Low-temperature Study of the Plastocyanin–Ferricyanide Electron-transfer Reaction in Aqueous Methanol Reveals an Unusual Energy Barrier

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Cryokinetic studies of the plastocyanin–ferricyanide redox reaction reveal a large enthalpy barrier that may be assigned to obligatory reorganisation of the electron-transfer assembly.

Cryosolvents[1,2] offer opportunities to study fast electron-transfer reactions of metalloproteins with greatly increased kinetic resolution and insight into accompanying solvent dynamics. We have examined a well-documented 'conventional' reaction;[3—5] oxidation of the 'blue' Cu centre of spinach plastocyanin (PCu) by ferricyanide [$Fe(CN)_6^{3-}$], in 50% (v/v) aqueous methanol ('50:50'); a low-viscosity cryosolvent suitable for fast stopped-flow measurements down to −45 °C.[6] We report here the detection of a large enthalpy barrier assignable to reorganisation of the electron-transfer assembly that is undetectable at ambient temperatures.

The Eyring plot for reaction (1) exhibits marked downwards curvature (Figure 1). With supporting data summarised as follows, this signifies a change in the rate-determining step of a consecutive reaction system.

Reaction (1), as followed at 597 nm, with $Fe(CN)_6^{3-}$ in pseudo first-order excess, is monophasic and exponential over the entire range +25 to −35 °C, pH* = 7.0,† $I = 0.10$ M (NaCl). The reaction is first order in [$Fe(CN)_6^{3-}$] throughout (Figure 1, inset).‡ At 25 °C, $k = 1.04 \pm 0.05 \times 10^4$ dm^3 mol^{-1} s^{-1}, to be compared with a value of $k = 8.0 \times 10^4$ dm^3 mol^{-1} s^{-1} obtained by Sykes and co-workers in H_2O.[5]

Cyclic voltammetry shows that $E_{1/2}$ [*vs.* normal hydrogen electrode (n.h.e.)] for the $Fe(CN)_6^{3-/4-}$ couple in '50:50' varies linearly with temperature between 25 (+305 mV) and −30 °C (+382 mV).

Comparison of the ^1H n.m.r. spectra of PCu^I in CD_3OD–D_2O [50:50 (v/v)] (pH* 7.0) and in D_2O (pH 7.0) at 20 °C shows persistence of amide NH and downfield-shifted backbone α-CH peaks indicating retention of β-sheet structure. The dispersion of ring current-shifted aromatic and methyl group resonances is similar for the two solvent systems. Chemical shift differences are generally small (<0.2 p.p.m.).§

The pH*–reactivity profile for the $PCu^I/Fe(CN)_6^{3-}$ reaction, obtained by pH* 'jumps' from pH* = 7.0, shows an activity switch-off, pK^* = 5.0 ± 0.2 at 0 °C, similar to that observed[5] in H_2O (pK = 4.9 at 25 °C). This is strong evidence that the Cu^I active-site structure and properties are retained.

The oxidation of PCu^I by ferrocenium ion, $Fe(cp)_2^+$, in '50:50' shows very similar kinetics to studies in H_2O reported by Pladziewicz *et al.*,[7] for which data are given in parentheses. At pH* = 7.0, $I = 0.10$ M, we obtain values of k/dm^3 mol^{-1} s^{-1} 1.9 × 10^6 (1.0 × 10^6) and the Eyring plot is linear between 25

$$PCu^I + Fe(CN)_6^{3-} \longrightarrow PCu^{II} + Fe(CN)_6^{4-} \qquad (1)$$

$$1/k_{obs} = 1/k_1[Fe^{III}] + k_{-1}/k_1k_2[Fe^{III}] + k_{-1}k_{-2}/k_1k_2k_3[Fe^{III}] \qquad (2)$$

$$k = k_BT/\mathrm{h} \div [\exp(\Delta H_H^{\ddagger}/RT)\exp(-\Delta S_H^{\ddagger}/R) + \exp(\Delta H_L^{\ddagger}/RT)\exp(-\Delta S_L^{\ddagger}/R)] \qquad (3)$$

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† pH* is the apparent pH in mixed aqueous solvents (see for example relevant discussions in refs. 1 and 2). We measured pH* directly using a glass electrode.

‡ At the lowest $Fe(CN)_6^{3-}$ concentrations, the reaction does not proceed to completion and second-order plots show a small but noticeable intercept. We have not corrected for this but at the high [$Fe(CN)_6^{3-}$] used to obtain Eyring data, the back reaction is negligible.

§ Preliminary two-dimensional n.m.r. experiments in '50:50' show retention of side-chain conformation around the Cu side. Nuclear Overhauser effects are operative for His-37(ε_1CH)—His-87(ε_1CH), His-37(δ_2CH)—Phe-35(δCH), and His-87(ε_1CH)—Leu-12(δ_2CH_3), exactly as observed for the protein in aqueous solution.

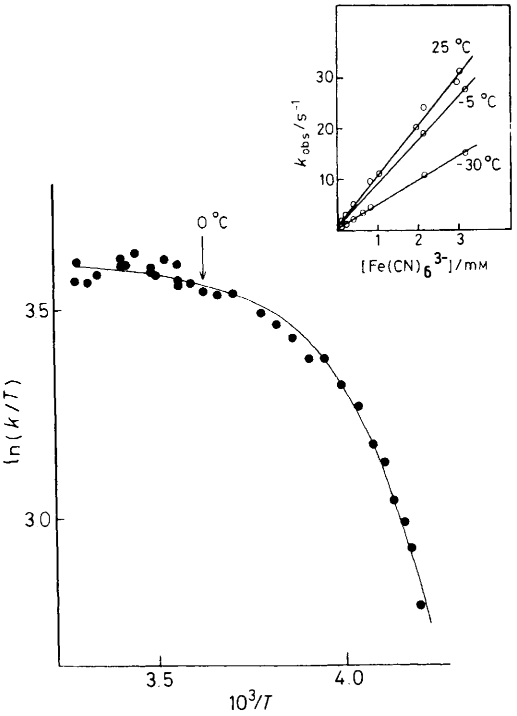

Figure 1. Eyring plot for the oxidation of PCu^I by $Fe(CN)_6^{3-}$ in 50 : 50 (v/v) $MeOH-H_2O$, pH* = 7.0 (5 mM HEPES-MES-acetate), $I = 0.10$ M (NaCl). Inset shows variation of rate constants k_{obs} with $[Fe(CN)_6^{3-}]$. Eyring data are for $[Fe(CN)_6^{3-}] = 3.02$ mM at which $k \simeq k_{obs}/[Fe(CN)_6^{3-}]$.

'Primitive' encounter; k_1, k_{-1}; K_1

$$\mathrm{PCu^{II} + Fe(CN)_6^{3-} \rightleftharpoons [PCu^{I} \sim Fe(CN)_6^{3-}]}$$

Reorganisation; k_2, k_{-2}; K_2

$$\mathrm{[PCu^{I} \sim Fe(CN)_6^{3-}] \rightleftharpoons [PCu^{I}{\cdot}Fe(CN)_6^{3-}]}$$

Electron transfer and completion; k_3

$$\mathrm{[PCu^{I}{\cdot}Fe(CN)_6^{3-}] \longrightarrow products}$$

Scheme 1

and −30 °C, $\Delta H^‡$/kJ mol^{-1} 21.5 (23.0) and $\Delta S^‡$/J K^{-1} mol^{-1} −52(−53).

We pre-empt the following discussion to propose a framework of consecutive steps for the $PCu^I/Fe(CN)_6^{3-}$ reaction, which under conditions of high $[Fe(CN)_6^{3-}]$ is essentially irreversible (Scheme 1). Assuming steady-state concentrations for the bimolecular complexes, the appropriate rate equation (2) is obtained. Notably each term retains first order dependence upon $[Fe(CN)_6^{3-}]$ (Fe^{III}).

The Eyring equation appropriate for two resolvable steps with high (H), or low (L) temperature dominance is given in equation (3). A computer fit of our data to equation (3) yields $\Delta H_H^‡ = 0.75 \pm 0.30$ kJ mol^{-1}, $\Delta S_H^‡ = -165 \pm 3$ J K^{-1} mol^{-1}, and $\Delta H_L^‡ = 61.8 \pm 1.0$ kJ mol^{-1}, $\Delta S_L^‡ = 90 \pm 5$ J K^{-1} mol^{-1}. The contrast in energetics is striking. Neither are appropriate for elementary diffusion (k_1) which is expected to follow, with some correction for the Debye term, the diffusional activation energy $E_a(\eta) = 28$ kJ mol^{-1} derived from the temperature dependence of viscosity.[6] Near-zero or negative $\Delta H^‡$ values coupled with a large negative $\Delta S^‡$ are associated with compact-assembly electron-transfer reactions with small Franck–Condon factors, and a contribution from exothermic pre-equilibria. We propose therefore that the 'normal' activation parameters $\Delta H_H^‡$ and $\Delta S_H^‡$ correspond to the composite electron-transfer rate constant $k_1k_2k_3/k_{-1}k_{-2}$ (*i.e.* $K_1K_2k_3$).

Our attention is now focussed upon $\Delta H_L^‡$ and $\Delta S_L^‡$, which demonstrate the potential for this type of study to reveal hidden detail. Extrapolated to 25 °C, this process occurs with $k = 5 \times 10^6$ dm^3 mol^{-1} s^{-1} and is thus undetectable. Such parameters must be assigned to a term that is dominated by a disordered and highly endothermic transition state. We propose that we are witnessing an essential reorganisation step identifiable with the composite term k_1k_2/k_{-1}. One interesting possibility is desolvation of the primary encounter complex to give a more compact and viable donor–acceptor assembly. It is believed[4,5] that $Fe(CN)_6^{3-}$ reacts at the relatively uncharged hydrophobic 'North' patch of plastocyanin adjacent to the Cu site.[8] At least part of the solvation shell of H_2O (or MeOH) molecules must be expelled; thus we anticipate (and indeed find) an interesting contrast with $Fe(cp)_2^+$, believed also to prefer the 'North' patch,[9] around which the solvation sphere should be relatively relaxed. Put another way, we observe two levels of encounter, solvated (inactive) and desolvated (active). Distinction between the two depends upon the height of the intervening energy barrier which reflects the strength of solvation.¶ Alternatively the barrier may represent a specific protein conformational change that is not required for the reaction with $Fe(cp)_2^+$. A further possibility is that $\Delta H_L^‡$ and $\Delta S_L^‡$ correspond to rate-determining dissociation of the product pair $[PCu^{II}{\cdot}Fe(CN)_6^{4-}]$. Stabilisation of this might arise through favourable local electrostatics (as Cu^{II}, the active site charge is formally 1+) and intermolecular hydrogen bonding (using perhaps $H-\varepsilon_2N$ of His-87)[8] to electron-rich CN^- ligands. Interestingly, Segal and Sykes[3] proposed exothermic precursory binding of this nature in their original study of PCu^{II} reduction by $Fe(CN)_6^{4-}$. With further studies, including low-temperature kinetics of the reverse reaction to provide a clearer picture of the free energy profile, we hope to establish more benchmarks in this area.

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References

1 P. Douzou, 'Cryobiochemistry,' Academic Press, London, 1977.
2 A. L. Fink and M. A. Geeves in 'Contemporary Enzyme Kinetics and Mechanism,' ed. D. L. Purich, Academic Press, London, 1983.
3 M. G. Segal and A. G. Sykes, *J. Am. Chem. Soc.*, 1978, **100**, 4585.
4 S. K. Chapman, I. Sanemasa, A. D. Watson, and A. G. Sykes, *J. Chem. Soc., Dalton Trans.*, 1983, 1949.
5 J. D. Sinclair-Day and A. G. Sykes, *J. Chem. Soc., Dalton Trans.*, 1986, 2069.
6 See ch. 2 of ref. 1.
7 J. R. Pladziewicz, M. S. Brenner, D. A. Rodeberg, and M. D. Likar, *Inorg. Chem.*, 1985, **24**, 1450.
8 J. M. Guss, P. R. Harrowell, M Murata, V. A. Norris, and H. C. Freeman, *J. Mol. Biol.*, 1986, **192**, 361.
9 J. R. Pladziewicz and M. S. Brenner, submitted for publication.

¶ Reconsideration of the temporal position of desolvation yields an alternative description involving free $Fe(CN)_6^{3-}$ in rapid equilibrium (k_1, k_{-1}) between solvated and desolvated forms. Only encounter of the desolvated species (k_2) gives a viable electron-transfer complex. Here $\Delta H_L^‡$ and $\Delta S_L^‡$ correspond to a composite diffusion-controlled rate constant.