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Direct electrochemistry of blue copper proteins at boron-doped diamond electrodes

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Abstract

Boron-doped diamond (BDD) is a promising electrode material for use in the spectro-electrochemical study of redox proteins and, in this investigation, cyclic voltammetry was used to obtain quasi-reversible electrochemical responses from two blue copper proteins, parsley plastocyanin and azurin from *Pseudomonas aeruginosa*. No voltammetry was observed at the virgin electrodes, but signals were observed if the electrodes were anodised, or abraded with alumina, prior to use. Plastocyanin, which has a considerable overall negative charge and a surface acidic patch which is important in forming a productive electron transfer complex with its redox partners, gave a faradaic signal at pre-treated BDD only in the presence of neomycin, a positively charged polyamine. The voltammetry of azurin, which has a small overall charge and no surface acidic patch, was obtained identically in the presence and absence of neomycin. Investigations were also carried out into the voltammetry of two site-directed mutants of azurin, M64E azurin and M44K azurin, each of which introduce a charge into the protein's surface hydrophobic patch. The oxidizing and cleaning effects of the BDD electrode pre-treatments were studied electrochemically using two inorganic probe ions, $Fe(CN)_6^{3-}$ and $Ru(NH_3)_6^{3+}$, and by X-ray photoelectron spectroscopy (XPS). All of the electrochemical results are discussed in relation to the electrostatic and hydrophobic contributions to the protein/diamond electrochemical interaction.

Keywords: Diamond; Azurin; Plastocyanin; Electrode surface treatment; Electrode surface characterisation

1. Introduction

Direct, unmediated electron transfer (ET) between a redox protein and an electrode has been achieved with many different combinations of proteins and electrode surfaces over the past 25 years (see for example [1–3] for recent reviews). The proteins studied in this manner range from small, water-soluble redox proteins, notably cytochrome *c*, blue copper proteins and ferredoxins, to large, sometimes multi-redox-centred enzymes. In contrast to protein redox titrations, direct electrochemical studies enable measurement both of redox potentials and the kinetic details of ET reactions involving protein redox centres. The field is also of particular interest with regard to the fabrication of selective biosensors based on electrochemical principles [1].

This work investigates the use of boron-doped diamond (BDD) as the electrode in the direct electron transfer to proteins. BDD is a relatively new electrode material, which offers a number of attractions in this type of application [4]. It displays a wide working potential window of around 3.5 V, because of high overpotentials for the decomposition of water, thus permitting the investigation of redox processes at extreme potentials. The background currents tend to be low as a result of the phase purity and low interfacial capacity, enabling the measurement of faradaic processes with high sensitivity. Diamond electrodes are inert chemically, and are thus resistant to poisoning by insulating deposits which can build up on more reactive electrode materials. The hydrophobicity or hydrophilicity of the electrode can be easily controlled by

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hydrogenation or oxidation of the surface, to promote interaction with active sites of proteins with different character. All of these features are particularly relevant to the problems that are commonly experienced in direct protein electrochemistry. Diamond also possesses excellent optical transparent properties from the near-UV to the far-IR making it possible to combine electrochemical measurements with UV and IR spectroscopies.

There have been two recent communications of quasi-reversible direct cyclic voltammetric signals obtained from cytochrome *c* on BDD electrodes [5,6]. The work presented here extends such studies to a different class of ET protein, the 'blue copper' proteins azurin (from *Pseudomonas aeruginosa* (*Pa*)) and plastocyanin (from parsley), which are electron transfer proteins that contain a single so-called 'type-1' or 'blue' copper site [7–9]. Many direct electrochemical and related spectroscopic studies have previously been made of plastocyanin and azurin (see, for example, Refs. [10–25]). They are thus ideal for use in the present study to assess the use of diamond electrodes for measurement of protein/electrode surface interactions.

Pa azurin has been found to associate and transfer electrons with both physiological and non-physiological ET partners via a so-called northern hydrophobic surface patch [26–29] and, in contrast to other small electron transfer proteins, displays voltammetry at both (hydrophobic) basal-plane graphite, as well as at (hydrophilic) edge-plane graphite [30]. Azurin's propensity to transfer electrons via the hydrophobic patch, along with its small overall charge at near neutral pH of −1 to −2 e (where e denotes the magnitude of the charge of an electron) [31], is thought to underlie the protein's activity at the hydrophobic basal-plane graphite electrode. It is thus of interest to establish how these factors manifest themselves at diamond electrodes, which, when terminated with chemisorbed hydrogen, are more hydrophobic than basal-plane graphite. In contrast, plastocyanin from spinach does not display a direct voltammetric signal at a basal-plane graphite electrode [30]. This is also true at a (negatively charged) edge-plane graphite electrode unless a positively charged promoter such as neomycin is added to neutralise the protein's large negative charge (roughly -7 e at neutral pH [30,31]. These findings confirm the importance of electrostatic interactions in plant plastocyanin ET. Thus *Pa* azurin and plastocyanin clearly have very different characters, raising the question of what differences are required in the preparation of the diamond electrode to accommodate these variations in protein properties.

2. Experimental materials and methods

The boron-doped diamond films used in this work were grown by hot filament chemical vapour deposition on tungsten substrates, and we have described the preparation and characterization of these electrodes in previous work [32]. In brief, the 1 mm diameter electrodes are polycrystalline in nature, with a random grain orientation, a sharply faceted morphology, a grain size in the range 5–10 μm, and a roughness of about 1 μm due to the irregular orientations of the grains. These are high-quality diamond films that display a negligible graphitic content as judged by Raman spectroscopy. In experiments where precisely defined electrode areas were required, the BDD electrodes were mounted in a heat-shrunk PTFE sheath which was sealed with insulating epoxy resin to expose a controlled electrode area of 7.9×10^{-7} m^2 to the electrolyte solution.

Electrodes were first used in their "as-grown" state, where X-ray photoelectron spectroscopy measurements indicate the diamond surface is terminated with covalently bonded hydrogen, and free of other impurities apart from weakly bonded hydrocarbons. Electrode pre-treatments were carried out to vary the surface composition. Alumina-abraded BDD electrodes were rubbed with a slurry of 1 μm alumina powder and ultrapure water supported on cotton wool. Electrodes were also polished when required with a commercial 1 μm diamond particle suspension (Kemet). Before an electrode was used, any cleaning material was removed by thorough rinsing and subsequent ultrasonication for 1 min in ultrapure water. Electrode anodisation was accomplished when required by holding the electrode at +1.24 V versus SHE in a 1 M HNO_3 solution for 60 s, followed by thorough rinsing with ultrapure water. The initial hydrogenation of bare BDD electrodes was restored when required by applying a microwave-generated hydrogen plasma (Penta Science and Technology, H_2 pressure 45 Torr, 1.2 kW power, substrate temperature ca. 600 °C) for ca. 10 min.

Cyclic voltammetry was carried out using a μ-Autolab II potentiostat (Eco-Chemie, Utrecht, The Netherlands) controlled by an IBM-compatible PC running Eco-Chemie GPES software (Version 4.7). A two-compartment glass electrochemical cell was used, comprising a reference compartment containing a saturated calomel reference electrode (Radiometer) linked via a luggin capillary to a smaller working compartment containing the BDD working electrode and a platinum wire (Advent Research Materials, 0.4 mm diameter) counter electrode. The reference compartment was filled with 0.1 M KCl, while the working compartment contained the analysed solution. All cyclic voltammetry was performed at room temperature (21 ± 1 °C). The protein solutions used were invariably between 100 and 250 μM concentration, containing 0.1 M KCl or NaCl background electrolyte, and 20 mM buffer (phosphate or HEPES) at near-neutral pH. Exact conditions are given in figure legends. The cell was enclosed in a steel mesh Faraday cage which was earthed via the potentiostat to minimize electrical noise. All scans were begun from the oxidative limit following an equilibration period of 5–30 s. Solutions were made up in ultrapure water. All chemicals were of the highest available purity and were bought from Sigma–Aldrich, except for $Ru(NH_3)_6Cl_3$ and citric acid which were bought from Alfa Aesar.

In the rotating disk electrode (RDE) experiments, an electrode rotor and its associated controller were used to spin