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Time-Resolved Surface-Enhanced Resonance Raman Spectroscopy for Studying Electron-Transfer Dynamics of Heme Proteins

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Investigations of redox processes of heme proteins at electrodes are of particular interest since the electrode/electrolyte interface may be regarded as a model for biological interfaces.[1] Thus, such studies may contribute to a better understanding of the mechanism of the biological electron-transfer (ET) reactions. Electrochemical methods such as cyclic voltametry (CV) probe the overall redox process of the bulk species and, only in special cases, can be used to determine kinetic constants of the ET reactions of the adsorbed molecules.[2] Moreover, these techniques cannot provide insight into the molecular processes in the electrical double layer. Such processes may include conformational transitions of the electroactive species coupled with the ET reactions as it has been shown for cytochrome *c* (Cyt-*c*).[3,4] Since the same conformational changes of Cyt-*c* have also been detected upon binding to cytochrome *c* oxidase, it has been suggested that the biological electron transfer is controlled by conformational gating.[5] To test this hypothesis, it is required to analyze the dynamics of the redox processes of both conformers of the adsorbed heme protein.

Thus, there is a need for a technique which monitors processes of the adsorbed species and which is capable to provide both structural and kinetic information about the interfacial processes. Surface-enhanced resonance Raman spectroscopy (SERRS)[6] fulfills these requirements inasmuch as it selectively probes the vibrational spectra of the heme group of cytochromes adsorbed on rough silver electrodes. So far this technique has been only employed to study potential-dependent equilibria of Cyt-*c* and other heme proteins.[3,7] In this paper, we report for the first time on time-resolved SERRS experiments of a cytochrome which provide insight into the ET dynamics and allow for the determination of the heterogeneous ET rate constants. In addition, the method we have developed is also of general importance for studying potential-dependent processes of adsorbates on electrodes.

In this study we have focused on the "thermoresistent" cytochrome c_{552} (Cyt-c_{552}), the counterpart of the eukaryotic Cyt-*c* in the respiratory chain of *Thermus thermophilus*.[8] The protein was isolated and purified as described elsewhere.[8] The SERR spectra were measured with a spectrograph equipped with a CCD detection system.[9] For the SERR experiments, a rotating Ag electrode was used to avoid laser-induced heating of the adsorbed protein molecules.[3] The electrode was in contact with a buffered solution (pH 7.6, 10 mM Tris-HCl) containing 50 mM KCl as a supporting electrolyte. The Cyt-c_{552} concentration was 10^{-7} M which ensured a saturation coverage of the Ag surface. At this low concentration, no resonance Raman (RR) scattering of the dissolved species interfered with the SERR spectra.

In stationary experiments it was found that upon adsorption on the Ag electrode Cyt-c_{552} reveals conformational equilibria similar to Cyt-*c*, i.e., between two states denoted as B1 and B2.[10] Whereas B1 exhibits the same structure and reduction potential as the dissolved species, the formation of B2 is associated with a structural change of the heme pocket leading to a coexistence of a six-coordinated low-spin (6cLS) and a five-coordinated high-spin (5cHS) configuration. These structural changes are accompanied by a drastic lowering of the reduction potential. At potentials < -0.1 V (all potentials cited in this work refer to the saturated calomel electrode), state B1 prevails whereas at more positive potentials the SERR spectra include substantial contributions from state B2. Thus, potential-dependent measurements at potentials above -0.2 V encounter the difficulty that SERR spectra include contributions from four different species, i.e., the oxidized and reduced forms of state B1 and the oxidized 6cLS and 5cHS forms of state B2. As their relative contributions vary with the potential, it was possible to determine the spectra of all species based on the component analysis.[11] In this way, the stationary SERR spectra measured as a function of the potential could be analyzed quantitatively allowing the determination of the redox potential of B1. The value of -0.045 V we have obtained is only slightly more negative than that determined for the dissolved Cyt-c_{552} (-0.013 V)[12] which further supports the view that both species exhibit the same (heme pocket) structures.

In time-resolved experiments, a rapid potential jump[13] from an initial potential E_i to a final potential E_f (ΔE_{if}) was employed and the SERR spectra were measured after (variable) delay times δ' for a time interval Δt of 21 ms. After this measuring interval, the potential was set back to E_i (ΔE_{fi}) in order to establish the initial equilibrium. The sequence of ΔE_{if} and ΔE_{fi} was repeated N times until the effective total accumulation time $N \cdot \Delta t$ was between 2 and 3 s as required for a sufficient signal-to-noise ratio. The time resolution was controlled by gated excitation rather than gated signal detection.[14] Gating of the cw-laser beam which was achieved by a rotating chopper wheel was synchronized with triggering the potential jump.[15]

A selection of time-resolved SERR spectra measured after a potential jump ΔE_{if} from 0.0 to -0.1 V are shown in Figure 1 along with the stationary SERR spectrum measured from the

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redox-equilibrated sample at −0.1 V. In the region of the ν_4 mode which is known to be a sensitive marker for the oxidation state of the heme iron,[16] we note an increase of the prominent peak at ca. 1361 cm^{-1} (ferrous heme) at the expense of the 1371 cm^{-1} shoulder (ferric heme) with increasing delay times. These time-dependent changes in the ν_4-band region are accompanied by an intensity decrease of other bands which are characteristic for a ferric heme, such as the ν_3 and ν_{10} modes at 1501 and 1634 cm^{-1}, respectively. At a delay time of 103 ms, the SERR spectrum (not shown) is already very similar to the stationary spectrum at −0.1 V implying that the relaxation of the redox process is nearly completed. The measured spectra normalized as described elsewhere[10] were quantitatively analyzed by complete component spectra of the various species as determined in stationary experiments.[11] This approach is significantly more accurate than a conventional band fitting analysis inasmuch as the degrees of freedom just correspond to the number of the underlying species which in this case were the oxidized and reduced forms of state B1 as well as two oxidized 6cLS and 5cHs forms of state B2. The results obtained in this way revealed that for the potential jump from 0.0 to −0.1 V the relative contributions of B2 were small and invariant in the time range from 30 to 110 ms. These findings indicate that under these conditions the ET reactions and conformational transitions occur on different time scales. Consequently, it is possible to analyze the data based on a simple one-step relaxation mechanism for the redox process of state B1 according to

$$\frac{\Delta[C_r]_{t=\delta'}}{\Delta[C_r]_{t=0}} = \exp(-(k_1 + k_2)t) \qquad (1)$$

where $\Delta[C_r]_{t=\delta}$ and $\Delta[C_r]_{t=0}$ denote the relative concentration differences of the reduced B1 state of Cyt-c_{552} with respect to the equilibrium value at E_f for the delay times $t = \delta'$ and $t = 0$, respectively. The rate constants k_1 and k_2 refer to the heterogeneous electron-transfer rate constants for the reduction and oxidation of the adsorbed Cyt-c_{552}(B1), respectively. Since the relative SERR intensities are proportional to the relative concentrations, $\Delta[C_r]_{t=\delta'}/\Delta[C_r]_{t=0}$ is equal to the ratio of the corresponding intensity differences. The latter data are obtained from the component analysis of the time-resolved SERR spectra measured at various delay times and the stationary SERR spectra of the (redox-)equilibrated Cyt-c_{552} adsorbed at E_i and E_f. As shown in Figure 2, the data plotted semilogarithmically as a function of the delay time δ, follow a straight line with a correlation coefficient of 0.97. From the slope, the rate constant k_1 is determined to 6.3 s^{-1} taking into account that k_2 is equal to $k_1/K(E_f)$ where $K(E_f)$ is the equilibrium constant at E_f.

The value for k_1 refers to a driving force of −0.058 eV. Assuming that the reorganization energy for Cyt-c_{552} is similar to that estimated for Cyt-c (i.e., ca. 0.6 eV),[17] the standard heterogeneous rate constant $k_1(E°)$ at the formal reduction potential is calculated to 2.0 s^{-1}. In previous CV and electroreflectance studies, these constants have been determined for the redox process of Cyt-c at Au electrodes with modified surfaces.[2] However, the reported values covering a range from 0.1 to 1000 s^{-1} strongly depend on the kind of the surface modifiers and can even vary substantially for Cyt-c from different sources. In addition, in contrast to SERR spectroscopy, the methods which were employed in these studies cannot distinguish between different electroactive conformers (i.e., B1 and B2). Thus, the comparison of these data with the present results which refer to a well-defined redox couple do not allow any conclusions concerning possible similarities or differences of the ET mechanism of Cyt-c_{552} with respect to Cyt-c.

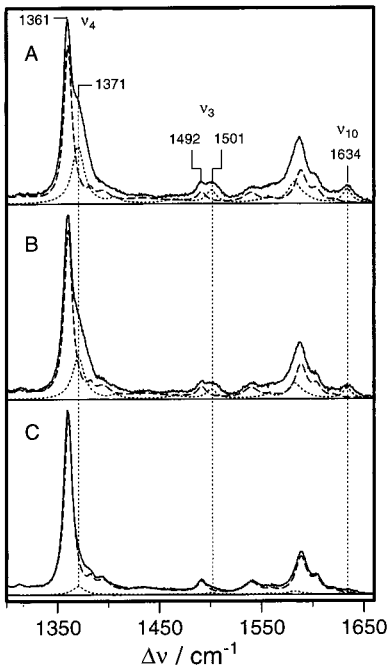

Figure 1. Time-resolved SERR spectra of Cyt-c_{552} measured at delay times of 21 (A) and 62 ms (B) following a potential jump from 0.0 to −0.1 V, compared with a stationary SERR spectrum measured at −0.1 V (C). Excitation wavelength: 413 nm (50 mW at the sample). Spectral resolution: 2.5 cm^{-1} with 0.5 cm^{-1} increments per data point. Accumulation time: 20 s for C and 3 s for A and B. The component spectra of the oxidized and reduced state B1 are denoted by the dotted and dashed lines, respectively. For simplicity, the component spectra of state B2 which contribute by less than 10% to the measured spectra are not displayed.

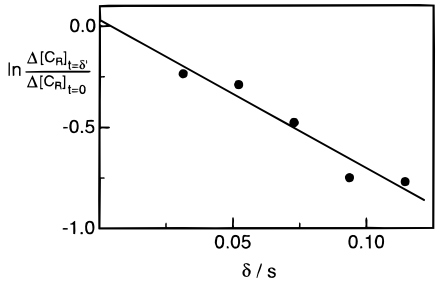

Figure 2. Semilogarithmical plot of $\Delta[C_r]_{t=\delta}/\Delta[C_r]_{t=0}$ versus the delay time δ which is given by $\delta = \delta' + \Delta t/2$ to account for the width of the measuring interval.

On the other hand, the present study has demonstrated that time-resolved SERR spectroscopy can simultaneously provide kinetic and structural information about ET reactions of adsorbed monolayers which is a prerequisite for elucidating the dynamics of the complex redox processes of cytochromes. Moreover, the time resolution of our experimental approach is only limited by the recharging time of the electrical double layer[18] so that heterogeneous ET reactions can be monitored in wide potential range, thereby providing an alternative access to reorganization energies.[19]

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