Specific Ion effects on the Electrochemical Properties of Cytochrome c

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

The range of salts used as supporting electrolytes in electrochemical studies of redox proteins and enzymes varies widely, with the choice of electrolyte relying on the assumption that the electrolyte used does not affect the electrochemical properties of the proteins and enzymes under investigation. Examination of the electrochemical properties of the redox protein cytochrome c (cyt c) at a 4,4′-bipyridyl modified gold electrode demonstrates that both the redox potential (E°) and the faradaic current are influenced by the nature of the electrolyte used, in a manner explained primarily by Hofmeister effects. The faradaic peak currents display an atypical trend on switching from kosmotropic to chaotropic anions, with a maximum current observed in the presence of Cl⁻. For a series of cations, the peak current increased in the sequence: Li⁺ (0.34 μA)<guanidinium⁺ (0.36 μA)<Na⁺ (0.37 μA)<K⁺ (0.38 μA)<Cs⁺ (0.40 μA), and for anions decreased in the sequence: Cl⁻ (0.37 μA)> Br⁻ (0.35 μA)> ClO₄⁻ (0.35 μA)> SCN⁻ (0.31 μA)> F⁻ (0.30 μA). E° decreased by a total of 24 mV across the series F⁻ > Cl⁻ > Br⁻ > ClO₄⁻ > SCN⁻ whereas no specific ion effect on E° was observed for cations. Factorisation of E° into its enthalpic and entropic components showed that while no specific trends were observed, large changes in ΔH° and ΔS° occurred with individual ions. The effect of anions on the faradaic peak current can be qualitatively explained by considering Collins’ empirical rule of ‘matching water affinities’. The effect of cations can not be explained by this rule. However, both anion and cation effects can be understood by taking into account the cooperative action of electrostatic and ion dispersion forces. The results demonstrate that the choice of supporting electrolyte in electrochemical investigations of redox proteins is important and emphasize that care needs to be taken in the determination and comparison of E°, ΔH° and ΔS° in different solutions.

1. Introduction

Understanding of the electrochemical properties of redox enzymes is intrinsic to their successful utilization as biocatalysts, biofuel cells or biosensors. Cytochrome c (cyt c) is an electron transport protein comprised of a heme group and a 104 amino acid residue organized into a series of five α-helices and six β-turns. It is one of the most extensively studied redox proteins and has been widely used as a model to study electron transfer in proteins. The protein contains a number of lysine residues clustered around the heme edge of the protein which allow the protein to dock with the negatively charged groups of its redox partners such as cytochrome c oxidase and cytochrome c peroxidase, a feature that has been exploited to probe the electrochemical properties of cyt c.

The charge distribution on the protein is heterogeneous, resulting in dipole moments of 308 and 325 D for the reduced and oxidized protein, respectively, at neutral pH. The relatively high value of E°, (258 mV vs. SHE (standard hydrogen electrode)), arises in part from the π-electron-acceptor character of the thioether sulfur atom of the axially bound methionine to iron, which preferentially stabilizes the ferrous state. This selective stabilization is further enhanced by the poor accessibility of the heme to solvent and burial of the heme within a hydrophobic pocket. Binding of the protein to the surface of an electrode surface prior to electron transfer can be promoted by a range of modifiers. A detailed investigation of the mechanism of reduction of cyt c at a 4,4′-bipyridyl modified gold electrode demonstrated that the binding step provides approximately half of the activation free energy for electron transfer and is a crucial factor in the enhancement of the rate of electron transfer. 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. Insight into the correlation between E° and structural properties of redox proteins can be obtained from the factorization of the enthalpic (ΔH°) and entropic (ΔS°)
components (eq. 1). Ligand-heme interactions dominate the enthalpic term, with the methionine ligand stabilizing the ferroheme. Solvent reorganization effects and the more compact structure of the ferroheme form contribute to the entropic term.  

\[ E^\circ = \frac{-\Delta H^\circ}{nF} + \frac{T\Delta S^\circ}{nF} \]  

(1)

Electrochemical measurements require the presence of an electrolyte whose sole function is ideally that of charge transport. Typical supporting electrolytes for cyt c electrochemical measurements are NaClO₄, KNO₃, or mixtures of salts (i.e. Na₂SO₄, KClO₄ and KCl). However, the nature of the ions present in a solution can affect the properties of an enzyme, including properties such as protein folding and enzymatic activity. The effect of the ions can follow the Hofmeister series which lists the ions in order of their relative effects on the properties of the protein. Such effects were first described by Hofmeister who observed that the nature of the salt used affected the solubility of egg-white proteins in aqueous solutions. The efficiency of salts in promoting protein precipitation was found to be:

- Anions: SO₄²⁻ > F⁻ > Cl⁻ > NO₃⁻ > Br⁻ > I⁻ > ClO₄⁻ > SCN⁻
- Cations: Cs⁺ > NH₄⁺ > K⁺ > Na⁺ > Li⁺ > Mg²⁺

Hofmeister 'specific ion effects' are ubiquitous in biology, chemistry and physics. In addition to protein precipitation, properties such as enzymatic activities, aggregation behavior of triblock copolymers, neutral lipid membrane interactions, anion affinities at the air-water interface, asymmetric partitioning of anions in lysozyme dispersions, protein adsorption on mesoporous materials, pH of buffers, are all ion specific.

It has been proposed that the ordering of the ions arises from changes in the hydrogen-bonding network of water in bulk solution as a result of electrostatic effects which depend on the charged nature of the ions. In this approach, ion specificity is associated with electrolyte induced changes in water structure that depend on the capacity of ions to form (kosmotropic ions), or to break (chaotropic ions) hydrogen bonds. Such correlations arise from a theoretical framework that includes only electrostatic and hydration forces between ions and water. However this theory cannot satisfactorily explain the range of effects that have been observed and recent data indicate that hydrogen-bonding networks in aqueous solutions are unaffected by the addition of different anions.

In more recent approaches, it has been shown that standard theories are deficient in that they omit non-electrostatic, ion specific electrodynamic fluctuation (dispersion) forces. The DLVO description of forces between colloidal particles can be separated into two types; an electrostatic, double layer component due to an inhomogeneous profile of ions at the interface and opposing attractive quantum mechanical forces which are described by van der Waals-Hamaker interactions. The latter ignores the ion profiles and the specific dispersion forces acting on ions. The ion specific dispersion potentials acting on ions can be included at the same level as electrostatic forces within a Poisson-Boltzmann description. Within this approach electrostatic and dispersion forces combine to induce structuring of the local water molecules around ions (kosmotropy, chaotropy). The interactions, long and short range, between such "modified" ions are reflected in bulk properties such as activity. The key parameter of this approach is the ion polarizability, α, which is usually large for ions with a large radius (i.e. Π, SCN⁻, Cs⁺) and small for ions with small radius and high electrical charge (i.e. F⁻, Li⁺, Mg²⁺). The common observation that ion specific effects are usually stronger for anions is due to the fact that they are more polarizable than cations, however a full description of Hofmeister effects also requires the inclusion of hydration effects and ionic size.

In this paper we report measurements of the faradaic peak currents and the E°' of cytochrome c in the presence of a range of cations and anions and demonstrate that the nature of the ion influences both of these parameters in a manner which can be ascribed to the Hofmeister effect. While the peak current is influenced by both anions and cations, E°' is affected only by anions. Specific ion effects on the peak current can be understood by considering the polarizability of the ion, which affect the interaction between cyt c and the modified-gold electrode. Elucidation of the enthalpic and entropic components of E°' demonstrate that the overall changes in E°' are masked by significant, opposing changes in ΔH° and ΔS°. These changes are analyzed and compared to ion-specific effects observed for other systems.

2. Experimental

2.1 Materials

Horse heart cytochrome c (type VI) was purchased from Sigma-Aldrich and used without further purifications. Potassium hydrogen phosphate, potassium di-hydrogen phosphate, sodium fluoride, sodium chloride, sodium bromide, sodium perchlorate, sodium thiocyanate, lithium chloride, potassium chloride, cesium chloride, guanidine in chloric acid, 4,4'-bipyridyl, sulfuric acid and hydrogen peroxide were obtained from Sigma-Aldrich. Agar was obtained from BDH. Deionised water was obtained from an Elga Maxima water purification system and had a resistivity of 18.2 MΩ cm on delivery.

2.2 Methods

A 2-mm-diameter gold work electrode (CH Instruments) was used for all experiments. The electrode was cleaned by immersion in piranha solution (7 H₂SO₄: 3 H₂O₂) for 10 min, mechanically polished with alumina slurry (1.0, 0.3, and 0.05 μm) and sonicated for 5 minutes prior to use. A stock solution of cytochrome c was prepared in 4.4 mM potassium phosphate
buffer (pH 7.0) and frozen in aliquots of 1 mL. Salts were dried in an oven at 110°C for 24 h prior to use. A stock solution of 4,4′-bipyridyl was prepared in potassium phosphate buffer (4.4 mM, pH 7.0). Electrochemical measurements were performed using solutions containing cyt c, the appropriate salt (200 mM), and 4,4′-bipyridyl (10 mM) made up to a total volume of 5 mL. The pH of the solutions was measured with an Orion 420A pH meter, calibrated with Thermo scientific buffers at pH 4.01, 7.00, 10.01. The average pH was 7.15 ± 0.15. Cytochrome c concentrations were determined spectrophotometrically (Shimadzu UV-11800) using an extinction coefficient of 106100 M cm⁻¹ at 410 nm. The concentration of cytochrome c in solution was 99 ± 5 µM.

Differential pulse voltammetry was performed on a CHI630A potentiostat (CH Instruments), using an increment of 0.001 V, amplitude of 0.05 V, pulse width of 0.06 s and a quiet time of 10 sec. A two-compartment, “nonisothermal” cell was employed in which the reference electrode was isolated from the working electrode compartment. Electrical contact between the compartments was maintained via a 1 M KCl/Agar salt bridge. The reference (Ag/AgCl|KCl sat) and counter electrodes (platinum wire) were placed in a solution of 1M KCl maintained at 20 ± 1 °C using a water bath (Clifton Bennet). Unless otherwise stated, all potentials reported here are referenced to the SHE (E°' SHE = E°' Ag/AgCl + 0.204 V at 20 °C). The working electrode was placed in the second compartment, which was comprised of a 10 mL cell with a heating jacket connected to a water bath (Lauda ecoline003). Each value of E°' represents an average of 3 ± 1 data points. The electrostatic surface potential of cyt c was calculated using APBS, PDB2PQR, which employs PROPKA, was used to calculate the partial charges on the protein residues. PRODRG was used to generate the parameters for the heme.

3. Results and discussion

3.1 Effect of ionic strength on E°' and peak current

Differential pulse voltammograms of cytochrome c at a 4,4′-bipyridyl modified electrode (Fig. 2) show reversible behavior with a peak width at half maximum of 92 mV.

Fig. 2 Differential pulse voltammograms of cyt c in a solution of potassium phosphate buffer (4.4 mM, pH 7.15) at 298 K in the presence of a range of anions.

![Figure 2](image-url)
The electrode surface is, at least at lower ionic strength, electrostatic in nature. The formal reduction potential of cyt c decreased as the ionic strength increased (Fig. 3B). Previous work has shown that, at low ionic strength (0.04 to 0.06 M) the $E^\circ$ of cyt c follows the Debye- Hückel equation. Using a Debye-Hückel model of cyt c as a low dielectric constant spherical cavity with a spherical surface charge distribution in a solvent of a continuum dielectric constant $\varepsilon$ gives:

$$E^\circ = E^\circ - 0.059A(Z_{\infty}^2 - Z_{&&}^2) f(I)$$

(2)

where

$$f(I) = \frac{\sqrt{I}}{1 + B_{0.329} \sqrt{I}}$$

(3)

$I$ is the ionic strength, $B = 0.329$ and $a_1=18$. The formal potential shows a dependence on $f(I)$ (Fig. 3C), though the response is not linear across the range examined, with deviations from linearity at high ionic strength which may arise from non-electrostatic effects.

### 3.2 Specific Ion Effects on peak current of cyt c

As the faradaic current is proportional to the amount of protein interacting with electrode, specific ion-protein interactions may alter the measured current. Specific ion effects were examined at an ionic concentration of 200 mM, a concentration reasonably close to that where specific ion effects are observed but sufficiently low to provide an observable faradaic response. Plots of peak current for a range of anions (Fig. 4A) and cations (Fig. 4B) display atypical trends when compared with the monotonic trends that are usually observed. The highest current for the anion series was obtained with Cl$^-$, while in the cation series, the highest current was obtained with Cs$^+$. A similar trend has been observed recently for cyt c.

Apart from the unexpected behavior of F$^-$, the observed results...
can be explained by Collins’ empirical rule of ‘matching water affinities’. Chaotropic anions can bind to chaotropic charged residues such as lysine on the surface of cyt c. In contrast to pure electrostatic effects, this approach considers the effect of the surface charge density of the ion on ion-water interactions. Small ions, such as Li\(^+\), Na\(^+\) and F\(^-\) have a high surface charge density (they are “hard” or “kosmotropic”) and bind water molecules strongly, whereas large ions, i.e. Cs\(^+\), ClO\(_4\)\(^-\), SCN\(^-\) have a low charge density (they are “soft” or “chaotropic”) and bind water molecules weakly. This rule states that ions prefer to pair with counterions or ionic groups which have comparable hydration enthalpies, i.e. similar water affinities. In this classification, the charged groups on a protein surface, i.e. carboxylates and alkyl ammonium, are kosmotropic and chaotropic respectively. At a pH of 7.1, cyt c has a net positive charge (Fig. 5). The chaotropic ammonium groups of lysine residues can form strong ion pairs with chaotropic anions, with the strength of interactions increasing in the order: Cl\(^-\) < Br\(^-\) < ClO\(_4\)\(^-\) < SCN\(^-\). These ion pairs can have a detrimental effect on the binding between cyt c and the 4,4\(^\prime\)-bipyridyl modified gold electrode, thus leading to a decrease in the peak current (Fig. 5A). Fluoride does not follow this trend, possibly because its strongly kosmotropic nature does not permit the formation of ion pairs with chaotropic cationic groups at the surface of cyt c. The effect of F\(^-\) on peak current is similar to that produced by a chaotropic anion, producing the observed atypical curve. A similar trend was previously observed by Sedlak et al. where the effect of a series of ions on the rate constant for cyanide association with the heme iron of cyt c was a result of modulation of the Met80–heme iron bond strength and/or conformational flexibility of the heme region. Such atypical behaviour has been frequently observed. As recently reported by Schwertz et al., a partial or total reversal in the series can be ascribed to changes in the polarity or charge of the surface. A reversal of the order of the series was observed for lysozyme on changing from low to high salt, concentrations arising from change in the surface charge due to ion specific adsorption. A transition region has usually been observed where the order of ions can be partially or fully re-arranged It is feasible that the ion concentrations of 0.2 M used here are in this transition region, accounting for the observed position of F\(^-\). The cation effects on the peak current (Fig. 4B) display a monotonic increase from Li\(^+\) to Cs\(^+\) followed by a decrease for guanidinium. Guanidinium is usually considered to be a strongly denaturing (chaotropic and poorly hydrated) ion. On the basis of this classification it should be placed close to either ammonium or cesium in the conventional Hofmeister series (as is shown here). For example, the surface charge density of a silica based porous material followed the series: Li\(^+\) > Na\(^+\) > K\(^+\) > guanidinium > Cs\(^+\). In this study, the effect of guanidinium lies between that of Li\(^+\) and Na\(^+\). Using the conventional classification of guanidinium as a strong chaotropic its atypical position in the series can be explained in the same manner as for F\(^-\), i.e. the effects of the ion are observed in the transition region between the direct and the reversed Hofmeister series. It is clear that the behavior of guanidinium cannot be classified in the context of the conventional Hofmeister series. The overall cation effects (Fig. 4B) cannot be rationalized using the approach described for anions. While cyt c has a net positive charge at pH 7.1, it also has a significant number of negatively charged residues which can act as adsorption sites for cations (Fig. 5B). These residues are clustered at the distal side of the protein, away from the heme edge. The order of binding of cations to kosmotropic carboxylate groups would, according to Collins, follow the trend: Li\(^+\) > Na\(^+\) > K\(^+\) > Cs\(^+\). However, binding of cations to the negatively charged residues would not be expected to affect the binding of cyt c to the modified gold electrode surface as electron transfer occurs through the heme edge of the protein. The effect of cations is significant, with the peak current following the trend: Cs\(^+\) > K\(^+\) > Na\(^+\) > Gd\(^3+\) > Li\(^+\). The increase in peak current, from 0.34 to 0.39 μA, in the presence of Li\(^+\) and Cs\(^+\), respectively, indicate that more subtle cation effects are occurring.
From the theory of the double layer, a charged colloidal particle will establish an inhomogeneous concentration profile of cations and anions, $\rho_i(x)$\textsuperscript{15}. The ionic distribution follows from the equation:

$$\nabla^2 \phi(x) = 4\pi \sum \frac{\rho_i(x)}{\varepsilon}$$ \hspace{1cm} (4)

where $\phi$ is the electrostatic potential and $\varepsilon$ the dielectric constant.

For a simple 1:1 electrolyte, such as the salts used in the present work, and for a positively charged surface, such as the heme edge of cyt c at pH 7.1, the ionic concentration profile ($\rho_i(x)$ and $\rho_o(x)$ for anions and cations respectively) can be described by the Boltzmann distribution:

$$\rho_i(x) = \rho_0 \exp \left( \frac{-e\phi}{kT} \right)$$ \hspace{1cm} (5A)

$$\rho_o(x) = \rho_0 \exp \left( \frac{e\phi}{kT} \right)$$ \hspace{1cm} (5B)

where $e$ is the unit charge and $\rho_0$ the bulk salt concentration. Under suitable boundary conditions the resulting Poisson-Boltzmann equation can be solved to show that a positively charged protein surface would adsorb counter ions (anions) and repel cations. Conventional double-layer theory yields the same result, irrespective of which 1:1 electrolyte used, i.e. no specific ion effects should occur.

A qualitative rationalization of both anion and cation effects can be made by considering ion dispersion forces, as described in detail by Ninham and Lo Nostro.\textsuperscript{39} Ion dispersion forces operate together with electrostatic forces to modulate ion binding at colloidal surfaces\textsuperscript{32}, introducing an additional term, $U_i^{\text{dispersion}}(z)$ (eq. 6 and 7) to the electrostatic potential, $\phi$ (eq. 5).

$$U_i^{\text{dispersion}}(x) = \frac{B_i}{x} f(x)$$ \hspace{1cm} (6)

$$\rho_i(x) = \rho_0 \exp \left( -\frac{\pm e\phi + U_i^{\text{dispersion}}(x)}{kT} \right)$$ \hspace{1cm} (7)

where $B_i$ is the dispersion coefficient, $x$ is the distance of the ion from the protein surface, and $f(x)$\textsuperscript{35} is a function of the reciprocal of the size of the ion ($a$), $B_i$ depends on the ion dynamic polarizability ($\alpha_i(\omega)$) and the dielectric properties of both the surface and the solvent. Hofmeister effects are the result of a delicate interplay between hydration, non-electrostatic potentials and ionic size effects.\textsuperscript{35} $B_i$ is affected by all three parameters, whereas $f(x)$\textsuperscript{35} depends only on ionic size. The size of the ion has two contrasting effects; polarizability increases with size, and so does $B_i$, whereas $f(x)$ decreases. The resulting value of $U_i$ is a subtle balance between these effects.\textsuperscript{35} Experimental verification of this theory has been hindered by the lack of accurate values of ion polarizabilities. Recently, progress in calculating $B_i$ coefficients \textit{ab initio} from ion polarizabilities for some surfaces (i.e. air-water,\textsuperscript{40} water-silica,\textsuperscript{41} water-alumina,\textsuperscript{42} and water-protein\textsuperscript{43}) has been made. While $B_i$ values for a range of anions at

### Table 1 List of ionic sizes (hard sphere radius), $a$,\textsuperscript{46} static ionic polarizabilities, $\alpha_0$,\textsuperscript{48} and dispersion coefficients (water-protein), $B$.\textsuperscript{46}

<table>
<thead>
<tr>
<th>Ion</th>
<th>$a$ (Å)</th>
<th>$\alpha_0$ (Å$^3$)</th>
<th>$B$ (10$^{-6}$ J m$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F$^-$</td>
<td>1.12</td>
<td>1.218</td>
<td>n.a.</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td>1.86</td>
<td>4.220</td>
<td>- .26</td>
</tr>
<tr>
<td>Br$^-$</td>
<td>2.16</td>
<td>6.028</td>
<td>-1.70</td>
</tr>
<tr>
<td>ClO$_4^-$</td>
<td>2.35</td>
<td>5.488</td>
<td>-1.53</td>
</tr>
<tr>
<td>SCN$^-$</td>
<td>2.39</td>
<td>7.428</td>
<td>-2.7</td>
</tr>
<tr>
<td>Li$^+$</td>
<td>0.42</td>
<td>0.028</td>
<td>n.a.</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>0.67</td>
<td>0.131</td>
<td>-0.20</td>
</tr>
<tr>
<td>K$^+$</td>
<td>1.06</td>
<td>0.795</td>
<td>n.a.</td>
</tr>
<tr>
<td>Cs$^+$</td>
<td>1.62</td>
<td>2.354</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

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the water-protein interface have been reported, similar values for all cations are not yet available. The ionic sizes, static ion polarizabilities and dispersion coefficients for some ions used in the present work are listed in Table 1.

Ion dispersion forces combine with electrostatic forces to modulate the ionic distribution profile at the surface of cyt c. Anions can be adsorbed on the positively charged surface of cyt c but to different extents according to the dispersion potential, \( U^\text{dispersion} \), calculated from eq. 6. A higher value of polarizability and, consequently, a more negative value of \( B \) will result in a more attractive dispersion force (Table 1), according to the series SCN\(^-\) > Br\(^-\) > ClO\(_4\)^- > Cl\(^-\). With the exception of the relative positions of Br\(^-\) and ClO\(_4\)^-, this series follows the experimental results (Fig. 4A). The relative ordering of Br\(^-\) and ClO\(_4\)^- is likely to arise from the fact that polyatomic ions, such as ClO\(_4\)^-, have a significant quadrupole moment that is not considered with the calculations only including induced dipole interactions. Cations will be repelled from the surface of the protein but, similarly to anions, the more polarizable Cs\(^+\) will be able to approach closer to the surface than the less polarizable Li\(^+\). Strong anion binding, as for SCN\(^-\), would reduce the positive surface potential of cyt c thus resulting in a decrease of peak current (Fig. 4A). On the contrary, cation adsorption, as for Cs\(^+\), would increase the surface potential of cyt c, thus favoring electrostatic binding to the modified gold electrode and increasing the peak current. The observed ion specific effects can be rationalized in this manner. While the effect of specific adsorption of the ions on the 4,4'-bipyridyl modified gold electrode surface can not be excluded, it is not likely that such an effect is significant as disruption of the 4,4'-bipyridyl layer would be expected to affect the peak currents, but not \( E^\circ\), \( \Delta H^\circ \) and \( \Delta S^\circ \). Specific ion effects on \( E^\circ\), \( \Delta H^\circ \) and \( \Delta S^\circ \) will be discussed in the next section.

### 3.3 Specific Ion Effects on \( E^\circ\) of cyt c

Plots of \( E^\circ\) of cyt c as a function of temperature (Fig. 6A and 7A) are in agreement with previously published data with \( E^\circ\) decreasing with increasing temperature. However, the temperature dependence of \( E^\circ\) is also dependent on the nature of the anion present in the solution. While cyt c display essentially the same value of \( E^\circ\) in solutions containing fluoride and chloride, in the presence of Br\(^-\), ClO\(_4\)^- and SCN\(^-\), \( E^\circ\) undergoes a progressive decrease. An exception to the linear dependence of \( E^\circ\) on temperature was observed for thiocyanate which showed a biphasic response with a break point at 308 K. This behavior may arise from a change in the conformation of the protein from a low to high-temperature conformer and has been observed previously in mixtures of solvents. The values of \( E^\circ\) as a function of the different salts are shown in Fig. 6B. \( E^\circ\) decreases according to the Hofmeister series, ranging from a higher redox potential for lower polarizable (kosmotropic) ions to a lower redox potential for highly polarizable (chaotropic). A difference of 22±2 mV was observed between the two limits, with \( E^\circ\) of 244 and 222 mV for NaF and NaSCN, respectively. Fig. 7A shows \( E^\circ\) as a function of temperature for a series of cations. Unlike the anion series, no clear trend could be ascribed to ion specific effects. For example, no significant difference was observed in \( E^\circ\) in LiCl and CsCl.

Analysis of the temperature dependence of \( E^\circ\) indicates that the enthalpy and entropy changes are compensative with the enthalpy term being the dominant component in all solutions examined (Fig. 8 and table 2). The observed changes in \( E^\circ\) mask significant changes in \( \Delta S^\circ \) and \( \Delta H^\circ \). On changing the anion from F\(^-\) to ClO\(_4\)^-, \( \Delta H^\circ \) decreased significantly by 78 mV while \( \Delta S^\circ \) increased by 49 mV. In contrast, little change was observed in the cation series with the exception of guanidinium.
This trend is not so surprising as cations are less polarizable then anions and their lower ionic dispersion forces can account for the small changes observed for the cation series. The enthalpy of reduction is a function of a range of effects including the nature of the axial ligand(s), the net charge (of both the heme and the peptide), extent of the hydrogen bond network, and the degree of solvent exposure. Reduction entropies of redox proteins are considered to be due to solvent induced reorganization effects, alteration of solvent dielectric about the metal redox centers, and the influence of ligation. It is not possible to identify a general trend in $\Delta H^{\circ -}$ and $\Delta S^{\circ -}$ for either the cation or the anion series.

For example, in the case of NaCl the entropic contribution ($T \Delta S^{\circ -}/nF$) is $-0.147$ V while the enthalpic term ($-\Delta H^{\circ -}/nF$) is 0.390 V. The entropic term in the presence of Cl$^-$, while less negative and indicative of preferentially stabilization of the reduced form of cyt c, is similar to that of ClO$_4^-$. The values for Br$^-$ and SCN$^-$ are lower and similar in magnitude. In contrast, the enthalpic term for Cl$^-$ is similar to those of Br$^-$, ClO$_4^-$ and SCN$^-$, indicative of no specific ion effects. For the cation series, Na$^+$, K$^+$ and Cs$^+$ display similar values of $-\Delta H^{\circ -}/nF$ which are slightly larger than that of Li$^+$, while the entropic contributions for all four cations are broadly similar.

4. Conclusions

The specific effects of both anions and cations on the $E^{\circ -}$ and the peak current of cyt c have been probed using differential pulse voltammetry at 4,4$'$-bipyridyl modified gold electrodes. The anions examined have a more pronounced effect on cations on the $E^{\circ -}$ of cyt c, with $E^{\circ -}$ decreasing in the sequence: F$^-$ > Cl$^-$ > Br$^-$ > ClO$_4^-$ > SCN$^-$. This effect can be explained by the tendency of kosmotropic anions to stabilize the reduced state of the iron in the heme, increasing $E^{\circ -}$. The peak currents showed an atypical trend in the presence of anions, with a maximum value obtained with CI$^-$ instead of the conventional monotonic series. Anion-protein interactions affect the peak current, since the latter is proportional to the amount of protein interacting with 4,4$'$-bipyridyl adsorbed on the electrode surface. Apart from the unexpected behaviour of F$^-$, chaotropic anions can bind to the chaotropic, charged lysine residues on the surface of cyt c, weakening the electrostatic interactions between the charged lysine residues of cyt c and the modified gold electrode. The presence of cations can also affect the response, with the peak current decreasing in the sequence Cs$^+$ > K$^+$ > Na$^+$ > Go$^+$ > Li$^+$. This trend can be explained by the existence of attractive dispersion forces between the ions and the protein, providing experimental corroboration to Ninham’s theory of ionic dispersion forces. The results obtained here demonstrate the importance of the choice of the electrolyte in examining the electrochemical properties of redox proteins. In addition to the effects established here, the nature of the ion may affect the kinetics the reaction. While this study has described the response of the model redox protein cyt c, it is feasible that similar changes can arise with other redox proteins and enzymes, which may ultimately be displayed as changes in sensitivity or response for such enzymes when utilized in applications such as biosensors and biofuel cells.

Acknowledgments

LM thanks the Master and Back project financed by the RAS (Regione Autonoma della Sardegna). This work was supported by the Programme for Research in Third Level Institutions (INSPIRE). The assistance of Dr. J. Cooney and Dr. T. Kawaga in preparing Fig. 5 is gratefully acknowledged.

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