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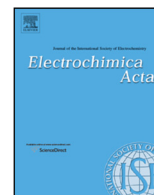

The impact of enzyme orientation and electrode topology on the catalytic activity of adsorbed redox enzymes

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ABSTRACT

It is well established that the structural details of electrodes and their interaction with adsorbed enzyme influences the interfacial electron transfer rate. However, for nanostructured electrodes, it is likely that the structure also impacts on substrate flux near the adsorbed enzymes and thus catalytic activity. Furthermore, for enzymes converting macro-molecular substrates it is possible that the enzyme orientation determines the nature of interactions between the adsorbed enzyme and substrate and therefore catalytic rates. In essence the electrode may impede substrate access to the active site of the enzyme. We have tested these possibilities through studies of the catalytic performance of two enzymes adsorbed on topologically distinct electrode materials. *Escherichia coli* NrfA, a nitrite reductase, was adsorbed on mesoporous, nanocrystalline SnO_2 electrodes. CymA from *Shewanella oneidensis* MR-1 reduces menaquinone-7 within 200 nm sized liposomes and this reaction was studied with the enzyme adsorbed on SAM modified ultra-flat gold electrodes.

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1. Introduction

Electrochemistry is a very powerful tool with which to study the catalytic mechanisms of redox enzymes [1–3]. A particularly useful method is protein-film electrochemistry (PFE), where the redox protein or enzyme is directly adsorbed on the electrode's surface. In PFE, interfacial electron transfer (ET) kinetics are not limited by diffusion of the enzyme to the electrode. When the flux of substrate to electrode is enhanced by, for instance, using a rotating-disk electrode, a platform is created in which individual steps in the catalytic cycle of enzymes can be deconvoluted. These organic–inorganic hybrid systems provide a platform for fundamental research and have application in biosensing and biofuel cells [4–6].

In order for PFE, biosensors or biofuel cells to achieve their optimal efficacy, the interfacial ET between the electrode and enzyme has to be fast compared to the enzyme's turnover. In turn, this requires the enzyme to be oriented with its electron-entry site towards the surface or, alternatively, the redox site of the enzyme can be 'wired' to the electrode [7,8]. The orientation of enzymes physisorbed (i.e., non-covalently bound) on an electrode depends on many factors, including the charge of the electrode. For over two decades, a common and very successful approach to control the properties of a gold interface has been to modify it with self-assembled monolayers (SAMs). The influence of different SAMs on the electrochemical properties of physisorbed enzymes have been extensively investigated (e.g. Refs. [9–11]). In many cases redox proteins and enzymes physisorb in a distribution of interconverting orientations that can be biased by the applied electrode potential [12–14]. Heterogeneity in the surface orientation of an adsorbed protein has been recognised to influence the electrochemical behaviour and various theories have been developed to account for distributions in electron transfer rates [1,15].

Although the orientation of an enzyme is known to influence the interfacial ET kinetics, it is in principle possible that the turnover number of an enzyme is reduced if substrate access to the active site is obstructed by the electrode. This consideration will be especially important if enzymes have bulky substrates (e.g. polymeric substrates) or if the substrates are encapsulated in bulky entities such as lipid vesicles (Fig. 1A). It may also become significant for smaller substrates when the enzyme is adsorbed on a nanostructured

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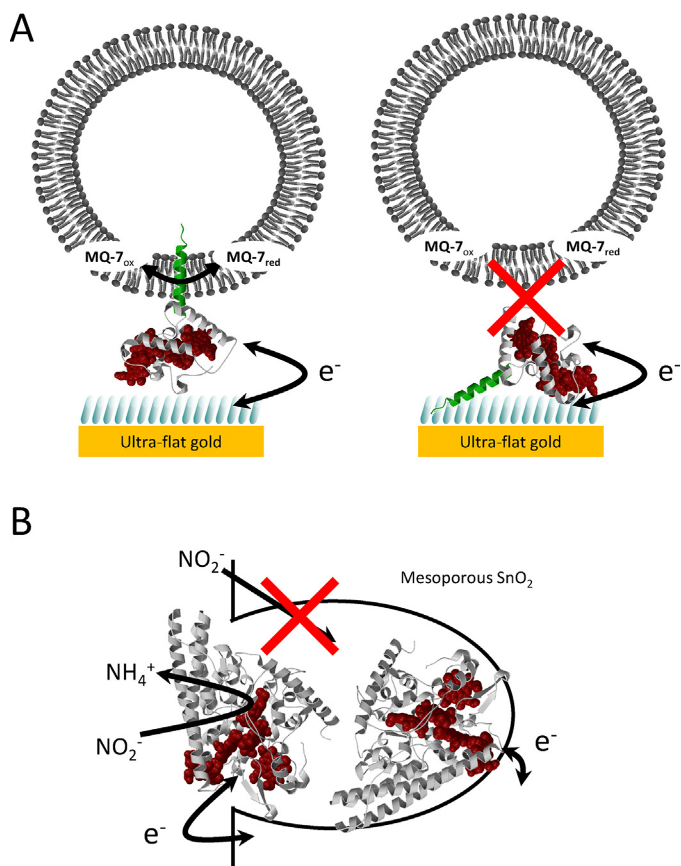

Fig. 1. Schematics of the protein film electrochemistry pursued in this work. (A) A ribbon presentation of NrfH, a homologue of CymA, immobilised on a SAM modified gold electrode together with a lipid vesicle with MQ-7. The 8OH and $8NH_3^+$ thiols in the SAM are shown as blue bars, the NrfH polypeptide in white (globular 'head' domain), green (lipophilic 'tail') and red (the prosthetic heme groups). Two possible orientations for the adsorption of CymA are illustrated. (B) A ribbon presentation of NrfA adsorbed within a mesoporous, nanocrystalline SnO_2 electrode with the polypeptide in white and hemes in red. (For interpretation of the references to color in the artwork, the reader is referred to the web version of the article.)

electrode material (Fig. 1B). Here we present PFE of two enzymes that suggests the electrode can indeed impact on the catalytic behaviour by obstructing substrate access to the active site of these enzymes.

The impact of a mesoporous electrode material on the catalytic activity of an adsorbed enzyme film was investigated through the properties of an *E. coli* periplasmic nitrite reductase, NrfA, adsorbed on mesoporous, nanocrystalline SnO_2 electrodes. NrfA contains five c-type hemes (Fig. 1B). Four of these have His/His ligation and the fifth has proximal ligation from lysine and distal ligation from water or hydroxide [16]. The lysine ligated heme is associated with the active site of NrfA where the six-electron reduction of nitrite to ammonium occurs. We have previously shown that NrfA adsorbs as an electroactive film on mesoporous, nanocrystalline SnO_2 electrodes [17,18]. Cyclic voltammetry (CV) and spectro-electrochemistry were used to define reduction potentials for the hemes in adsorbed NrfA and the values were found to be in good agreement with those displayed by the protein in solution. Catalytic reduction was reported by CV when nitrite was included in the experiment and this confirmed the functional integrity of the adsorbed enzyme. Here, we describe how the apparent nitrite reductase activity of NrfA depends on the amount of enzyme adsorbed on the SnO_2 electrode, i.e., the enzyme coverage.

The influence of enzyme orientation on the catalytic conversion of bulky substrates was studied with CymA, which is a membrane-bound tetraheme c-type cytochrome from *Shewanella oneidensis* MR-1 (Fig. 1A). CymA contains one transmembrane α-helix localised in the inner membrane and a heme-containing globular head domain facing the periplasm. Recent characterisation of CymA by electronic absorbance, magnetic circular dichroism and electron paramagnetic resonance spectroscopies identifies three low-spin bis-histidine coordinated hemes in CymA and one high-spin heme with His/H_2O coordination [19]. We have shown that CymA can be immobilised on an electrode surface where it can catalyse the reduction of hydrophobic menaquinone-7 (MQ-7) that is located in 'bulky' lipid vesicles [20]. Here, we aimed to control the orientation of the adsorbed CymA by changing the charge on a flat gold electrode modified with self-assembled monolayers (SAMs) of 8-mercapto-octanol (8OH) and 8-amino-1-octanethiol ($8NH_3^+$). Changes in the SAM can in principle affect the surface coverage, the interfacial electron transfer and also accessibility of enzyme to substrate and its rate of catalytic transformation. As a consequence we have monitored each of these properties separately for CymA using quartz-crystal microbalance with dissipation (QCM-D) and CV.

2. Methods

2.1. Studies of NrfA

NrfA purification and (spectro-)electrochemical characterisation after adsorption on SnO_2 electrodes were performed as described previously [16,18]. The optically transparent SnO_2 working electrodes were comprised of a 4 μm thick layer of SnO_2 nanoparticles annealed to a fluorine-doped tin oxide coated (TEC 15) glass slide [21]. NrfA films of different surface coverage were formed by soaking SnO_2 electrodes for different times in 22.4 μM NrfA, 50 mM Hepes, 2 mM $CaCl_2$, pH 7.0. Prior to characterising the electrodes by electronic absorption spectroscopy and CV they were rinsed extensively with 50 mM Hepes, 2 mM $CaCl_2$, pH 7.0 to remove any loosely bound material. Electrochemical experiments were performed in 50 mM Hepes, 2 mM $CaCl_2$, pH 7.0 and the desired nitrite concentration while stirring the solution at 20 °C.

2.2. Studies of CymA

The preparation of ultra-flat gold electrodes (template-stripped gold), QCM-D crystals (gold surface) and CymA have been described in detail elsewhere [19,20]. Briefly, CymA was expressed in *Shewanella oneidensis* MR-1 and purified in 0.1% *n*-dodecyl β-D-maltoside (DDM) using standard chromatography methods [19]. SAMs were formed on cleaned gold electrodes by incubating them in mixtures of 8OH and $8NH_3^+$ (total concentration 1 mM) in propanol for 16 h. It is presumed that the relative amounts of 8OH and $8NH_3^+$ in the SAM is identical to that in the solution used to make the SAM. The electrodes were incubated with either 0.1 or 0.5 μM CymA (monomer concentration) in 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 30 mM Na_2SO_4, pH 7.4 (buffer) for 20 min. The CymA-modified electrode was rinsed two times with buffer supplemented with 0.01% DDM and then three times in with buffer without DDM.

The QCM-D crystals were incubated with CymA as described in the Results section, where data of the 7th overtone are shown. The QCM-D frequency is converted to adsorbed mass using the Sauerbrey equation. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) vesicles containing 1% (w/w) MQ-7 were prepared using extrusion through 200 nm track-etched membranes and added to the electrolyte at a final concentration of 1 mg/ml POPC. After incubating the CymA-modified electrodes for 20 min, the non-surface associated vesicles are removed from the electrochemical cell by

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