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Bioelectrochemistry 63 (2004) 55–59
Bioelectrochemistry
www.elsevier.com/locate/bioelechem

Cyclic voltammetry and voltabsorptometry studies of redox proteins immobilised on nanocrystalline tin dioxide electrodes

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Received 25 July 2003; received in revised form 8 September 2003; accepted 15 September 2003

Abstract

Protein film cyclic voltammetry is a well-established technique for the study of redox proteins immobilised on electrode surfaces. In this paper, we use nanostructured SnO_2 electrodes to demonstrate that cyclic voltabsorptometry is an effective, complimentary approach to such studies of protein redox function. We exemplify this approach using two different redox systems: microperoxidase-11 (MP-11) and flavodoxin *Desulfovibrio vulgaris* Hildenborough (Fld). Both systems were immobilised on nanocrystalline SnO_2 electrodes and the resulting films investigated by simultaneous cyclic voltammetry and voltabsorptometry. We demonstrate that cyclic voltabsorptometry allows the unambiguous and background free observation of redox reactions for both systems studied.

Keywords: Cyclic voltabsorptometry; Spectroelectrochemistry; Redox proteins; Nanostructured; SnO_2 electrodes

1. Introduction

Bioelectrochemistry is now a well-established discipline both for investigating protein redox chemistry and for developing electrochemical biosensors. The electrode can replace the physiological electron transfer partners, allowing both the driving of protein redox chemistry by the application of electrical potentials and the electrochemical sensing of changes in the protein redox states due to substrate binding. A key factor for the successful bioelectrochemical investigation of redox proteins remains the efficient interaction with the electrode surface. Protein immobilisation upon the electrode surface is an attractive approach to achieving this, thereby avoiding contributions to the experimental data deriving from protein diffusion in the solution. Protein film cyclic voltammetry, where current is monitored as function sweep potential, is well established as a powerful probe of the redox chemistry of such immobilised proteins [1]. A wealth of information about thermodynamic and kinetics of electrochemically driven protein reactions can be extracted using this technique.

Whilst undoubtedly a powerful approach, electrochemical studies of protein redox function have significant limitations. The experimental observable, the current flowing in the external circuit, is only an indirect probe of protein redox transitions. Such currents may also derive from electrode charging and/or faradaic currents associated with other redox species adsorbed to the electrode surface or present in the electrolyte, significantly complicating the unambiguous observation of protein redox chemistry. The technique is moreover susceptible to contamination by partially denatured proteins on the electrode surface [2]. Spectroelectrochemical approaches have been widely employed to overcome these limitations, exploiting the characteristic changes in optical absorption resulting from many protein cofactor redox transitions to allow the unambiguous assignment of the experimental data. One such technique of particular interest is cyclic voltabsorptometry, in which the optical absorbance at a wavelength selected to indicate the electron transfer process of interest is monitored as a function of sweep potential [3,4]. Since the optical signal is relatively free from background contribution, a much cleaner signal is observed than the corresponding cyclic voltammetry current response. However, such studies to date have been limited to protein electrochemistry in solution, due to the low optical density of protein monolayers adsorbed to conventional electrodes.

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doi:10.1016/j.bioelechem.2003.09.014

Herein we will demonstrate the use of cyclic voltasorptometry to investigate interfacial electron transfer reactions between a layer of immobilised protein and an electrode, an approach which, to our best knowledge has not been reported previously. For this purpose, nanostructured metal oxides, in particular SnO_2, are ideally suited as an electrode because of its optical transparency and electrical semiconductivity [5]. In addition, the advantage of having the protein under studies immobilised on the electrode is also obtained. The protein can be immobilised onto the surface after preparation of the electrode itself. As a result, immobilisation conditions can be tuned specifically to suit each biomolecule by prior control of film surface properties. We have shown that several redox proteins (cytochrome *c* (Cyt-*c*) and haemoglobin (Hb)) can be readily adsorbed on such electrodes with the binding mode being largely controlled by electrostatic interactions [5–7]. Facile direct interfacial electron transfer between a range of immobilised proteins and this electrode is achieved without the use of any redox mediators [5]. Moreover, UV absorption spectra imply that the immobilised proteins retain their native conformational states [5–7].

Two redox systems are chosen to exemplify both a simple and a more complex redox process. Firstly, we will investigate the one-electron ferric/ferrous redox couple of heme centre in microperoxidase-11 (MP-11). This heme undecapeptide is obtained from enzymatic digestion of Cyt-*c*, it is of biotechnological interest due to its catalytic activity towards H_2O_2 reduction [8,9]. Secondly, a more complex protein, flavodoxin (Fld), will be investigated, involving two consecutive electron transfers in a flavin mononucleotide (FMN) redox centre [10]. We note that previous electrochemical studies of the redox chemistry of Fld have been hampered by ambiguity resulting from the protein free FMN [2].

2. Experimental

2.1. Materials and reagents

Flavodoxin *Desulfovibrio vulgaris* strain Hildenborough were expressed and purified following the published procedure [10], yielding a holoprotein with RZ value of 5.4. MP-11 from horse heart cytochrome *c* and poly-L-lysine hydrobromide (PLL) were purchased from Sigma-Aldrich and were used without further purification. All aqueous solutions were prepared in distilled, deionised water of resistance $R = 10$ MΩ.

2.2. Immobilisation

Nanostructured SnO_2 electrode was prepared following the published procedure [5]. An aqueous solution of protein was prepared using a pH 7 and 8, 10 mM phosphate buffer (sodium or potassium salts). Protein immobilization was achieved by the immersion of PLL modified SnO_2 electrodes in a 2 ml of protein solution at 4 °C for at least 30 min and 1–2 days for MP-11 and Fld, respectively. Protein adsorption onto the SnO_2 electrodes was monitored by recording the UV–VIS absorption spectra of the electrodes using a Shimadzu UV-1601 spectrophotometer. Contributions to the spectra from the scatter and absorption by the SnO_2 films alone were subtracted by the use of protein free reference electrodes. Prior to all spectroscopic measurements, films were removed from the immobilisation solution and rinsed in buffer solution to remove non-immobilised protein.

2.3. Spectroelectrochemistry

Electrochemical and spectroelectrochemical experiments were performed using an Autolab PGStat 12 potentiostat. The spectroelectrochemical cell was a 4 ml, three-electrode cell with quartz windows, employing a platinum mesh flag as the counter electrode, a Ag/AgCl, KCl [3.5 M] reference electrode and the SnO_2 electrode as the working electrode. All potentials are reported against Ag/AgCl. The electrolyte, a protein free buffer, was thoroughly deaerated by bubbling with Argon prior to experiments. For spectroelectrochemistry, the above cell was incorporated in the sample compartment of the spectrophotometer and the absorption changes monitored as a function of step or sweep potential. Then, the absorbance at any wavelength indicating electron transfer were differentiated with respect to the potential, followed by smoothing with a fast Fourier transform smoothing algorithm. All experiments were carried out at room temperature.

3. Results and discussion

3.1. Immobilisation

Employing the extinction coefficient of the hemepeptide and protein in calculation, a loading of 9 and 4 nmol were achieved for MP-11 and Fld, respectively, on 4 μm thick, 1 cm^2 SnO_2-PLL electrodes. These values correspond to monolayer coverage of the electrodes, which was calculated using the approximate dimension of protein as well as surface area of the electrode.

3.2. Cyclic voltabsorptometry

3.2.1. One electron transfer process

Having monolayer coverage of protein on optically transparent electrode surface, the optical signals may reveal the interfacial processes. Inset of Fig. 1 shows the formation of α and β bands (520 and 550 nm) of MP-11/SnO_2-PLL electrode under application of potential at −0.4 V. These bands are typical for ferrous MP-11, establishing one electron exchange process between the hemepeptide and electrode. This process was furthermore investigated by sweeping the potential between 0 and −0.7 V and simultaneously monitoring current and absorbance at 550 nm.

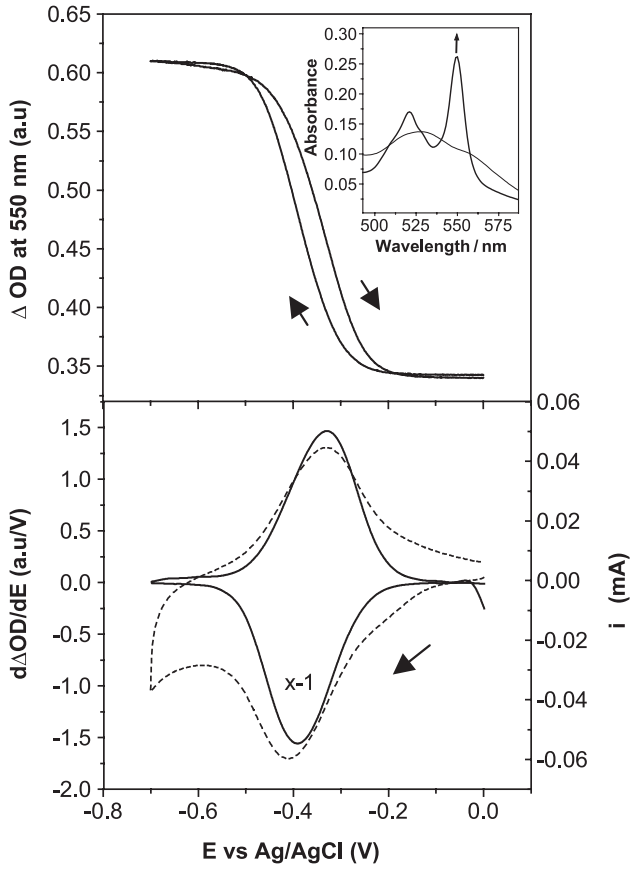

Fig. 1. CVA (top), CV (bottom,- - -) and DCVA (bottom,—) of MP-11/sno_2-PLL. Arrows show the scan directions. Absorption spectra of ferric (—) and ferrous (—) MP-11 are shown in inset. All experiments were measured at 10 mV/s in a protein free 0.01 M NaPi buffer solution pH 8.

The resulting absorbance (cyclic voltabsorptommogram, CVA) and current (cyclic voltammogram, CV) are displayed as a function of potential in Fig. 1. The increase and decrease of absorbance in the CVA, corresponding to the heme reduction and oxidation, respectively, are in good correlation with the nearly reversible, well defined anodic and cathodic peak currents in the CV, verifying the heme oxidation and reduction. The CV anodic and cathodic peaks are superimposed upon a background current attributed to film charging/discharging, with the ratio between the forward and reverse peak amplitudes, i_f/i_r, of 0.75, which is less than 1 as expected for a simple redox process. Since the CV signal is results from the differential change in redox state whilst the CVA signal results from the integrated population change, direct comparison of the CVA and CV data is facilitated by differentiation of the CVA data. The corresponding derivative voltabsorptomogram (DCVA) (Fig. 1, bottom) shows an excellent agreement with the CV data. It is however apparent that the DCVA data is background free, due to, as expected, the optical measurement being insensitive both to film charging and faradaic currents, with an anodic/cathodic peak ratio of ~ 1, in agreement with theoretical expectations. The scan rate dependence of the DCVA signal allows the calculation of interfacial electron transfer kinetics in an analogous manner to that well established for CV data [11], as we detail elsewhere [12].

3.2.2. Two consecutive electron transfer process

We turn now to study of the two redox transitions of Fld. This FMN co-factor of protein undergoes two redox transitions of interest: from the oxidised species (Fldq) to the semiquinone (FldqH), and a further reduction to the anionic hydroquinone ($FldqH^-$). Fig. 2 (top) shows a CV

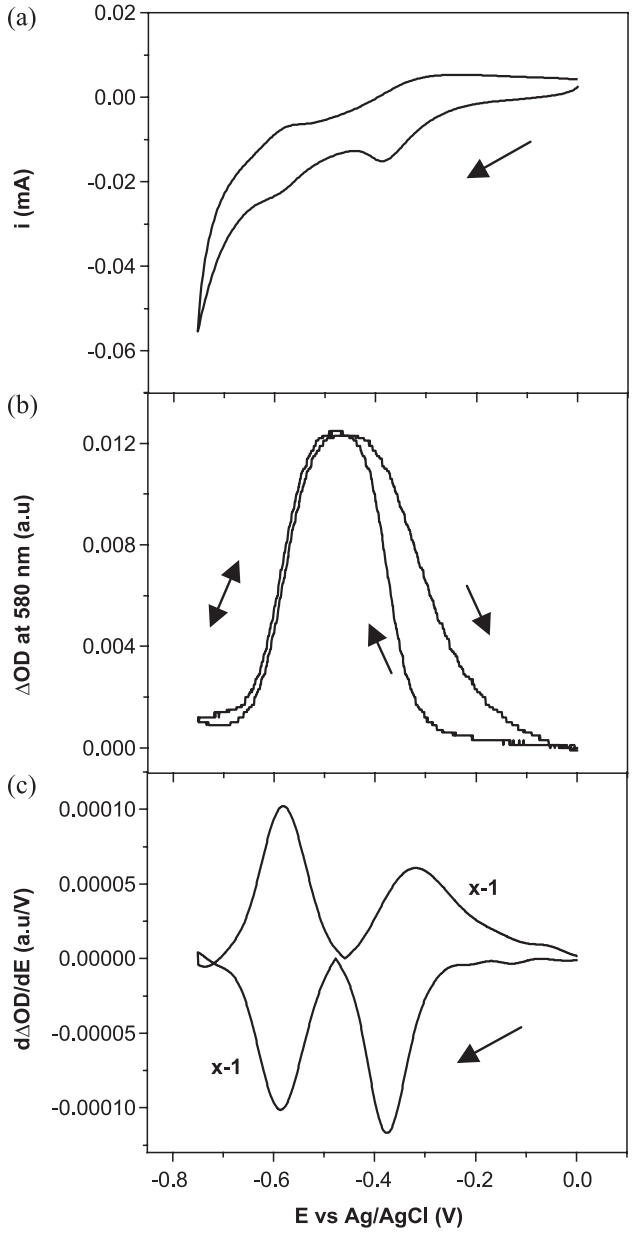

Fig. 2. CV (a), CVA (b) and DCVA (c) of Fld/SnO_2-PLL. Arrows show the scan directions. In order to make it readily comparable to CV, the second reduction and the first oxidation in DCVA data were multiplied by − 1. All experiments were measured at 5 mV/s in a protein free 0.014 MKPi buffer solution ph 7.

of Fld/SnO_2-PLL electrode, in which both redox transitions can be resolved of peaks centred at −0.34 and −0.585 V, indicating the presence of two redox couple in the electrochemical process. These peaks position are in good agreement with the midpoint potentials of the first (E2, −0.337 V) and second (E1, −0.634 V) electron transfer of Fld determined in solution, forming the consecutive FldqH and $FldqH^-$, respectively [13]. The corresponding CVA and DCVA are shown in Fig. 2 (middle and bottom), following the absorbance at 580 nm, a wavelength characteristic for the formation of FldqH. It is apparent that there is a periodical increase and decrease of absorbance at this wavelength subject to the sweep potential, corresponding therefore to the reduction of Fldq to FldqH and the subsequent further reduction of FldqH to $FldqH^-$ and providing direct evidence for the assignment of each redox transition. Spectroelectrochemical data and control studies of free FMN alone (not shown) confirmed the assignment of the first reduction transition to undenatured protein, with negligible contributions to the data from free FMN. Moreover, it is apparent from comparison of the CV and DCVA signals that the DCVA signal is essentially background free, giving reduction and oxidation peaks which are much more clearly resolved than the corresponding CV data. A full analysis of these data, and their implications for the physiological function of Fld, will be presented elsewhere [14].

We note that the observation of the Fldq/FldqH redox couple in the protein film CV and CVA data shown here is striking since, except at mercury electrode [15], this couple was not observed in previous electrochemical investigations of Fld at pyrolitic graphite, polished edge-plane graphite, and glassy carbon disc electrode using a wide range of binding promoters [16–18]. This observation is consistent with our previous observations that nanostructured metal oxide electrodes, such as those employed here, provide a non-denaturing electrode surface for immobilisation of a wide range of proteins [5–7].

In conclusion, we have demonstrated the versatility of cyclic voltabsorptometry as an alternative technique to diffusionless cyclic voltammetry for studies of protein film redox chemistry. Compared to more established film cyclic voltammetry, film cyclic voltabsorptometry has advantages of unambiguous observation of specific redox transitions and background free data collection. We note that the experimental approach employed does have significant limitations, most obviously being limited to redox transitions resulting in optical absorption changes in the visible (the electrodes being absorbing for wavelengths <400 nm). We further note that the semi-conducting nature of the electrodes limits the conductivity window to potentials $<+0.3$ V vs. Ag/AgCl. Nevertheless, the approach presented here represents a novel and powerful approach to the study of protein redox function which we believe should be applicable to the study of a wide range of protein redox chemistry.

Acknowledgements

We are very grateful to the financial support from BBSRC and EPSRC, Michael Gratzel, Franscine Duriaux, Alex Green and Emilio Palomares for the synthesis of SnO_2 paste, Paul Briscoe for helping in protein preparation and purification, and Andrea Fantuzzi for helpful discussions.

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