Cytochrome C as a benchmark system for a two-layer gold surface with improved surface-enhancement for spectro-electrochemistry

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Abstract. A two-layer gold surface is developed for use with electrochemistry followed by surface-enhanced infrared absorption spectroscopy (SEIRAS) consisting of a conducting underlayer onto which Au nanoparticles (AuNPs) are grown by self-catalyzed electroless deposition. AuNPs are grown on protruding substructures of the 25 nm thin underlayer. The enhancement factor of the two-layer gold surface is controlled by the growth conditions. Cytochrome c adsorbed to a self-assembled monolayer of mercaptoethanol is used as a benchmark system for the investigation of complex heme proteins from the respiratory chain such as cytochrome c oxidase and the bc1 complex. Under optimum conditions the absorbance of the amide I band of cytochrome c is increased by a factor of 5 vs. classical SEIRAS surface. Reversible reduction/oxidation of cytochrome c on the two-layer gold surface is shown to take place by cyclic voltammetry.

Keywords: Surface-enhancement, SEIRAS, cytochrome C, spectro-electrochemistry, two-layer gold surface

1. Introduction

Electron transfer (eT) kinetics at metal–solution interfaces can be investigated by electrochemical methods with time resolutions down to nanoseconds depending on the size of the electrodes [3]. However, data thus obtained do not provide information on the molecular species involved in the eT. Spectro-electrochemistry is a way out of this dilemma, although there are only a few spectroscopic techniques available to meet the requirements regarding time resolution and sensitivity. Surface-enhanced infrared absorption spectroscopy, SEIRAS [6,9–11], has the potential to meet these requirements, particularly as it is applied in the ATR-FTIR mode where the bulk of the electrolyte adjacent to the metal surface is not a problem and, moreover, data acquisition is facilitated by Fourier transform infrared (FTIR) interferometry. With the help of the step-scan method, time resolutions down to the nanosecond time scale can be obtained [2].

In our attempts to investigate the redox kinetics of heme proteins, we developed a two-layer gold film with an improved enhancement factor and stability for use with SEIRAS [8]. In the present investigation,

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this system was applied for time-resolved studies of cytochrome c as an otherwise electrochemically and spectroscopically well-characterized system.

Note: Experimental methods can be found in [7] and [8].

2. Results and discussion

The absorption of cytochrome c to the mercaptoethanol-modified two-layer gold surface was shown to take 60 min as indicated by SEIRAS [8]. Saturation was measured by the amplitude of the most prominent bands at 1661 and 1551 cm^{-1} , which represent the amide I and II vibrations of the protein. After immobilization of cytochrome c spectral changes were then recorded as a function of applied potential. Such measurements will henceforward be named potentiostatic titrations. During such titrations difference spectra were taken at different potentials with the fully reduced state as the reference. As deduced from the CV measured of immobilized cytochrome, the fully reduced state is attained at a potential of -103 mV vs. SHE. For the potentiostatic titration, the potential was varied in steps of 50 mV between $+497$ and -103 mV vs. SHE.

Changes in the spectra were observed at wavenumbers in the range of 1500–1800 cm^{-1} . It could be shown that the observed changes in the spectra were correlated to the changes in the redox-state of the species involved in the electron transfer between the redox center of the cytochrome c and the used two layer gold surface.

Which of the bands is more or less correlated with the redox transition can be deduced from a plot of the amplitudes vs. potential. For example, the band at 1692 cm^{-1} yielded a sigmoidic curve, which can be fitted to the Nernst equation with a slope of 36 mV and an inflection point at 217 mV in reasonable agreement with the theoretical slope of 25 mV and the standard redox potential of cytochrome c, $E^0 = 230$ mV [5]. The band at 1552 cm^{-1} yielded also a sigmoidic function with an inflection point at 220 mV, but a shallow slope of 130 mV. This is in agreement with a band, the peptide group of which is located at a greater distance from the redox center and is therefore less correlated with the redox transition. An entirely unspecific orientational or other change of a peptide in an electric field would be indicated by a straight line as was observed for molecules, which do not undergo any faradaic process [2].

Finally, potential-controlled time-resolved (tr)-SEIRA measurements were conducted in the step-scan mode with a square wave excitation at frequencies between 0.7 Hz and 1 kHz. We could show that the optimal excitation frequency for cytochrome c is 500 Hz as deduced from the time-dependent changes of the bands at 1692, 1663, 1644 and 1625 cm^{-1} [7]. These bands could also be observed by the potentiostatic titration of the protein. According to [1] these bands are well established for redox-processes in the secondary structure of cytochrome c.

The isolation of spectral changes out of a noisy background due to external perturbation can be improved by the application of phase-sensitive detection (PSD). The bands changing with time can be discriminated effectively. Although a purely mathematical procedure, PSD has an effect similar to the analysis of the detector signal with the help of a lock-in amplifier, suggested to be applied for tr-SEIRAS earlier [2,4]. Only the signals that change with the phase angle need to be considered to depend on the excitation, whereas noise is not phase-shifted and therefore suppressed compared to the original spectrum. By curve fitting, using Lorentzian curves, we used PSD spectra to obtain the band parameters of absorbing components such as frequency, bandwidth, and amplitude.

Finally, the area of the band at 1692 cm^{-1} obtained from tr spectra was plotted as a function of time for the reductive and oxidative branch of the square-pulse excitation (Fig. 1). Measurements were carried out

Fig. 1. Peak area of the band at 1692 cm^{-1} plotted as a function of time. Monoexponentials were fitted to the data, and the corresponding time constants are $k_{0,\text{red}} = 1880 \pm 232\text{ s}^{-1}$ for the reductive branch and $k_{0,\text{ox}} = 1810 \pm 239\text{ s}^{-1}$ for the oxidative branch.

on four samples measured three times each. The data sets were fitted to the monoexponential functions

$$\Delta A_{\text{ox}}(t) = \Delta A_{\text{max,ox}} \{1 - \exp[-k_{\text{ox}}(t - t_{0,\text{ox}})]\} + \Delta A_{0,\text{ox}}, \quad (1)$$

$$\Delta A_{\text{red}}(t) = \Delta A_{\text{max,red}} \exp[-k_{\text{red}}(t - t_{0,\text{red}})] + \Delta A_{0,\text{red}} \quad (2)$$

for the oxidative and reductive branch, respectively, yielding a rate coefficient of $k_{\text{ox}} = 1810 \pm 239\text{ s}^{-1}$ for the oxidative branch and $k_{\text{red}} = 1880 \pm 232\text{ s}^{-1}$ for the reductive branch. $\Delta A_{\text{ox}}(t)$ and $\Delta A_{\text{red}}(t)$ are the absorbances as a function of time; $A_{\text{max,ox}}$ and $A_{\text{max,red}}$ are the maximal amplitudes of the absorbance; ΔA_0 are the amplitudes at the starting point of oxidation/reduction; and t_0 are the start times for the oxidative and reductive branch, respectively. The overall error includes the error due to sample preparation, the repeatability of the measurement, as well as the fitting.

3. Conclusion

Using cytochrome c as a benchmark system for complex heme proteins, we could show that the enhancement factor obtained by the newly developed two-layer gold surface was appreciably improved over that of the classical chemical (electroless) deposition technology. Reduction and oxidation of cytochrome c adsorbed onto the two-layer gold surface had been shown by cyclic voltammetry. Spectro-electrochemistry then enabled us to investigate the kinetics of electron transfer of cytochrome c using electrochemically controlled tr-SEIRAS in combination with the phase-sensitive detection (PSD). These results indicate that using these techniques, the analyses of more complex heme proteins such as the cytochrome c oxidase should be also possible.

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