are very attractive for investigations of the structure and dynamics of intermolecular electron transfer within the native cell. Infrared (IR) spectroscopy is a very powerful method to resolve the structural dynamics of proteins on the atomic level. For the first time, we succeeded in recording infrared difference spectra of a protein monolayer by exploiting the surface enhancement exerted by a modified gold surface.

The electrochemically induced oxidation and reduction process of cytochrome c (cyt c), a soluble 12.5 kDa protein that mediates single-electron transfer between the integral membrane protein complexes of the respiratory chain, is regarded as the ideal model system for such a study. Enormous efforts have been made using classical electrochemical methods, and considerable contributions have been achieved for the understanding of the thermodynamics and kinetics of this system. Yet, the lack of structural information from these methods has limited the understanding of the correlation between the protein structure and the reaction kinetics. Vibrational spectroscopy, such as Raman scattering or IR absorption spectroscopy, can fill this gap. However, for the study of biomolecules at the solid/liquid interface these methods are still in their infancies. The information provided by Raman spectroscopy is mostly restricted to the chromophore due to the necessity of resonance enhancement. The vibrations of the whole protein are probed by Fourier transform infrared (FT-IR) spectroscopy where the functionally relevant vibrational changes are resolved by recording the reaction-induced difference. This approach has been extremely successful in the elucidation of the catalytic mechanism of many enzymes.

Conventional IR spectroscopy suffers from sensitivity too poor to detect the minute spectral changes from a monolayer. To overcome this difficulty, we employ surface enhanced infrared absorption (SEIRA) spectroscopy. The rough gold surface crucial for the SEIRA effect, serves as the working electrode permitting full control of the applied voltage across the self-assembled protein monolayer. Briefly, a thin gold film (~10 nm thick) is formed on a silicon hemicylinder prism (surface area: 1.5 cm$^2$) by an electroless deposition technique. The gold surface is exposed for 10 min to a 1 mM solution of the surface modifier mercaptopropionic acid (MPA) to form a self-assembled monolayer (SAM). After rinsing the SAM with water, the prism was inserted into a spectroelectrochemical cell. The sample solution was 10 mM K$_2$SO$_4$ and 10 mM phosphate buffer, pH 7.0, with 2 μM horse heart cyt c (Sigma). The electrode was left at the open circuit potential for 1 h to adsorb cyt c onto the MPA layer. The adsorption kinetics were followed by continuously recording IR spectra with the attenuated total reflection (ATR)-Kretschmann configuration (modified setup from), and maximum coverage was reached within 30 min. Figure 1 depicts the cyclic voltammogram of a cyt c monolayer adsorbed onto the Au film electrode. Reversible oxidation and reduction currents are observed at peak positions of +55 and +9 mV, respectively. Thus, the formal potential is +32 mV vs Ag/AgCl (228 mV vs NHE), which is in the range of what is observed for cyt c in solution. This result demonstrates that cyt c is structurally and dynamically not impaired by the adherence to the SAM of MPA. The electronic charge which is transferred during a full redox reaction is 2.3 μC. According to Miyake et al., the roughness factor of the gold surface is 2.5. This yields a surface coverage of 6.2 × 10$^{-12}$ mol/cm$^2$, i.e., only about half of the full coverage (13.5 × 10$^{-12}$ mol/cm$^2$). Therefore, the signals detected in the SEIRA difference spectra (Figure 2) arise from only partial coverage of the full redox current displayed in (a). Open circles are the intensities of the band at 1692 cm$^{-1}$ of the difference spectra displayed in Figure 2.

Figure 1. (a) Cyclic voltammogram of horse heart cytochrome c adsorbed Au electrode. Scan rate is 50 mV s$^{-1}$. The dashed line corresponds to the double layer capacitance. (b) Total charge pass during oxidation and reduction of cytochrome c (solid curve) calculated by integration of the redox current displayed in (a). Open circles are the measurements of the band at 1692 cm$^{-1}$ of the difference spectra displayed in Figure 2.

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Electrochemically Induced Surface-Enhanced Infrared Difference Absorption (SEIDA) Spectroscopy of a Protein Monolayer

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Smaller bands assigned to other than amide I are observed at 1514, 1419 cm\(^{-1}\). The frequencies of the IR difference bands agree well with those reported for cyt \(c\) adsorbed to modified gold surface have been observed with excellent signal-to-noise ratio. The surface modification preserves the full functionality of cyt \(c\). The frequencies of the vibrational bands are identical to cyt \(c\) in solution, whereas the observed discrepancy in relative peak intensity can be solely attributed to the characteristics of SEIRAS. The optical near-field effect of SEIRAS provides the opportunity to select those vibrators from the background of a large molecule (e.g., a protein) that are close to the gold surface. Utilization of the ATR-SEIRAS configuration is advantageous not only for increased sensitivity but also for electrochemical analysis because the presence of a bulk solution phase reduces the contribution from solution resistance as compared to that in the conventional IR technique in thin layer configuration.\(^9,10\) ATR-SEIRAS provides fast electrochemical response\(^12\) and will enable time-resolved IR studies of biological samples at solid/liquid interfaces. Step-scan experiments\(^13\) to study the dynamics of membrane protein monolayers are currently underway. Finally, this methodology represents a nanotechnological approach toward the investigation of single native membranes and the molecular changes of their constituents upon an external trigger.

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