Reversible Increase in the Redox Potential of Cytochrome c in Methanol

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The E° of cytochrome c on a self-assembled monolayer (SAM) modified gold electrode increased by 300 mV immediately on immersion in methanol. On re-immersion of the electrode in aqueous buffer the original faradaic response was restored, but over a period of 120 min, indicating that methanol causes a significant change in conformation/orientation of the protein.

Much effort has been devoted to understanding the electrochemical properties of redox proteins and enzymes. For applications such as biosensors and biofuel cells, it is desirable to have direct and fast electron transfer kinetics between the electrode and the biological component of interest. However, the rate of direct electron transfer (DET) may be inherently slow due to a range of factors including: inaccessibility of the electroactive group in the protein structure; adsorption of the protein in a manner which does not promote fast electron transfer or denaturation of the protein. Modified electrodes can enhance the rate of electron transfer, and provide the opportunity to conduct detailed thermodynamic and kinetic studies. The use of self-assembled monolayers on electrodes to promote electron transfer between the redox protein of interest and the electrode has been widely used.

Factors that influence the E° of haem proteins include the nature of the axial ligands; the polarity of the haem environment and the extent of haem surface exposure. Despite numerous studies, the full range and effects of the factors that contribute to E° and their relative contribution has not yet been determined. While the majority of enzyme use occurs in aqueous media e.g. glucose analysis, enzymes can also catalyse reactions in nonaqueous media and can be used in synthetic reactions. However, there have been relatively few studies of the electrochemical properties of enzymes in organic media. Cyt c is one of the most extensively studied redox proteins and has been widely used as a model for larger, more complex systems. The reduction of the immobilised haem fragment of cytochrome c, microperoxidase-11, has been examined in aqueous buffer and glycerol. The increase of 30 mV in E° in glycerol was dominated by entropic changes. Immobilised cytochrome c showed a smaller increase (7 mV) in E° on immersion in glycerol, however the change was dominated by enthalpic changes. Faradaic responses were observed for MP-11 in acetoinitrile and ethanol, with increases of 60 and 64 mV in E°, respectively. The reduction of cyt c in solution was examined in a range of mixed solvents, with reversible responses (E° ranging from 199 – 274 mV vs SHE) obtained in methanol, acetonitrile, DMF and DMSO. Here we describe the direct electrochemical response of cyt c in methanol at a SAM modified gold electrode. The E° increased immediately on immersion in methanol, returning to the original value in aqueous solution, but on a much longer time scale, indicating that methanol causes a significant, reversible perturbation to the conformation and/or orientation of the protein at the surface of the electrode.

The electrochemical response of cyt c was examined at a mixed SAM (HS(CH2)10COOH/HS(CH2)3OH), which has been previously shown to provide faster and more reversible kinetics than a C12COOH SAM. In aqueous buffer (10 mM phosphate, pH 7.0) E° (234 ± 3 mV vs. SHE) and ΔEp were found to be in good agreement with literature values (Fig 1A). Plots of ipa and ipc vs. scan rate (ν) were linear, indicating that cyt c was immobilised on the electrode surface.

Cyt c, like the vast majority of proteins, is insoluble in organic solvents. Cyt c modified electrodes were placed in solutions of methanol containing 3% (v/v) added buffer, the minimum amount of water required to observe a faradaic response (no faradaic current was observed in the absence of added water). On immersion of the electrode in methanol, a significant increase in E° of 302 mV was immediately observed (Fig 1B), with the response being quasireversible (ipa/ipc = 1.77 and ΔEp = 144 mV). Plots of ipa and ipc vs. scan rate (ν) were linear. The increase in E° observed here is substantially larger that the values reported for immobilised MP-11 and cyt c in glycerol. A significant increase in the oxidation peak potential of cyt c adsorbed onto TiO2 modified SnO2 electrodes occurred in 95% acetonitrile (800 mV vs Ag/AgCl); however the process was irreversible.

When cyt c modified electrodes were removed from methanol and placed in buffer solution, no faradaic response was immediately evident over the potential range expected. A faradaic response developed over time, on immersion of the electrode in aqueous solution. The original response restored in 120 ± 20 min (Fig 2). The reappearance of the faradaic response followed zero order kinetics with a rate constant of 6.2 x 10⁻³ ± 1.2 x 10⁻³ pmol cm⁻² min⁻¹. Compton has previously reported that the faradaic response of cyt c modified electrodes disappeared on exposure to high salt concentrations, reappearing in buffer on a shorter time scale of 31 ± 14 min.

The loss of faradaic response of redox proteins frequently arises from desorption or denaturation of the protein at the electrode surface. It is not likely that cyt c is desorbed from the electrode surface as proteins are not soluble in nonaqueous media. While denaturation of the protein is possible, if it does occur it appears to be reversible as the faradaic response is restored on re-immersion in buffer. To examine this change...
further, experiments were performed in solutions containing urea (Fig 3A) and as a function of pH (Fig 3B). There was a gradual decrease in the peak currents as the concentration of urea was increased. In 5 M urea, there was a complete loss of response, which was not reversed on immediate re-immersion in aqueous buffer solution and did not appear after 120 min. ΔEp increased slightly (from 30 ± 3 mV to 38 ± 2 mV) in 1M and 3M urea solutions but no change in Eº was observed. At pH 3.0 there was a complete loss of the faradic response, a change that was partially reversed upon replacing the electrodes in pH 7.5 aqueous buffer. Both ΔEp and Eº were unchanged. In urea and in solutions at varying pH, no faradic currents were observed on scanning over a wider potential range i.e. 400-1000 mV. Urea is a known protein denaturant and in this case produces an irreversible change in the faradic response, while pH induces partially reversible changes. Methanol causes a different reversible change to cyt c. To investigate this further, the thermodynamics of reduction of cyt c were determined in aqueous buffer and methanol (Table 1).22 The observed increase in Eº arises from a significant increase in ΔSº which is counteracted by a large increase in ΔHº. The values of both ΔHº and ΔSº in aqueous solution compare well with previous reports on immobilised cyt c.22 The enthalpic effect is thought to be mainly related to the stabilisation of the Feº' state by ligand binding interactions (in particular the thioether sulfur ligation of the axial methionine), to the hydrophobic environment of the haem, and to the accessibility of the haem to the solvent. From the observed increase in ΔHº, the Feº' form of cyt c is of the reduced protein (metal ligation), and to the accessibility of the haem to the solvent. From the observed increase in ΔHº, the Feº' form of cyt c is preferentially stabilised in methanol, a stabilisation which may reflect the displacement of methionine from the stabilisation of the Feº' state by ligand binding interactions (in particular the thioether sulfur ligation of the axial methionine). From the observed increase in ΔHº, the Feº' form of cyt c is preferentially stabilised in methanol, a stabilisation which may reflect the displacement of methionine from

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Eº'(mV)</th>
<th>ΔEp (mV)</th>
<th>ΔHº (kJ mol⁻¹)</th>
<th>ΔSº (J K⁻¹ mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>234</td>
<td>39</td>
<td>-37.5±1.4</td>
<td>-33.6±1.9</td>
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<tr>
<td>97% MeOH</td>
<td>536</td>
<td>144</td>
<td>-7.3±0.2</td>
<td>44.9±8.9</td>
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*Eº' is reported vs SHE*

**Table 1** Thermodynamic data for cyt c modified electrodes in aqueous buffer and MeOH

**Notes and references**

Fig 1. Cyclic voltammogram of cyt c at C$_{10}$COOH/C$_7$OH gold electrodes in (A) 10 mM phosphate buffer, pH 7.0 and (B) 97% methanol ($\nu = 100$ mVs$^{-1}$).

Fig 2. Cyclic voltammograms of cyt c at C$_{10}$COOH/C$_7$OH gold electrodes in: aqueous buffer solution (---), 97% MeOH (---), buffer solution at $t = 20$ mins after exposure to methanol (---); and buffer solution at $t = 120$ mins after exposure to methanol (---). The inset shows the increase in cyt c redox surface coverage as a function of time after exposure to 97% methanol.

Fig 3. Cyclic voltammograms of cyt c at C$_{10}$COOH/C$_7$OH gold electrodes in aqueous buffer solution (A) 10 mM phosphate buffer, pH 7.0 (---), 1 M (---), 3 M (---) and 5 M (---) urea ($\nu = 100$ mVs$^{-1}$) and (B) 10 mM phosphate buffer, pH 7.5 (---) pH 3.0 (---) and on re-immersion in pH 7.5 (---) ($\nu = 100$ mVs$^{-1}$).