Electrochemistry of Cytochrome *c* at the Liquid−Liquid Interface

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The potential-controlled electron-transfer reaction between cytochrome *c* and 1,1′-dimethylferrocene at a liquid−liquid interface is reported. In this new approach to protein electrochemistry, the electron transfer process is apparently transport controlled, rather than adsorption limited. Furthermore, electron transfer at the liquid−liquid interface more closely resembles the situation of the protein in vivo.

Introduction

Cytochrome *c* is one of most important and extensively studied electron transfer proteins, partly because of its high solubility in water compared with other redox-active proteins. In vivo, cytochrome *c* transfers an electron from complex III to complex IV, membrane-bound components of the mitochondrial electron-transfer chain.[1] The electrochemical interrogation of cytochrome *c* has, however, been hindered because the redox-active heme center is buried beneath the surface of the protein.[2] This difficulty has been overcome by the introduction of modified electrode surfaces, with monolayers able to interact with both the heme center and the underlying electrode. Following the observation of reversible electron transfer,[3] a variety of functional groups have been employed to interrogate the electrochemical response of cytochrome *c*,[4,5] providing the platform for further research into the structure−function relationship of the protein[6] and the electrochemical characterization of monolayer covered electrodes.[7] Direct electron transfer to cytochrome *c* can also be achieved by using electrode materials such as oxides.[8] Alternatively, direct electrochemical studies of redox-active proteins can be made using the protein-film voltammetry approach, pioneered by Armstrong and co-workers, where proteins are adsorbed on rough hydrophilic surfaces such as edge-plane pyrolytic graphite.[9,10]

The interface between two immiscible electrolyte solutions (ITIES) has been proposed as a model for biological membranes.[11] Polarization of the ITIES can drive interfacial charge transfer,[12] thus electron transfer between hydrophilic and hydrophobic redox couples (denoted 1 and 2, respectively) located on either side of the ITIES can be induced:

$$O_{1(aq)} + R_{2(org)} \rightleftharpoons R_{1(aq)} + O_{2(org)}$$

As has been noted previously,[13] interfacial electron transfer across the ITIES represents an intermediate case between direct electron transfer at the electrode/electrolyte interface and homogeneous electron transfer in the solution phase. An extension of ITIES electron transfer would be the use of a redox-active protein as one of the electron-transfer components (i.e., either 1 or 2 above). Given the level of interest in cytochrome *c* and its hydrophilic nature, we report the initial electrochemical study of cytochrome *c* at the liquid−liquid interface in this letter. We believe this work ought to provide further insight into the electrochemical behavior of the protein, given its location in vivo.[1]

Electron transfer at the ITIES has been reported previously for molecules such as quinones and ascorbate that are involved in biological electron transfer[14,15] and recent studies using the scanning electrochemical microscope (SECM) have highlighted the use of liquid−liquid electrochemical techniques as probes of biological systems.[16,17] The SECM has also been employed as an indirect probe of electron transfer between the aqueous phase enzyme glucose oxidase and an organic phase electron donor, 1,1′-dimethylferrocene ($DMFeCp_2$),[18] although the direct measurement of electron transfer across the ITIES involving proteins has not been reported hitherto.

Experimental Section

Reagents. The electrochemical cell employed (cell 1) had the general composition:

$$Ag_{(s)}|AgTPB_{(s)}|BTPPA\ TPB\ (0.02\ M)_{(DCE)}||NaCl\ (0.1\ M),\ Na_2B_4O_7\ (0.0125\ M),\ NaOH\ (0.088\ M)_{(aq)}|AgCl_{(s)}|Ag_{(s)}$$

where the double bar corresponds to the polarized interface and DCE denotes the organic solvent, 1,2-dichloroethane (HPLC grade, supplied by Aldrich Corp, Gillingham, U.K.). Water was obtained from a Milli-Q purification system (Millipore, Watford, U.K.). Horse heart cytochrome *c* (Sigma, Poole, UK) and $DMFeCp_2$ (Aldrich Corp.) were added to the aqueous and DCE phases, respectively, as appropriate. Bis(triphenylphosphoranylidene) tetraphenylborate (BTPPA TPB), synthesized according to a literature procedure,[19] was employed as the organic electrolyte with the borate buffer as the aqueous electrolyte.

Instrumentation. A glass cell of 1.2 cm internal diameter was employed, with Pt gauze as the counter electrode in each phase. All potentials quoted for ITIES experiments are versus the reference electrodes of the above cell. Voltammetry was performed using an Autolab PGSTAT 30 potentiostat (Ecochemie BV, Utrecht, Netherlands). Spectrophotometric experiments were performed using a UV-2101PC spectrophotometer (Shimadzu Inc., Columbia, MD).

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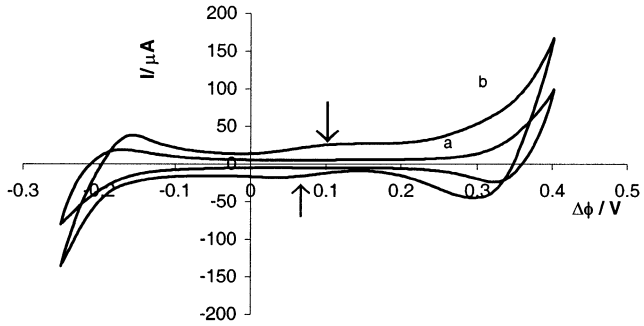

Figure 1. Cyclic voltammetry of (a) cell 1 with 0.02 M $DMFeCp_2$ added to the organic phase, (b) the cell 1 with 0.02 M $DMFeCp_2$ added to the organic phase and 4×10^{-4} M ferri-cytochrome *c* present in the aqueous phase. Voltage scan rate was 0.05 Vs^{-1}. The arrows denote the electron-transfer process.

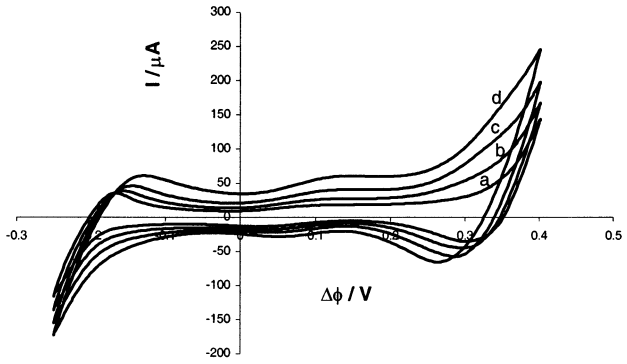

Figure 2. Cyclic voltammetry of cell 1, with an aqueous phase concentration of ferri-cytochrome *c* of 4×10^{-4} M, and 0.02 M $DMFeCp_2$ present in the organic phase. The voltage scan rates are (a) 0.02 V s^{-1}, (b) 0.05 V s^{-1}, (c) 0.10 V s^{-1}, and (d) 0.20 V s^{-1}.

Results

Figure 1a shows the voltammetric response obtained from cell 1 with 0.02 M $DMFeCp_2$ added to the DCE phase. At the extreme of the potentials applied, the current flow is due to the transfer of electrolyte across the polarized ITIES.[12] Between the extremes, the existence of the potential window demonstrates that none of the cell components undergo electron or ion transfer within this region. The addition of cytochrome *c* to cell 1 in the absence of $DMFeCp_2$ also demonstrated that no charge transfer occurred within the potential window (not shown).

The initial oxidation state of the protein was confirmed from the UV−vis spectrum, by recording the absorption peak centered on 540 nm. In its oxidized state, cytochrome *c* shows a single peak, but a split peak is seen for the reduced protein.[20] The spectra of the cytochrome *c*, following dialyzes with potassium ferricyanide and potassium ferrocyanide, respectively, clearly demonstrated that the oxidized and reduced forms of the protein predominated in each case. Additionally, the cytochrome *c*, as purchased, was confirmed to be in an initially reduced state.

No charge transfer was observed for voltammetry at the ITIES when reduced cytochrome *c* was present in the aqueous phase of cell 1, with $DMFeCp_2$ added to the organic phase. On replacement of the reduced cytochrome *c* with its oxidized form, a charge transfer was seen on both the forward and reverse scans, with the peak on the forward scan attributed to the heterogeneous transfer of an electron from the $DMFeCp_2$ to the cytochrome *c*, Figure 1b. The separation between the new voltammetric features was approximately 0.07 V, and the midpoint potential was 0.08 V. UV−visible absorption spectroscopy confirmed that the reaction between the cytochrome *c* in the aqueous phase and the $DMFeCp_2$ in the organic phase was not spontaneous, since the protein remained in the oxidized state over a 24 h period where no interfacial potential difference was applied. In contrast, confirmation that interfacial polarization (at +0.2 V) for 24 h led to reduction of the protein was obtained from the associated appearance of the split peak, centered at 540 nm, in the UV−vis spectrum. Despite the ubiquity of ferrocene as an electron donor, reports of electron transfer between ferrocene derivatives and cytochrome *c* are scarce because of the lipophilic nature of the former and hydrophilic nature of the protein. Kuwana and co-workers have, however, reported electron transfer between micelle-solubilized ferrocene and cytochrome *c*.[22,23]

The absorption peak in the UV−visible spectrum at 410 nm is due entirely to the heme moiety, hence the actual aqueous phase concentration of the protein was calculated to be 4×10^{-4} M (using ϵ_o at 415 nm = 109500 M^{-1} cm^{-1}).[20] The data in Figure 2 indicates that the charge transfer is diffusion limited, since the currents observed were found to depend linearly on the square root of voltage scan rate, in accord with the Randles−Sevčik equation.[21] Combining this analysis with the concentration derived from the spectro-photometry gave an aqueous phase diffusion coefficient for cytochrome *c* of 1.2 (± 0.5) $\times 10^{-6}$ cm^2 s^{-1}. The Randles−Sevčik analysis is only strictly valid where both forms of the organic redox couple are in excess. Fulfillment of this condition was attempted by the prior, partial oxidation of the $DMFeCp_2$: the resultant voltammetry was, however, complicated by an apparent coupling between the electron-transfer process and the transfer of the dimethylferricenium cation (vide infra). Stewart et al have performed numerical integrations to obtain the simulated cyclic voltammetric response for electron transfer at the ITIES for various concentration ratios.[24] For the particular case employed in this series of experiments (excess organic phase species all in reduced form, aqueous phase species all in oxidized form) a square root dependence of peak current on scan rate can still be obtained, although a peak current separation exceeding 0.06 V ensues. The analysis by Stewart et al cannot be compared directly to the experiments reported here since the numerical simulations assumed that the diffusion coefficients of all species were equal. However, the simulations showed that the peak current function tended to a limiting value, which was around 85% of the value from the classical Randles−Sevčik/Nicholson−Shain analysis.[21,24] Use of this current function increases the effective diffusion coefficient of the protein to 1.5 (± 0.5) $\times 10^{-6}$ cm^2 s^{-1}. Much of the uncertainty in the diffusion coefficient values quoted thus derives from the extent of applicability of the mass-transport model. The experimental diffusion coefficients compare with values from the literature ranging from 0.5×10^{-6} to 1.5×10^{-6} cm^2 s^{-1}.[8,20,25]

Prior oxidation of the organic phase, at a platinum disk electrode, generated a 3.4×10^{-4} M solution of the dimethylferricenium cation. Introduction of this solution to the organic phase of cell 1 gave a charge-transfer process at ca. −0.1 V, in the absence of any aqueous phase electron acceptor, Figure 3. This process is attributed to the transfer of the electro-generated dimethylferricenium ion from DCE to water: Randles−Sevčik analysis[21] of the ion transfer data gave a diffusion coefficient value of 2.8×10^{-6} cm^2 s^{-1} in agreement with the value reported for this species in DCE.[26] Introduction of oxidized cytochrome *c* to the aqueous phase of cell 1, in the presence of preoxidized $DMFeCp_2$ (1×10^{-4} M), gave the voltammetric response shown in Figure 4a. Comparison of the cyclic voltammogram with that obtained with no dimethylferricenium

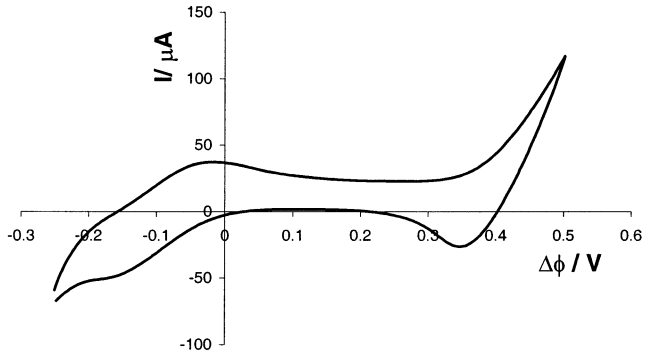

Figure 3. Cyclic voltammogram of cell 1, with the dimethylferricenium cation (3.4×10^{-4} M) added to the organic phase, recorded at a scan rate of 0.05 V s^{-1}.

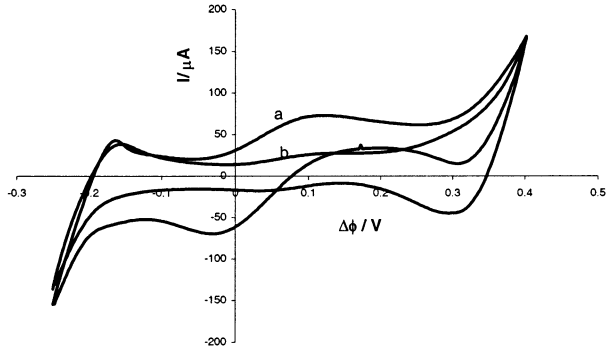

Figure 4. Cyclic voltammograms of cell 1 with 4×10^{-4} M ferricytochrome *c* in the aqueous phase, (a) in the presence of, and (b) in the absence of, 1×10^{-4} M dimethylferricenium ion in the organic phase. Scan rate employed in both cases is 0.05 V s^{-1}.

cation initially present (Figure 4b) shows that the two charge-transfer processes have merged into a single voltammetric feature, with an enhanced current. This observation suggests that there may be a coupling between the ion and electron-transfer processes, as found in biological systems.[26,27]

Finally, comparison of the midpoint potential, for the electron transfer observed at the ITIES between $DMFeCp_2$ and cytochrome *c*, and the corresponding value reported for $DMFeCp_2$ and ferricyanide ions[26] reveals that the former is ca. 0.08 V positive of the latter. This shift compares with the difference in standard reduction potential values of 0.085 V for ferricyanide,[21] compared to cytochrome *c*.[25]

Conclusion

Electrochemical and associated spectrophotometric measurements have demonstrated that direct electron transfer, between cytochrome *c* and a ferrocene derivative, occurs at the polarized liquid−liquid interface. This heterogeneous charge transfer is analogous to the role of cytochrome *c* in vivo.[1] The ability to induce such processes using voltammetry at the ITIES is of significance. The influences of driving force and adsorption on protein charge transfer at the ITIES are presently being evaluated. The ITIES approach represents an alternative method to study protein electron transfer, complementary to existing approaches,[2,8−10] but which may offer additional information because of its resemblance to electron transfer in vivo. Importantly, the extension to systems involving protein−protein electron transfer[28] (e.g., with aqueous phase cytochrome *c* and organic phase cytochrome *c* oxidase) is readily envisaged, with the variation in driving force accessible via the interfacial potential being used as a probe of such processes.

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