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Direct Electron Transfer Between Immobilised Cytochrome C and Gold Electrodes

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DIRECT ELECTRON TRANSFER BETWEEN IMMOBILISED CYTOCHROME C AND GOLD ELECTRODES.

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Abstract

In this paper presents data showing the electrochemistry of immobilised cytochrome c at an N-acetyl cysteine modified gold electrode. The electron transfer rate between the immobilised protein and the gold electrode was estimated as $3.4 \pm 1.2\ s^{-1}$ and the formal potential of the immobilised protein electrode was calculated as 2 mV vs. SCE. Studies were also carried out to investigate the nature of the protein-electrode interface.

FT-IR and scanning tunneling microscopy were used in a semi-quantitative manner in order to optimise the immobilisation procedure, and thereby maximise the surface coverage of the electrode. FT-IR studies showed that the protein-electrode exhibited characteristic peak absorptions at 1703 cm^{-1}, 1494 cm^{-1}, 1367 cm^{-1} and 1166 cm^{-1}. The peaks at 1703 cm^{-1} and 1494 cm^{-1} were attributed to amide I and II bands (respectively). The 1367 cm^{-1} and 1166 cm^{-1} peaks were associated with the amide III band of the immobilised protein.

The application of this electrode was investigated as a biosensor for the determination of superoxide ion production.

INTRODUCTION

A ring of positively charged lysines, which extend away from the surface of cytochrome c, can form salt bridges with negatively charged carboxylate groups e.g. aspartate residues of cytochrome c peroxidase. The electrostatic interaction between these two proteins draws them together such that their two haem groups are only 25 Å apart, thus facilitating electron transfer (1).

This biophysical model has previously been adapted to study electrochemical reactions of *soluble* proteins at modified electrodes. Hill *et al.* have demonstrated direct electron transfer between soluble cytochrome c and negatively charged modified gold electrodes (2). More recently, this approach has been used to study the electrochemistry of adsorbed cytochrome c at self-assembled alkanethiol monolayers on gold electrodes, although extremely slow electron transfer rates were measured (3).

In this paper, we make use of this established charge matching between cytochrome c and modified electrodes, but immobilise the protein at the surface with the aim of making a 'solid-state' bioelectronic interface. The significance of achieving direct electron transfer between an *immobilised* protein and an electrode is that there is potential for developing devices with applications either in molecular electronics, or molecular sensors.

To this end, cytochrome c was immobilised at an N-acetyl cysteine modified electrode using a 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) initiated condensation reaction. The direct electrochemistry of the immobilised protein was studied and preliminary results towards the future application of the electrode as a biosensor for the measurement of *in vivo* production of superoxide radicals were obtained.

EXPERIMENTAL

Horse heart cytochrome c was re-purified by CM 32-Sephadex cation exchange chromatography, and was dialysed exhaustively against 10 mM sodium phosphate buffer, pH 7.0. Prior to each experiment, gold electrodes (2 mm diameter) were cleaned using a 0.3 μm alumina slurry and were sonicated in distilled water for 30 seconds. The electrodes were modified by immersion in a 2 mM solution of N-acetyl cysteine in 10 mM phosphate buffer, pH 7.0 for 2 hours and were then washed in distilled water.

Cytochrome c was immobilised at the modified electrodes using the water soluble condensing reagent, EDC. The N-acetyl cysteine modified electrode was first incubated in a 10% (w/v) solution of EDC in water for 2 hours at 25°C, and was then washed in water. The EDC activated electrode was placed in a solution of cytochrome c (4.2 g l^{-1}) in 10 mM sodium phosphate buffer, pH 7.0 at 4°C for six hours. The electrode was finally washed exhaustively with 10 mM sodium phosphate buffer, pH 7.0.

The electrochemistry of the immobilised protein was investigated using a conventional two compartment, three-electrode system with a saturated calomel electrode (SCE) as reference, a platinum gauze counter electrode and the modified gold working electrode. Electron transfer reactions were investigated using cyclic voltammetry over the potential range -150 to +100mV vs SCE at scan rates between 5 and 50mV s^{-1}.

FT-IR was performed on cytochrome c immobilised onto evaporated gold (100 nm) on a silicon substrate using a Bomem MB 120 Infra-red spectrophotometer. The whole system was purged with nitrogen for 20 minutes prior to scanning, in order to reduce interference from both CO_2 and water vapour. Measurements were made between 500 and 4000 cm^{-1}

STM images showing the coverage of cytochrome c on the surface of the gold electrodes were obtained using a WA Technology STM. The scanning tip was made by electrolytically etching 0.5 mm tungsten wire in 2 M KOH. Images were taken in the constant current mode.

Electroanalytical experiments for the detection of the superoxide anion were performed using a purpose built four electrode electrochemical cell and bi-potentiostat, as described previously (4). The electrochemical cell contained a gold working and background electrode, a platinum counter electrode and a Ag/AgCl reference electrode.

The working gold electrode was modified with immobilised cytochrome c, as described above, whilst the background electrode was modified using the same methods but with a solution of 5 g l^{-1} human serum albumin, in place of the cytochrome c. Both the working electrode and the background electrode were poised at +200 mV vs. Ag/AgCl. Superoxide was produced using a xanthine/ xanthine oxidase generating system. The reaction was initiated by the addition of xanthine oxidase to give a final enzyme concentration in the range 0 - 0.48 μM. The response at the electrode was measured as the initial (linear) rate of current generated by the re-oxidation of reduced cytochrome c at the immobilised electrode.

RESULTS AND DISCUSSION

The Electrochemistry of Immobilised Cytochrome c.

The modification of the working electrode by N-acetyl cysteine occurs through the formation of a strong gold-thiol bond, leaving an exposed carboxylate group, and an acetyl-protected amino group extending away from the electrode. Cytochrome c was immobilised at the gold modified electrode using a carbodiimide initiated condensation procedure. After covalent attachment, the electrode was thoroughly washed and the electrochemistry of the immobilised protein was investigated. It was observed that maximum peak currents, $i_{p,a}$ and $i_{p,c}$ were obtained in sodium phosphate buffer at pH 7.0.

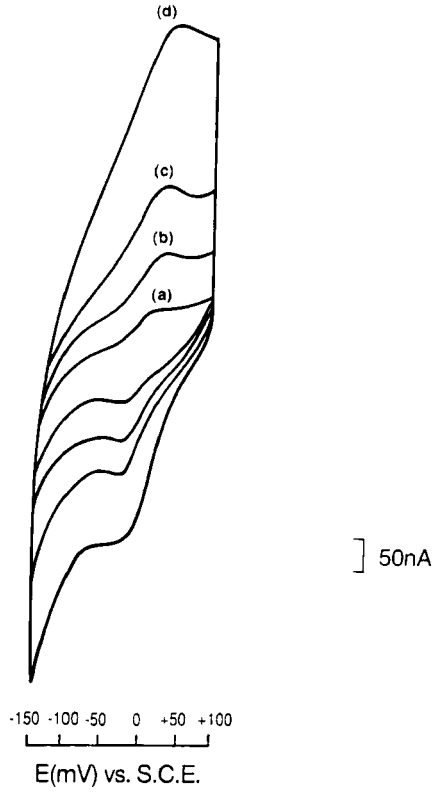

Figure 1: Cyclic voltammogram of immobilised cytcohrome c at an N-acetyl cysteine modified gold electrode (see text for details).

Figure 1 (above) shows cyclic voltammograms at the immobilised cytochrome c N-acetyl cysteine electrode in 10 mM sodium phosphate buffer pH 7.0 at scan rates at (a) 5, (b) 10, (c) 20 and (d) 50 mV s^{-1}. The formal potential of the cytochrome c at the electrode (E'^{O}) was calculated using the Laviron model as +2 mV (5). This value was 23 mV negative of the $E_{1/2}$ of cytochrome c at the same modified electrode in solution, but was in close agreement with the value for cytochrome c bound to tin oxide electrodes (6).

Previously, electrochemical rate constants (k_{et}) have been estimated from surface voltammetric experiments for simple reversible redox systems (5). These methods have also been applied to quasi-reversible protein electrochemistry (3), although, in practice, the model does not account for any departure from the ideal wave behaviour predicted at a Langmuir isotherm. The voltammograms which we have obtained exhibit quasi-reversible electrochemistry, with an increasing ΔEp with increasing scan rate. Under the limiting case $\Delta Ep < 200/n$ mV, k_{et} was estimated as 3.4 ±1.2 s^{-1}. These results compare with $k_{et} = 30\ s^{-1}$, obtained using a diffusionless analysis for soluble cytochrome c at bipyridyl modified gold electrode (2) and $k_{et} = 0.1\ s^{-1}$, obtained at alkanethiol modified gold electrodes (3).

FT-IR and STM Analysis of Immobilised Cytochrome c.

All absorbance spectra showed six peaks observed after cytochrome c was immobilised at the gold surface. There was a peak at 2978 cm^{-1}, which was attributed to C-H stretches, as well as a minor absorbance peak at 2350 cm^{-1} which was assigned to residual atmospheric CO_2. In addition, there were four major peaks at 1703 cm^{-1}, 1494 cm^{-1}, 1367 cm^{-1} and 1166 cm^{-1}. The peaks at 1703 cm^{-1} and 1494 cm^{-1} were attributed to amide I and II bands (respectively) of the immobilised protein. The 1367 cm^{-1} and 1166 cm^{-1} peaks were associated with the amide III band. The high background absorbance suggested that cytochrome c did not form a monolayer.

This observation was supported by images of immobilised cytochrome c obtained using STM, which showed clearly that large amounts of protein were immobilised on the gold to give total coverage of the surface (7).

The Development of a Superoxide Biosensor.

Superoxide radicals (O_2^-) are highly reactive species which are generated by the partial ($1e^-$) reduction of O_2. In aqueous solution, their redox capability has been attributed to the perpetuation of inflammation and tissue damage

in disease states, including rheumatoid arthritis (8). The amperometric assay described in this paper is an adaptation of an electrochemical assay using soluble cytochrome c (9), but is based upon the O_2^- specific reduction of *immobilised* cytochrome c, and its subsequent re-oxidation at the electrode surface at +200 mV vs. SCE. O_2^- was produced using a xanthine/xanthine oxidase generating system. Results show a linear calibration for the rate of production of O_2^- by xanthine oxidase (XOD) in the presence of saturating xanthine over the range 0-0.48 μM XOD according to the relationship $y = 2.2\ \mu A\ min^{-1}\ cm^{-2}\ \mu M + 0.01\ \mu A\ cm^{-2}\ min^{-1}$, $r = 0.99$.

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