Journal of Electroanalytical Chemistry 660 (2011) 367–376

Contents lists available at ScienceDirect

Journal of Electroanalytical Chemistry

journal homepage: www.elsevier.com/locate/jelechem

Electric-field effects on the interfacial electron transfer and protein dynamics of cytochrome c

H. Khoa Ly [a], Nattawadee Wisitruangsakul [a,b], Murat Sezer [a], Jiu-Ju Feng [a,c], Anja Kranich [a], Inez M. Weidinger [a], Ingo Zebger [a], Daniel H. Murgida [d], Peter Hildebrandt [a,*]

[a] *Technische Universität Berlin, Institut für Chemie, Sekr. PC 14, Straße des 17. Juni 135, D-10623 Berlin, Germany*

[b] *Iron and Steel Institute of Thailand, 1st-2nd Floor, Bureau of Industrial Sectors Development Building, Trimitr Soi., Pharam 4 Rd. Phakhanong, Klongtoey Bangkok 10110, Thailand*

[c] *School of Chemistry and Environmental Science, Henan Normal University, Xinxiang, Henan 453007, China*

[d] *Departamento de Química Inorgánica, Analítica y Química Física/INQUIMAE-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pab. 2, Piso. 1, C1428EHA-Buenos Aires, Argentina*

ARTICLE INFO

Article history:
Received 18 May 2010
Received in revised form 8 October 2010
Accepted 15 December 2010
Available online 21 December 2010

Keywords:
Surface enhanced Raman spectroscopy
Surface enhanced infrared spectroscopy
Electron transfer
Cytochrome c
Electric field

ABSTRACT

Time-resolved surface enhanced resonance Raman and surface enhanced infrared absorption spectroscopy have been employed to study the interfacial redox process of cytochrome *c* (Cyt-c) immobilised on various metal electrodes coated with self-assembled monolayers (SAMs) of carboxyl-terminated mercaptanes. The experiments, carried out with Ag, Au and layered Au–SAM–Ag electrodes, afford apparent heterogeneous electron transfer constants (k_{relax}) that reflect the interplay between electron tunnelling, redox-linked protein structural changes, protein re-orientation, and hydrogen bond re-arrangements in the protein and in the protein/SAM interface. It is shown that the individual processes are affected by the interfacial electric field strength that increases with decreasing thickness of the SAM and increasing difference between the actual potential and the potential of zero-charge. At thick SAMs of mercaptanes including 15 methylene groups, electron tunnelling (k_{ET}) is the rate-limiting step. Pronounced differences for k_{ET} and its overpotential-dependence are observed for the three metal electrodes and can be attributed to the different electric-field effects on the free-energy term controlling the tunnelling rate. With decreasing SAM thickness, electron tunnelling increases whereas protein dynamics is slowed down such that for SAMs including less than 10 methylene groups, protein re-orientation becomes rate-limiting, as reflected by the viscosity dependence of k_{relax}. Upon decreasing the SAM thickness from 5 to 1 methylene group, an additional H/D kinetic isotope effect is detected indicating that at very high electric fields re-arrangements of the interfacial or intra-protein hydrogen bond networks limit the rate of the overall redox process.

1. Introduction

Interfacial electron transfer (ET) reactions play a key role in various processes of technological importance such as catalysis, corrosion and energy conversion or storage [1–3]. Furthermore, they are constitutive for a large number of biological functions and essential for most of the biotechnological applications that utilize redox enzymes [4]. This wide range of fundamental and applied aspects of heterogeneous ET has motivated numerous experimental and theoretical studies for decades. It is, therefore, quite surprising that these elementary reactions are yet not comprehensively understood.

One reason for this quite remarkable gap is related to methodological shortcomings. For a long time, electrochemical methods have been the only techniques for determining thermodynamic and kinetic data of redox processes although the nature of the participating molecules could not be directly identified [5,6]. Thus, analyses of mechanistic aspects of interfacial redox processes had to rely upon indirect evidences.

In this respect, surface enhanced Raman (SER) and surface enhanced infrared absorption (SEIRA) spectroscopy represent powerful complementary techniques since the vibrational spectra of molecules in close vicinity of metal electrodes can be selectively probed due to the resonant coupling of the radiation field with the surface plasmons of the metallic support, which may be Ag or Au [7,8]. These methods can be coupled with the potential jump technique such that they provide information of the kinetics and thermodynamics of the interfacial processes and the nature of the molecule species involved [9–13].

SER and SEIRA spectroscopy have been employed to study interfacial processes of redox proteins [14–17]. In these studies, the SER

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[*] Corresponding author. Tel.: +49 30 314 21419; fax: +49 30 314 21122.
E-mail address: hildebrandt@chem.tu-berlin.de (P. Hildebrandt).

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doi:10.1016/j.jelechem.2010.12.020

effect is combined with the molecular resonance Raman (RR) effect (surface enhanced resonance Raman – SERR) such that it is possible to selectively probe the redox site solely of the immobilised proteins by tuning the excitation line in resonance with both the electronic transition of the cofactor *and* the surface plasmons of the metal [7,8]. The sensitivity and selectivity of SEIRA spectroscopy is increased upon operating in the difference mode such that only potential-dependent changes of the vibrational bands of the protein *and* the cofactor are detected [13,16,17].

A widely used model system, appropriate for employing SERR and SEIRA spectroscopy as well as electrochemical methods, is based on Au or Ag electrodes coated by self-assembled monolayers (SAM) of mercaptanes [14,15]. Such coatings on Au surfaces have been first characterised by Nuzzo and Allara [18] and later used for biocompatible immobilisation of redox proteins [19–21]. The terminal tail groups of the mercaptanes can be varied to allow for different modes of protein binding and the thickness of the SAM is defined by the number of methylene groups in the alkyl chain of the mercaptanes. These devices allow systematic variation of important parameters controlling the heterogeneous ET, such as the ET distance, driving force, charge distribution in the SAM/protein interface, and ionic strength, viscosity, and pH in the bulk solution.

A particularly large body of experimental and theoretical data has been accumulated for the redox process of the heme protein cytochrome *c* (Cyt-c) electrostatically immobilised on electrodes coated with carboxyl-terminated SAMs [11–17,20–50]. The results obtained so far indicate that the overall redox process is determined by the coupling of protein dynamics with electron tunnelling [42,46–48]. This coupling results from the fact that the energetically preferred electrostatic binding domain corresponds to an orientation of the bound protein that exhibits a distinctly lower tunnelling probability than a lower affinity binding site [46,48]. Optimum electron tunnelling efficiency, therefore, requires rotational motions of the protein on the SAM. This conclusion seems to provide a satisfactory explanation for the unique distance-dependence of the experimentally observed ET rate which first increases exponentially with decreasing distance, i.e. the SAM thickness, but then levels off to a plateau region for SAMs shorter than 10 methylene groups [12–15,23,25,27,29–32,42]. This region has, hence, been attributed to a kinetic regime in which protein-re-orientation becomes the rate-limiting step [42]. This interpretation is consistent with the experimental finding that the relaxation constants for protein re-orientation decrease substantially with decreasing distance in contrast to the exponential increase of the electron tunnelling rate. The slow-down of the protein dynamics with decreasing distance has been attributed to the concomitant increase of the electric field strength at Cyt-c/SAM interface, thereby increasing the activation energy for protein re-orientation. Thus, the interfacial redox process *in toto* has been suggested to be modulated by the electric field. Evidently, this scenario holds for quite different proteins which have been shown to exhibit a similar non-exponential distance dependence of the heterogeneous ET rate constant [51–55].

However, there are still some observations which do not fully fit into this scheme. First, in the limit of highly restricted protein mobility, one would expect at least a slight increase of the ET rate with further decreasing the electron tunnelling distance due to the exponential increase of the electronic coupling parameter in each protein orientation. However, all experimental data reported so far indicate an essentially distance-independent regime for short distances, i.e. high electric fields. Second, although experimental data obtained for SAM-coated Au and Ag electrodes display a qualitatively similar distance dependence, even for the same protein (i.e. Cyt-c) the absolute values for the experimentally determined ET rate constants are generally higher for Au than for Ag [12,13,23,25,27–29,36,38,45]. Third, in the plateau region, the ET rate constants have been shown to be lower in D_2O compared to H_2O, which has been ascribed to a kinetic isotope effect due to the coupling of electron transfer with redox-linked proton translocation in the protein or in the protein/SAM interface [12,43]. Although recently a part of this isotope effect was attributed to the intrinsically higher viscosity of D_2O solutions [43], it remains to be clarified why the ratio of the rate constants in H_2O and D_2O increases with decreasing distance to the electrode.

In the present work we have addressed these three questions by employing time-resolved (TR) SERR and SEIRA spectroscopy to probe the distance- and overpotential-dependence of the interfacial ET on Ag, Au, and Au–SAM–Ag hybrid electrodes. Special emphasis is laid on the analysis of viscosity and H/D effects on the kinetic constants. The results lead to the conclusion that not only protein re-orientation but also the electron tunnelling step itself is controlled by the interfacial electric field.

2. Experimental

2.1. Materials

6-mercaptohexanoic acid (C_5) from Dojindo, 16-mercaptohexadecanoic acid (C_{15}), 11-mercaptoundecanoic acid (C_{10}), and mercaptoacetic acid (C_1), all purchased from Sigma–Aldrich, were used without further purification. Formation of SAMs on the metal electrode followed the protocol described previously [26]. Horse heart Cyt-c from Sigma–Aldrich was purified by HPLC. The water used in all experiments was purified by a Millipore system and its resistance was more than 18 MΩ. All other chemicals were of highest purity grade available.

2.2. Surface Enhanced Resonance Raman spectroscopy

The spectroelectrochemical cell for SERR spectrosocpy has been described elsewhere [26]. All potentials cited in this work refer to the Ag/AgCl (3 M KCl) electrode. A rotating Ag ring served as the working electrode. SER-activation, coating of the electrode by SAMs and subsequent protein immobilization followed the protocol described previously with minor modifications [10,26]. Briefly, prior to the spectroscopic experiments, the electrolyte solution (30 mM phosphate buffer, pH = 7.0) was bubbled with catalytically purified oxygen-free argon for ca. 20 min. Consecutively, Cyt-c solution was added yielding a final concentration of ca. 0.2 μM. Protein adsorption was achieved by incubating the working electrode for 30 min into the protein containing buffer solution at open circuit potential. All experiments were performed in the presence of protein in solution and Ar overpressure.

For experiments in D_2O, the protein was dissolved in buffered D_2O solutions adjusted to pD = 7.0 [12] and subsequently incubated for further 18–24 h for complete H/D exchange. To account for the intrinsically higher viscosity of D_2O, comparative experiments in H_2O buffer were carried out at a viscosity of 1.2 cp adjusted by addition of sucrose [42]. All SERR measurements were carried out with a roughened Ag electrode [10] or an Au–SAM–Ag electrode fabricated as described previously [45].

SERR spectra were measured in back-scattering geometry using a confocal microscope coupled to a single stage spectrograph (Jobin Yvon, LabRam 800 HR) equipped with a liquid-nitrogen cooled back illuminated CCD detector. The 413-nm line of a cw Krypton ion laser (Coherent Innova 300c) was focused onto the surface of the rotating Ag electrode by means of a long working distance objective (20×; NA 0.35). Typically, experiments were performed with laser powers of ca. 1 mW. Effective acquisition times were between 3 and 10 s. All experiments were repeated several times to