Direct Electrochemistry of the Photosynthetic Blue Copper Protein Plastocyanin. Electrostatic Promotion of Rapid Charge Transfer at an Edge-Oriented Pyrolytic Graphite Electrode

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Abstract: Direct (unmediated) electrochemistry of the photosynthetic "blue" copper protein plastocyanin may be readily achieved at an edge-oriented pyrolytic graphite electrode over the pH range 4–8. Promotion of rapid charge transfer by Mg^{2+} ions (<5 mM) or by mild acidification (pH 4–6) of the medium closely parallels in vivo regulatory characteristics for plastocyanin at the thylakoid membrane. The edge face of pyrolytic graphite, subjected to standard polishing procedures in air, contains a variety of hydrophilic C–O functional groups which are expected to interact favorably with plastocyanin once electrostatic repulsion has been overcome. Optimal heterogeneous rate constants at 3 °C range between $10 \pm 5 \times 10^{-3}$ cm s^{-1} at pH 4 and $2 \pm 1 \times 10^{-3}$ cm s^{-1} at pH 8. Apparent $E_{1/2}$ values at decreasing low pH increase by ca. 55 mV per pH unit, with pK ~ 5.5. This may be identified with the protonation of active-site histidine-87 in accordance with established crystallographic and kinetic evidence. Plastocyanin behaves as an effectively symmetrical redox system ($i_{pa} \sim i_{pc}$) throughout the pH range 4–8. At scan rates of up to 500 mV s^{-1} at pH 4, an effective equilibrium between redox-active and redox-inactive (protonated) forms of reduced plastocyanin is maintained at the electrode. Accordingly, $t_{1/2}$ for deprotonation of H^+–Cu^I plastocyanin is estimated to be <1 ms.

Photosynthetic electron transport between cytochrome *f* and the $P700^+$ reaction center is mediated by plastocyanin, a soluble "blue" copper protein of molecular weight 10 500.[1] Unique structural,[2] spectroscopic,[3] and electron-transfer properties[4] associated with the active site have in recent years attracted widespread and diversified attention. The Cu atom, located in a hydrophobic region at one end of the molecule,[2] is coordinated by cysteine thiolate, methionine thioether, and two histidine imidazole ligands in a distorted tetrahedral geometry. Plastocyanins from higher plants carry a significant overall (and conservatively localized) negative charge.[1,2] This feature bears profound mechanistic implications with regard to in vivo regulation of electron transfer at the negatively charged thylakoid membrane,[5] of which cytochrome *f* and the P700 center are integral components. Regulatory effects of divalent metal ions and intrathylakoid pH have indeed been well documented and discussed for the plastocyanin-dependent reduction of $P700^+$.[6,7]

Interfacial electron-transfer processes, as exemplified by those occurring at the thylakoid membrane, have been of particular interest in our endeavors[8,9] to achieve, and capitalize upon, the direct (unmediated) electrochemistry of redox proteins and enzymes. We have now found that rapid heterogeneous electron transfer to spinach plastocyanin may be readily achieved at a polished edge-oriented pyrolytic graphite surface. Promotion by addition of Mg^{2+} or mild acidification highlights a simple, yet intriguing, analogy with electron-transfer processes at biological membranes.

Experimental Section

Plastocyanin was prepared from spinach leaves essentially according to literature procedures[10] except that final purification by gel filtration was carried out by using a column of LKB Ultragel AcA 202. The product was oxidized with $K_3Fe(CN)_6$, extensively desalted, and concentrated by using an Amicon 8MC diafiltration unit and then frozen and stored as pellets in liquid N_2. The resulting purity index A_{278}/A_{597} was 1.33. Identical electrochemical results were obtained by using samples of plastocyanin that had been subjected to further chromatograhy to give $A_{278}/A_{597} = 1.16$. All reagents were of Analar or Aristar grade, and doubly deionized water was used throughout the studies. Solutions for electrochemistry were made up to include 5 mM buffer–1 mM KCl as the basic supporting medium. Buffers used were as follows: acetate, pH 4.0–5.5; MES (2-[*N*-morpholino]ethanesulfonate), pH 6.0–6.3; HEPES (*N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonate), pH 7.0; Tricine (*N*-[tris(hydroxymethyl)methyl]glycine), pH 8.0. All pH standardizations were made at room temperature, and stock buffer adjustments were made with KOH or HCl.

Cyclic voltammetry was carried out using an Oxford Electrodes potentiostat and an all-glass cell (600 μL) with a conventional three-electrode configuration. At room temperature, measurement of plastocyanin electrochemistry was troubled by "impersistence" (a general deterioration in faradaic response). This problem was largely overcome by thermostating the cell at 3 °C. The counter electrode was a semi-cylindrical piece of Pt gauze while the reference was saturated calomel with contact achieved through a Luggin capillary. From a graph of values for the saturated calomel electrode (SCE) potential at various temperatures,[11] we adopted a value of E (SCE) = +258 mV vs. NHE at 3 °C. Working electrodes were constructed from 5-mm disks of standard pyrolytic graphite (Le Carbone, Portslade, Sussex, England) cut with the *a*–*b* plane perpendicular to the disk face and housed in Teflon sheathes. Prior to each experiment, electrodes were polished with a 0.3-μm alumina–water slurry (high purity Al_2O_3, B.D.H. or Banner, U.K.) and sonicated thoroughly. Small additions of a 0.5 M $MgCl_2$ solution were made by using a Hamilton syringe following which the sample solution was stirred briefly with a magnetic micro-"flea". With solutions of low ionic concentration, it was necessary to take precautions to minimize problems arising from uncompensated resistance.[12] Consequently the working electrode surface was reproducibly positioned close (ca. 2 mm) to the

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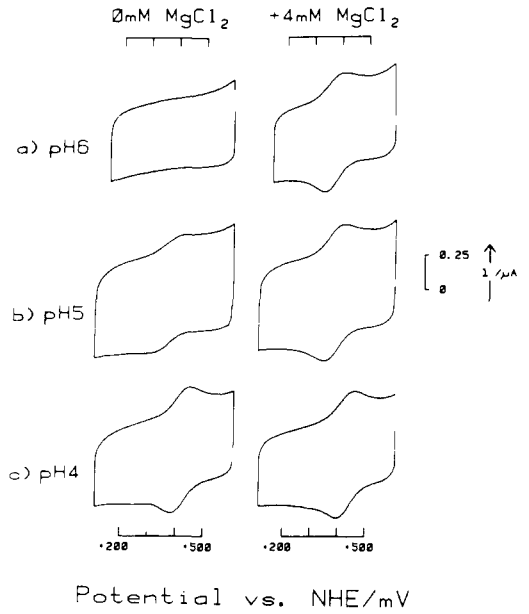

Figure 1. Dc cyclic voltammograms for plastocyanin (25 μM) at a polished edge-oriented pyrolytic graphite electrode, showing the effects of pH and addition of $MgCl_2$: (a) pH 6.0, 5 mM MES–1 mM KCl as background electrolyte; (b) pH 5.0, 5 mM acetate–1 mM KCl as background electrolyte; (c) pH 4.0, 5 mM acetate–1 mM KCl as background electrolyte. Results shown are for initial scans at 20 mV s^{-1}. Temperature = 3 °C. A faint broad feature frequently appearing at ca. 200 mV on the cathodic scan at pH 4 could be identified as the reduction of trace amounts of free Cu(II). The wave could be removed by the addition of 1 μM EDTA, without any change in the faradaic responses due to plastocyanin.

Luggin tip, and most measurements (at scan rates of 20 mV s^{-1}) were made with low protein concentrations (25 μM) for which faradaic peak currents were <0.2 μA. An assessment of the magnitude of apparent increases in peak separation ΔE_p, due to uncompensated resistance, was made by scanning a solution of ferrocene–monocarboxylic acid (10 μM in 5 mM Tricine–1 mM NaCl, pH 8.0). Under these conditions, currents were obtained that were similar to those measured[13] in our protein experiments. Typical results (ΔE_p) were 60 mV (20 mV s^{-1}) and 110 mV (200 mV s^{-1}). Addition of 4 mM $MgCl_2$ gave 60 mV (20 mV s^{-1}), 70 mV (200 mV s^{-1}), and 80 mV (500 mV s^{-1}), without increases in peak currents. In each case, plots of peak current density vs. (scan rate)$^{1/2}$ for this test system were linear. As this indicated that errors due to uncompensated resistance were likely to be significant at the lowest electrolyte concentrations, all estimations of kinetic parameters for plastocyanin electrochemistry were carried out by using data obtained with solutions containing at least 4 mM $MgCl_2$.

Results and Discussion

Promotion of plastocyanin electrochemistry at edge-oriented pyrolytic graphite is clearly illustrated by the set of dc cyclic voltammograms shown in Figure 1. Measurements here correspond to initial scans at 20 mV s^{-1} with 25 μM plastocyanin (oxidized form) in an initial background medium of 5 mM buffer–1 mM KCl. Well-defined cathodic and anodic waves (with peak separations ΔE_p <65 mV) are generated upon mild acidification or the addition of millimolar amounts of $MgCl_2$. Similar behavior was observed with use of higher (25 mM) buffer concentrations. Further measurements, intended to define more closely the pH and Mg^{2+} requirements for faradaic response, yielded the numerical data as complied in Table I. The resulting profile is outlined in Figure 2. Here the electrochemical response is represented in terms of initial-scan cathodic peak current densities based upon geometric electrode surface area. There is

(13) Our cyclic voltammograms generally contained relatively high charging contributions. Peak currents for initial and steady-state scans were most conveniently estimated by using base lines drawn by extrapolation of the slope at the commencement of sweep. For the case of scans at 500 mV s^{-1}, estimations of the ratio i_{pa}/i_{pc} were made, after subtraction of the contribution from capacitance, using a published semiempirical procedure (Nicholson, R. S. *Anal. Chem.* **1966**, *38*, 1406).

Table I. Initial-Scan Cathodic Peak Currents[a] Obtained for Plastocyanin at Edge-Oriented Pyrolytic Graphite under Various Conditions of pH and Mg^{2+} Concentration (Temperature = 3 °C; Background Medium is 5 mM Buffer–1 mM KCl)

pH	Mg^{2+}, mM	i_{pc}, μA	pH	Mg^{2+}, mM	i_{pc}, μA	pH	Mg^{2+}, mM	i_{pc}, μA
4.0[b]	0	0.20	5.0	5	0.22	7.0[d]	0	*f*
	0.5	0.21		7	0.19		0.5	0.10
	1	0.19					1	0.14
	2	0.22	5.5[b]	0	0.09		2	0.16
	4	0.20		0.5	0.15		3	0.16
	10	0.21		1	0.16		4	0.17
				2	0.17		5	0.17
4.5[b]	0	0.21		5	0.18		6	0.17
	0.5	0.24		10	0.19		10	0.17
	1	0.21						
	2	0.22	6.0[c]	0	*f*	8.0[e]	0	*f*
	5	0.21		0.5	0.12		1	0.10
	10	0.21		1	0.15		3	0.14
				2	0.19		5	0.14
5.0[b]	0	0.12		4	0.18		6	0.14
	0.5	0.16		5	0.22		8	0.15
	1	0.20		6	0.21		10	0.15
	2	0.18		10	0.18			
	4	0.19						

[a] Electrode surface area 0.20 cm^2, scan rate 20 mV s^{-1}. The concentration of plastocyanin is 25 μM. [b] Acetate. [c] MES. [d] HEPES. [e] Tricine. [f] Faradaic peak current too low to measure.

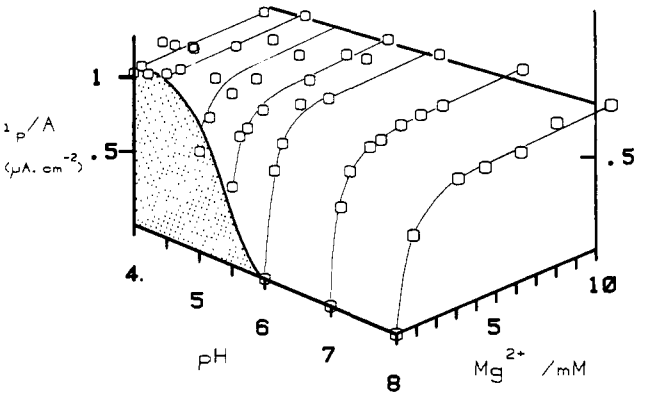

Figure 2. Three-dimensional representation illustrating the effects of pH and Mg^{2+} concentration upon observed (initial scan, 20 mV s^{-1}) cathodic peak current densities for plastocyanin, 25 μM in 5 mM buffer (acetate, MES, HEPES, and Tricine), with 1 mM KCl, at 3 °C.

a clear transition from pH-promoted (pH 4) to Mg^{2+}-promoted (pH 6–8) electrochemistry. At low plastocyanin concentrations, i.e., 25 μM, saturation requirements for Mg^{2+} are <5 mM at pH 7. We have found that with use of increasing protein concentrations, i.e., above 50 μM, higher levels of Mg^{2+} are required in order to achieve and maintain well-defined faradaic activity. It is likely that protein–protein repulsions at the electrode surface and competing less-reversible adsorption processes become more pronounced at higher protein concentrations. At the low-pH limit, additions of Mg^{2+} do not markedly increase current density. The monovalent electrolyte KCl is considerably less effective, and concentrations of around 0.1 M and above are required for comparable promotion at pH 7. The substitution-inert trivalent cation $Cr(NH_3)_6^{3+}$ is, by contrast, effective at submillimolar amounts, an observation that lends further support to views previously expressed[9] concerning the importance of electrostatically dominated interactions in the promotion of protein direct electrochemistry.

The series of steady-state dc cyclic voltammograms shown in Figure 3, obtained at pH 6.3 with the addition of $MgCl_2$, illustrates the typical dependence of current magnitude on scan rate observed throughout the pH range of study. For these measurements, voltammograms at different scan rates were recorded (generally at or around the tenth cycle) without intervening repolishing of the electrode. The stability of the promoted electrochemistry is

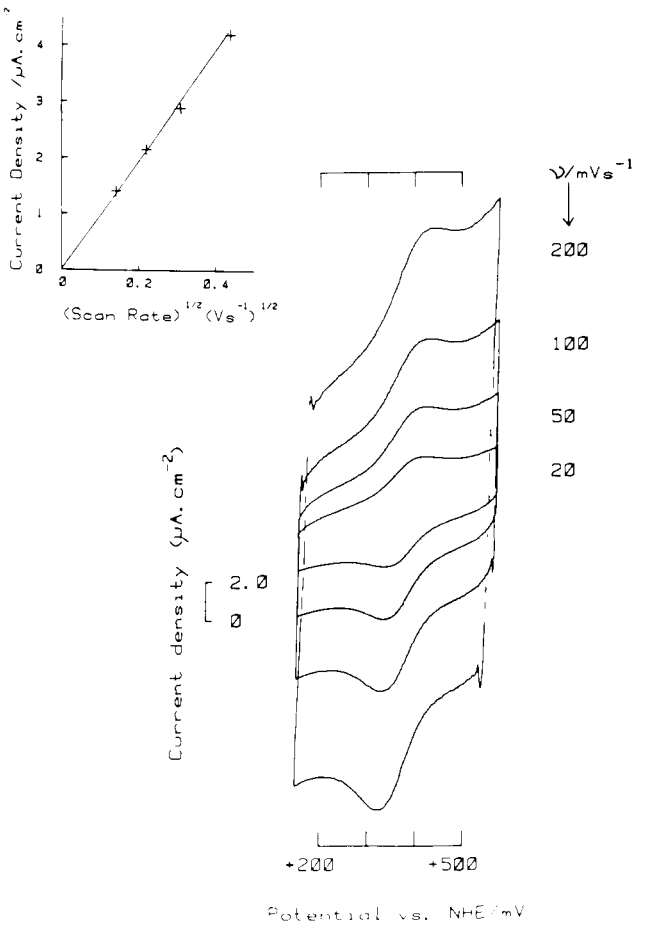

Figure 3. Steady-state dc cyclic voltammograms for plastocyanin (50 μM in 5 mM MES–1 mM KCl, pH 6.3, with 15 mM $MgCl_2$) at various scan rates, 3 °C. Inset shows plot of cathodic peak current density vs. (scan rate)$^{1/2}$. Scans were typically recorded on the tenth cycle.

thus clearly evident. As further indicated in the inset, plots of peak current density against (scan rate)$^{1/2}$ are linear, consistent with charge transfer dominated by diffusion of species to the electrode surface.[14] Calculated diffusion coefficients, based upon geometric surface areas, show a decrease from $7 \pm 1 \times 10^{-7}$ cm^2 s^{-1} at pH 4 to $4 \pm 1 \times 10^{-7}$ cm^2 s^{-1} at pH 8. This trend may reflect some increase in hydrodynamic resistance due to higher levels of ion pairing or solvation or, possibly, diminishing effective surface area. A comparable theoretical diffusion coefficient $D = 8.7 \times 10^{-7}$ cm^2 s^{-1} at 3 °C may be obtained[15] by use of the Stokes–Einstein equation. Rate constants for heterogeneous charge transfer, as estimated from initial scan CV peak separations at scan rates 20–100 mV s^{-1}, according to the procedure of Nicholson,[16] range from $10 \pm 5 \times 10^{-3}$ cm s^{-1} at pH 4 to $2 \pm 1 \times 10^{-3}$ cm s^{-1} at pH 8.[17] By inspection of observed anodic and cathodic wave shapes and current amplitudes at 20 mV s^{-1}, and comparison with computer simulations of Nicholson's solution for quasi-reversible electrode reactions, values for the electrochemical charge-transfer coefficient α were estimated to be close to 0.5. In agreement with previous potentiometric[18] and kinetically determined[19] pH profiles for redox potentials, values of $E_{1/2}$ increase below pH 5.5 with a limiting slop $-d(E_{1/2})/d(pH) \sim 55$ mV. At pH 7, the redox potential in the presence of 5 mM $MgCl_2$ is 375 ± 10 mV, whereas at pH 4, the corresponding value is 430 ± 10 mV.

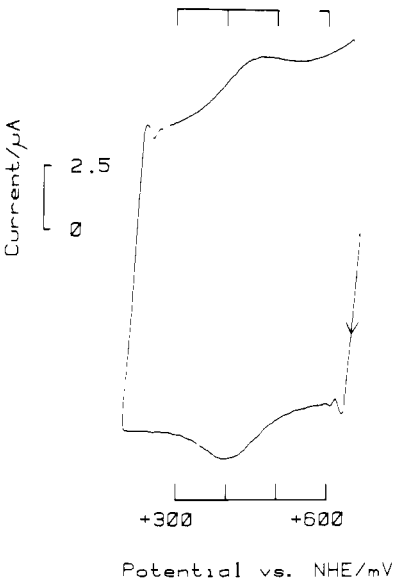

Figure 4. Initial-scan cyclic voltammogram at 500 mV s^{-1} obtained for oxidized plastocyanin (28 μM) at pH 4.0 (5 mM acetate–1 mM KCl, with addition of 10 mM $MgCl_2$). Temperature = 3 °C.

Our studies indicate that plastocyanin behaves as a quasi-reversible system uncomplicated by coupled chemical reactions throughout the pH range 4–8. Further investigations showed also that cyclic voltammograms at pH 4 were similar whether using oxidized or reduced (addition of ascorbate followed by dialysis) plastocyanin solutions. These observations demand discussion in light of evidence for the formation of a kinetically *inactive* protonated form of the reduced protein. Detailed crystallographic measurements made over a range of pH values have shown[20] that protonation (pK 5–6) of the histidine-87 δ-nitrogen at the active site, which may be correlated with the pH-dependent increase in redox potential, leads to the effective formation of three-coordinate Cu(I). Kinetic manifestation of the resulting high Franck–Condon reorganization barrier for electron transfer has been established by Sykes and co-workers[19,21,22] in studies of the rapid bimolecular oxidation of parsley plastocyanin by small inorganic redox probes. Our apparently contradictory results may be reconciled with these observations upon consideration of the electrochemical characteristics for a E_rC_r system as treated by Nicholson and Shain.[23] For the system as written

$$PCu^{II} + e^- \rightleftharpoons PCu^{I} \text{ (electrode)}$$

$$PCu^{I} + H^+ \rightleftharpoons H^+\text{–}PCu^{I} \qquad k_f[H^+]\ (=k_f'),\ k_b,\ K[H^+]\ (=K')$$

normal reversible electrochemistry, with $E_{1/2}$ displaced by (RT/F) ln $(1 + K')$, may be observed if $k_f[H^+]$ and k_b are sufficiently fast to maintain equilibrium at the electrode surface (case V of

(14) Bard, A. J.; Faulkner, L. R. "Electrochemical Methods"; Wiley: New York, 1980; Chapter 6.

(15) See, for example: Tanford, C. "Physical Chemistry of Macromolecules"; Wiley: New York, 1960; Chapter 6. From the Stokes–Einstein equation written as

$$D = \frac{kT}{6\pi\eta}\left(\frac{4\pi N}{3M\bar{v}}\right)^{1/3}$$

where M is the molecular weight and v is the partial specific volume of plastocyanin = 0.74 cm^3 g^{-1} (see ref 2); inserting $\eta_{H_2O} = 16 \times 10^{-3}$g cm^{-1} s^{-1} (3 °C) gives $D = 8.7 \times 10^{-7}$ cm^2 s^{-1}, in good agreement with the electrochemical values at low pH.

(16) Nicholson, R. S. *Anal. Chem.* **1965**, *37*, 1351.

(17) These estimations were derived from data obtained over several experimental sessions. Within the range of scan rates used, there were no marked trends toward larger rate constants at lower scan rates that would be indicative of effects due to uncompensated resistance. It is most likely that the observed scatter arises from difficulties in maintaining rigorous electrode surface reproducibility.

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(23) Nicholson, R. S.; Shain, I. *Anal. Chem.* **1964**, *36*, 706.

ref 23). For initial-scan cyclic voltammograms, the use of working curves showing the variation of the anodic-to-cathodic peak current ratio i_{pa}/i_{pc} with the function ψ ($=K'(nvF/RT(k_f' + k_b))^{1/2}$, where v = scan rate (volts per second), $k_f' = k_f[H^+]$, and $K' = K[H^+]$, permits an estimation of the deprotonation rate constant k_b to be made. As $\psi \rightarrow 0$, i.e., as $(k_f' + k_b)$ increases, the voltammetric response approaches the form expected for a normal uncoupled system with the corresponding $E_{1/2}$ displacement. In order to reaffirm our observations and to estimate a lower limit for k_b, we carried out a series of experiments at pH 4 using scan rates up to 500 mV s^{-1}. High charging currents, and the necessarily low protein concentrations which yielded relatively small faradaic responses, limited our ability to examine faster scan rates. Apparent values of $E_{1/2}$ were independent of scan rate, and general faradaic wave shapes did not change significantly as the cathodic switching potential was varied between −150 and +50 mV vs. SCE (+108 and +308 mV vs. NHE). A typical initial-scan cyclic voltammogram measured at a scan rate of 500 mV s^{-1} is shown in Figure 4. By inspection, observed i_{pa}/i_{pc} ratios lie close to 1; with adoption of a cautious limit $i_{pa}/i_{pc} > 0.75$, use of the published working curve gives $\psi < 1.0$. Consequently from our results at v = 500 mV s^{-1}, and using pK = 5.5, we conclude that k_b must exceed 640 s^{-1}; that is, $t_{1/2}$ for deprotonation is <1 ms. A similar result was obtained by using reduced plastocyanin and treating as for a C_rE_r system (case III of ref 23). This limiting value may be compared with recent evidence,[24] based upon NMR line-broadening measurements with *Anabaena variabilis* plastocyanin, which indicates that the lifetime of a proton on the δ nitrogen of histidine-87 is about 0.4 ms. Thus the observation of well-behaved electrochemistry at low pH is due to the comparatively rapid proton transfer which maintains effective equilibrium between protonated (inactive) and unprotonated (active) forms of the reduced protein.

Our results so far have served to demonstrate further the mechanistic information and insight that may be drawn from direct electrochemical studies on redox proteins. A number of surface studies[25] have demonstrated the presence of various C–O functional groups, including carboxylates and phenol, on samples of graphite subjected to oxidative pretreatments. Recent ESCA studies carried out in this laboratory have furthermore shown[26] that C–O functionalities are generated on our "edge" surfaces by routine polishing with alumina slurries. These groups are expected to interact favorably with hydrophilic residues on the protein surface once electrostatic repulsions have been overcome. The promotion of electrochemistry by mild acidification may arise from protonation of electrode surface groups or perhaps of amino acids on the protein leading to relief of electrostatic repulsion. Plastocyanins from higher plants have isoelectric points at or below pH 4,[1,27] and estimations based upon published amino acid sequence data[28] indicate that the total protein charge at pH 7 may be as high as −9. Specific amino acid protonation equilibria, in addition to protonation at the Cu(I) active site, have been detected kinetically in small reagent studies[22,29] and may indeed be mechanistically important in electrochemical promotion. Support for the suggestion that protonation equilibria at the electrode surface are important stems from pH titration studies[30] carried out with the hexacyanoferrate(II/III) system at low ionic strength, for which a similar "cut-off" in faradaic response at pH >6 is observed. Consequently, since the reduction potential of hexacyanoferrate (400 mV vs. NHE) is very similar to that of plastocyanin, this suggests that at pH 8 the electrode surface is negatively charged within the potential range used in our experiments. The effects of Mg^{2+} may be attributed to reduction of the Stern layer potential due to surface adsorption, Gouy–Chapman diffuse layer "screening", protein–cation binding, or moreover (and most interestingly perhaps) formation of transient "electrostatic bridging" between negatively charged zones on the protein and the electrode surface. The case for predominantly electrostatic influences in preference to more specific chemical effects is supported by our above-mentioned observations concerning the increased effectiveness of substitution-inert $Cr(NH_3)_6^{3+}$. A cation-binding domain, conserved in higher plant plastocyanins, has furthermore been identified in high-resolution ^1H NMR studies,[31,32] from observations of the paramagnetic line broadening of assigned proton resonances by Cr(III) complexes.

The analogy with fundamental in vivo processes at the chloroplast thylakoid membrane, for which divalent metal ions[6,7] and pH[7] are similarly implicated in plastocyanin–$P700^+$ electron-transfer regulation, is striking. Recent studies[7] of electron transfer between plastocyanin and $P700^+$ in highly purified reaction center complexes indicate that *localized* interactions between complex and plastocyanin are stimulated by increases in Mg^{2+} concentration (0–20 mM) or by mild acidification (pH <6) which may occur in the intrathylakoid space upon illumination. It is significant that we now observe plastocyanin electron-transfer activity to be similarly stimulated at an electrode surface, though the topography of the latter is unlikely to approach the expected levels of sophistication or specificity of the $P700^+$ reaction center interaction domain. In our continuing studies we are seeking to establish a mechanistic framework for protein interfacial electron-transfer processes. It will furthermore be of considerable interest to examine the scope for protein direct electrochemistry as a probe for intrinsic redox properties.

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