The Effect of Bulk Solvent Structure on the Temperature Dependence of the Reduction Potential of Cytochrome c*

by GEORGE P. KREISHMAN, C. WILLIAM ANDERSON, CHIH-HO SU, II. BRIAN HALSALL and WILLIAM R. HEINEMAN

Department of Chemistry, University of Cincinnati, Cincinnati, Ohio 45221, U.S.A.

Manuscript received December 1st 1977

Summary

The reduction potential of horse heart cytochrome c in various sodium halide solutions in H₂O and D₂O has been measured over the temperature range of 25 to 50 °C. In aqueous chloride solutions the temperature dependence was biphasic with the intersection point at 42 °C. This biphasic behavior is interpreted in terms of chloride-induced bulk destructuring of water at 42 °C. All samples in D₂O and samples in H₂O not containing chloride ion gave a linear temperature dependence. The decrease in reduction potential with increasing temperature for solutions containing F⁻, Cl⁻, Br⁻ and I⁻ correlates with the extent of anion binding to the oxidized form of cytochrome c.

Introduction

Several physical parameters exhibit a characteristic biphasic behavior when measured as a function of the temperature in aqueous NaCl solutions. The mean ionic activity coefficients of NaCl,¹ the conductance of Cl⁻,² the reduction potentials of the saturated calomel and the silver-silver chloride reference electrodes,³ the aggregation properties of purine,⁴ and the absorbance of purine⁵ all exhibit this biphasic behavior in their temperature dependence. The biphasic phenomenon has also been observed in biological systems such as the aggregation properties of membrane vesicles,⁶ the doubling time of A. hydrophila,⁶ the amount of saturated fatty acids in E. coli membranes⁷ and of penicillinase activity in B. cereus.⁸ In all of these systems the transition temperature for biphasic behavior is 42 °C.

In a previous communication, we reported the temperature dependence of the formal reduction potential, $U'_0$, for cytochrome $c$. In aqueous NaCl solution, the temperature dependence of $U'_0$ exhibited biphasic behavior with the inflection at 42 °C. However, a linear temperature dependence was obtained in $D_2O$-NaCl solutions.

To better understand the reasons for these dependencies, we have measured the temperature dependence of $U'_0$ for cytochrome $c$ as function of solvent structure ($H_2O$ vs. $D_2O$) and in the presence of a series of sodium halides (NaBr, NaCl, NaF and NaI).

**Experimental**

The formal reduction potentials for cytochrome $c$ were measured by a spectroelectrochemical technique using an optically transparent thin layer electrode, OTTLE. The OTTLE was constructed by sandwiching a 1 x 3 cm piece of 100 wires-per-inch gold minigrid (BUCKBEE-MEARS Co., St. Paul, Minnesota) between two quartz plates as shown in Fig. 1. The quartz plates were separated by strips of 2-mil teflon tape (DILETRIX CORP., Farmingdale, N. Y.) around the edges to give an optical cell thickness of ca. 0.2 mm. This assembly was attached with epoxy to a plexiglas block (1 cm x 3 cm x 6 cm) with appropriate holes drilled for the optical path, solution entrance, thermistor, and contact with reference and auxiliary electrodes. The cell was filled by injecting solution into the solution entrance port until it overflowed into the auxiliary-reference-electrode-port. The reference and platinum wire auxiliary electrode were then immersed in the overflow solution. The cell required about 200 mm$^3$ of solution, which was sufficient for a complete experiment for temperature dependence. A thermistor (YELLOW SPRINGS Instruments) was positioned in the thermistor port for temperature measurements with a digital voltmeter (FLUKE 8000 A DMM). The thermistor was previously calibrated against a 0.1 °C precision mercury thermometer. The OTTLE was clamped between two water circulating type thermal blocks and the temperature regulated with a HAAKE Modell J of thermostat regulator. The potentials were measured versus a S.C.E. of the H-cell design. The temperature of the S.C.E. was maintained equivalent to that of the OTTLE. Control experiments$^{11}$ in which the potential difference between an S.C.E. maintained at room temperature and an S.C.E. the temperature of which was varied under the conditions employed for the measurements on cytochrome $c$ gave potential changes of less than 2 mV over the temperature range of 25–55 °C. Similar behavior has been observed in other laboratories.$^{12}$ Optical measurements were made with a HARRICK rapid scan spectrometer, RSS-B. The potential of the electrochemical cell was controlled with a potentiostat of conventional operational amplifier design.

All solutions were prepared to be 1 mM cytochrome $c$, 0.1 mM 2,6-dichlorophenolindophenol, 0.10 M sodium halide and 0.10 M sodium phosphate buffer at pH 7.0 or pD 7.0. The 2,6-dichlorophenolindophenol
served as a mediator-titrant for coupling the cytochrome c with the electrode potential. $U^{\circ}$ values were measured using a previously described thin-layer spectropotentiostatic technique. This method involved incrementally converting the cytochrome c from its fully oxidized to fully reduced state by a series of applied potentials. For each potential a spectrum was recorded after equilibrium was attained. The formal redox potential was obtained from a NERNST plot. The temperature of the solution was then changed and allowed to equilibrate for 30 minutes at the new temperature before repeating the sequence.

![Diagram of optically transparent thin layer electrode used for spectroelectrochemical measurements of $U^{\circ}$](image)

Chemicals and their sources were as follows: Na$_2$HPO$_4$, AR grade, MALLINCKRODT; NaH$_2$PO$_4$·H$_2$O, certified A.C.S., FISHER SCIENTIFIC; Cytochrome c. Type VI, 95-100% pure, SIGMA CHEMICAL Co.; 2,6-Dichlorophenolindophenol, 99+ % pure, FLUKA COLUMBIA ORGANIC CHEMICALS, Columbia, S. C.; D$_2$O, Gold Label grade, DIAPREP, ALDRICH CHEMICAL Co.; NaBr, NaF, NaI, NaCl, KCl, Suprapur, E. M. LABORATORIES or certified A.C.S., FISHER SCIENTIFIC. Doubly distilled, deionized water was used to prepare all aqueous solutions.
Results

The variation in $U^{0'}$ for horse heart cytochrome $c$ as a function of temperature in aqueous solutions containing different sodium halides is shown in Fig. 2. All solutions which do not contain Cl$^-$ exhibit a linear temperature dependence between 25 and 50 °C. However, in the presence of Cl$^-$, biphasic behavior occurs with a transition temperature of 42 °C. The temperature dependence in D$_2$O solutions of bromide and chloride exhibit a linear behavior as shown in Fig. 3. The thermodynamic parameters of entropy and enthalpy obtained from the plots in Fig. 2 and 3 are listed in Table 1.
The temperature dependence of $U^0$ for horse heart cytochrome $c$ in 0.10 M NaCl and NaBr solution in D$_2$O. Solutions contain 0.10 M sodium phosphate pH 7.00 buffer.

Table I. Thermodynamical parameters for the solutions of cytochrome $c$ in 0.10 M alkali halide, 0.10 M sodium phosphate, pH or pHD 7.00.

<table>
<thead>
<tr>
<th>Alkali halide</th>
<th>Solvent</th>
<th>$\Delta H^o$ kcal/mole</th>
<th>$\Delta S^o$ cal/mole °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaBr</td>
<td>H$_2$O</td>
<td>-14.0</td>
<td>-27.1</td>
</tr>
<tr>
<td>NaF</td>
<td>H$_2$O</td>
<td>-12.0</td>
<td>-20.3</td>
</tr>
<tr>
<td>NaI</td>
<td>H$_2$O</td>
<td>-12.7</td>
<td>-23.0</td>
</tr>
<tr>
<td>NaCl ($&lt;42$ °C)</td>
<td>H$_2$O</td>
<td>-9.1</td>
<td>-10.2</td>
</tr>
<tr>
<td>NaCl ($&gt;42$ °C)</td>
<td>H$_2$O</td>
<td>-29.4</td>
<td>-75.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>D$_2$O</td>
<td>-12.0</td>
<td>-19.5</td>
</tr>
<tr>
<td>NaBr</td>
<td>D$_2$O</td>
<td>-13.4</td>
<td>-19.2</td>
</tr>
</tbody>
</table>
Discussion

When measured in aqueous chloride solutions, the temperature dependence of the $U^0'$ of horse heart cytochrome $c$ exhibits biphasic behavior with the transition temperature at 42 °C. As described in the introduction, this same biphasic behavior occurs in a variety of physical parameters when measured in aqueous Cl$^-$ solutions. Consequently, a fundamental change (which occurs at 42 °C) in the nature of aqueous Cl$^-$ solutions is a probable cause of the observed biphasic behavior in such a variety of physical parameters.

Studies of aqueous Cl$^-$ solutions have indicated the presence of two main ionic species in aqueous HCl solutions: a very weakly solvated ion, Cl$^-$, and a trihydrate, $[\text{Cl(H}_2\text{O)}_3]^-$.

The equilibrium $\text{Cl}^- + 3\text{H}_2\text{O} \rightleftharpoons [\text{Cl(H}_2\text{O)}_3]^- \ $ was found to exhibit a marked discontinuity in the entropy change at 42 °C. These results were interpreted in terms of a change in the extent of the bulk water structure with the bulk water being more structured below 42 °C than above this temperature. This postulated change in the structure of bulk water was found to be consistent with the degree of purine self-association in aqueous Cl$^-$ solutions.

A change in the structure of bulk water also accounts for the biphasic behavior in the $U^0'$ of cytochrome $c$. Since cytochrome $c$ undergoes a size change, the oxidized form being slightly larger than the reduced form, the reduction process is accompanied by water compensation. This process, as described by Lumry, occurs in two steps: (a) contraction of the protein leaving a hole and (b) water filling that hole. This process allows more hydrogen bonds between water molecules to be formed. Consequently, it is accompanied by a decrease in both enthalpy and entropy. The magnitude of the enthalpy and entropy changes are directly proportional to the decrease in size of the protein. Using Lumry's value of $\Delta S^0/\Delta V = 1.2 \text{ e.u./cm}^3$ and the thermodynamic parameters for the reduction process, the decrease in volume of cytochrome $c$ in going from the oxidized to the reduced form is about 25 to 50 Å$^3$ for the various halide solutions.

The biphasic temperature dependence shown in Fig. 2 indicates a significant change at 42 °C in entropy for the reduction of cytochrome $c$. This change can be related to differences in the bulk water structure above and below 42 °C. As the bulk solvent becomes less structured above 42 °C, the larger oxidized form becomes more favored and $U^0'$ decreases. The large slope of $dU^0'/dT$ for cytochrome $c$ in NaCl–H$_2$O solution is attributed to this bulk water destructuring above 42 °C. The water is less structured, and the oxidized form is more favored.

No biphasic behavior is obtained in aqueous bromide, fluoride and iodide solutions. Thus, the change in water structure at 42 °C does not occur in these solutions. Likewise, linear behavior is observed in D$_2$O chloride solutions. This is consistent with the more structured nature of D$_2$O compared to H$_2$O.
Ion binding has been shown to be involved in the reduction of cytochrome c as given by the following equation:

\[
\text{cyt } c^{III} - X^- + e^- \rightleftharpoons \text{cyt } c^{II} + X^- \tag{1}
\]

where the oxidized form binds a halide anion. Binding of a negatively charged ion to the net positively charged oxidized form would be expected to reduce charge repulsion in the cytochrome c resulting in a more compact protein. Thus, in the presence of ion binding the net size change upon reduction would be diminished. It has been shown that of the halides, Cl\(^-\) binds very strongly to the oxidized form whereas Br\(^-\) and I\(^-\) ions bind much more weakly.\(^{18}\) For the reduction process in H\(_2\)O, the \(\Delta S^0\) values, which are directly proportional to the size change, are in the expected order with \(\Delta S^0\) (NaCl, \(T < 42^\circ C\)) > \(\Delta S^0\) (NaF) \(\approx\) \(\Delta S^0\) (NaBr), \(\approx\) \(\Delta S^0\) (NaI). The larger halide ions (Br\(^-\) and I\(^-\)) might be expected to exhibit weaker binding because of their increased size. F\(^-\) would not be expected to bind strongly because of very strong solvation by H\(_2\)O causing the effective ionic size in water to be large.\(^{20}\) Thus, below 42 \(^\circ C\), the size change between the oxidized and reduced forms correlates well with the known binding constants of the ions.

The biphasic temperature dependence of the redox potential of cytochrome c is consistent with the concept of salt-induced bulk water destructuring at 42 \(^\circ C\). Since most advanced forms of life cannot exist much above 42 \(^\circ C\), this phenomenon may have important ramifications on the properties of many biological systems.

Acknowledgment

This investigation was supported in part by National Science Foundation Grant CHE77-04399 (W.R. H.). C. W. Anderson acknowledges support provided by a Procter and Gamble Fellowship and a University of Cincinnati Summer Research Fellowship.

References

1. R.P. Smith, J. Am. Chem. Soc. 61, 497 (1939)
Reduction Potential of Cytochrome c

14 L. Leifer and K. Inoue, manuscript in preparation
20 J. O'M. Bockris, Quart. Rev. Chem. Soc. 3, 173 (1949)