Voltammetry of Plastocyanin at a Graphite Electrode: Effects of Structure, Charge, and Electrolyte

Dharmini D. Niles McLeod,[†] Hans C. Freeman,*[,†] Ian Harvey,[†,‡] Peter A. Lay,[†] and Alan M. Bond*[,§]

School of Chemistry, University of Sydney, NSW 2006, Australia, and Department of Chemistry, Monash University, Vic. 3168, Australia

Received May 24, 1996[⊗]

Comparative voltammetric studies on *Anabaena variabilis* plastocyanin (positively charged at neutral pH) and spinach and poplar plastocyanins (negatively charged at neutral pH) have been undertaken at an edge-plane graphite electrode as a function of ionic strength, pH, and Mg^{2+} concentration at 3 °C. The aim was to provide a more detailed understanding of the influence of the electrode−protein (solution) interfacial characteristics, as well as the variation of the formal potential with both the nature of the plastocyanin species and the pH. As might be expected, some of the interfacial properties associated with the positive charge on *A. variabilis* plastocyanin are the opposite of those observed with the negatively charged plastocyanins. For example, the linear diffusion component of the mass transport process for *A. variabilis* plastocyanin under the conditions of cyclic voltammetry is decreased and the radial diffusion component is increased by the addition of Mg^{2+}, whereas the reverse occurs with poplar and spinach plastocyanins. The voltammetrically determined reversible potentials for *A. variabilis* plastocyanin are considerably less positive than those for spinach and poplar plastocyanins, in agreement with values calculated from chemically based redox studies. Ionic strength effects, as determined by addition of $NaClO_4$ over the concentration range 0.005−0.20 M, are negligible for all three proteins. The addition of Mg^{2+} causes a significant shift in the reversible potential toward more positive values for spinach and poplar plastocyanin but only a small positive shift for *A. variabilis* plastocyanin. The difference is attributed to a specific binding effect. The addition of Mg^{2+} also dramatically alters the pH dependence of the reversible potential, indicating that the equilibrium between the protonated and unprotonated forms of reduced plastocyanin is modified by binding of Mg^{2+} to the protein. It is concluded that the effects of biologically relevant redox-inactive cations such as Mg^{2+} or Ca^{2+} have to be considered carefully in studies of the redox chemistry of metalloproteins.

Introduction

Plastocyanin belongs to a class of metalloprotein in which the redox-active site consists of a single coordinated metal atom. The plastocyanin molecule (M_r ~10 500) contains a single "blue" or "type-1" copper active site, so that electron transfer involves an alternation between an oxidized (Cu^{II}) and a reduced (Cu^{I}) state of the protein. The polypeptide not only 'tunes' the metal centre for its redox role, but also provides a molecular configuration that is appropriate for interaction with specific redox partners. The redox properties of plastocyanin are of biological interest because the protein has an essential electron-transfer function in the photosynthetic reaction centers of higher plants as well as some green and blue-green algae.[1−3]

The amino acid sequence and the conformation of the single polypeptide chain of plastocyanin vary slightly, depending on the source. Typically there are about 100 amino acid residues, among which 35 are invariant or conservatively substituted.[1] The structures of Cu^{II}-plastocyanins from three higher plants (poplar,[4] cucumber[5] and oleander[5]), two green algae (*Enteromorpha prolifera*[6] and *Chlamydomonas reinhardtii*[7]), and a blue-green alga (*Anabaena variabilis*[8]) have been determined crystallographically. There is a high degree of structural homology among all these plastocyanins. The major features that they have in common are illustrated by the structures of poplar and *A. variabilis* plastocyanins, which are particularly relevant to the present work (Figure 1). The molecules have the shape of a slightly flattened cylinder with approximate dimensions 40 × 32 × 28 Å. The Cu atom is located in a hydrophobic pocket near one end of the molecule. It is not directly accessible to the solvent, chemical or biological redox reagents, or electrodes. The four Cu-binding residues—two histidines, a cysteine, and a methione—are strictly conserved. The Cu-site dimensions are identical within the crystallographic limits of precision.

While Figure 1 shows the close similarities between poplar and *A. variabilis* plastocyanins, it also shows that the conformations of the polypeptide backbone are not identical. There are some significant conformational differences, and these are associated with differences between the amino acid sequences.

* Corresponding authors.
† University of Sydney.
‡ Present address: School of Applied Sciences, De Montfort University, Leicester LE1 9BH, U.K.
§ Monash University.
⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1996.

(1) Sykes, A. G. *Chem. Soc. Rev.* **1985**, *14*, 283.
(2) Haehnel, W. In *Photosynthesis III*, Staehelin, L. A., Arntzen, C. J., Eds.; Encyclopedia of Plant Physiology, New Series, Vol. 19; Springer Verlag: Berlin, 1986; p 547.
(3) Redinbo, M. R.; Yeates, T. O.; Merchant, S. *J. Bioenerg. Biomembr.* **1994**, *26*, 49.
(4) (a) Colman, P. M.; Freeman, H. C.; Guss, J. M.; Murata, M.; Norris, V. A.; Ramshaw, J. A. M.; Venkatappa, M. P. *Nature (London)* **1978**, *272*, 319. (b) Guss, J. M.; Freeman, H. C. *J. Mol. Biol.* **1983**, *169*, 521. (c) Guss, J. M.; Bartunik, H. D.; Freeman, H. C. *Acta Crystallogr.* **1992**, *B48*, 790.
(5) Church, W. B.; Garrett, T. P. J.; Guss, J. M.; Tong, H.; Freeman, H. C. Unpublished work.
(6) Collyer, C. A.; Guss, J. M.; Sugimura, Y.; Yoshizaki, F.; Freeman, H. C. *J. Mol. Biol.* **1990**, *211*, 617.
(7) Redinbo, M. R.; Cascio, D.; Choucais, M. K.; Rice, D.; Merchant, S.; Yates, T. O. *Biochemistry* **1993**, *32*, 10560.
(8) Fields, B. A.; Govinderaju, K.; Guss, J. M.; Jackman, M. P.; Sykes, A. G.; Freeman, H. C. Manuscript in preparation.

S0020-1669(96)00620-9 CCC: $12.00

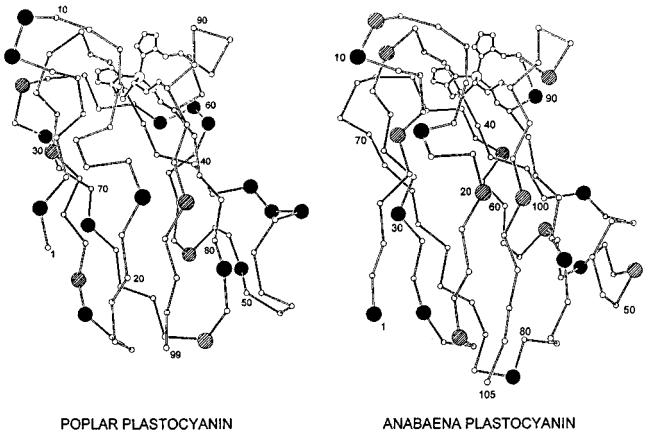

Figure 1. Comparison between the molecular structures of poplar (left) and *Anabaena variabilis* (right) plastocyanins. The blue-green algal protein lacks the "negative patch" of conserved acidic residues found in the plastocyanins of higher plants and most other algae. The molecular structures are represented by the C^{α} atoms, the Cu atom, and the Cu-binding side chains. Filled circles: C^{α} atoms of Asp and Glu residues (side chains negative at pH 7). Shaded circles: C^{α} atoms of Lys and Arg residues (side chains positive at pH 7).

Table 1. Electrostatic Charges on Plastocyanin Molecules at pH 7

plastocyanin	negative charges (Glu^-, Asp^-, Cys^-)	positive charges (Lys^+, Arg^+, free His^+, Cu^+)	formal charge on $Cu^{I}Pc$	formal charge on $Cu^{II}Pc$
poplar	16	7	−9	−8
spinach	16	7	−9	−8
A. variabilis	11	12	+1	+2

The sequences of poplar,[9] spinach,[10] and *A. variabilis*[11] plastocyanins are shown in Figure 2. The higher plant polypeptides have 99 residues, compared with 105 residues in the blue-green algal plastocyanin. The algal polypeptide actually has additional amino acid residues at 8 positions, but it also has a deficiency of 2 residues at positions 58−59−60 of the plant plastocyanin sequences. The shortening of the polypeptide backbone by 2 residues results in the straightening of a prominent kink, which is relatively close to the Cu site and may have a functional significance (see below). On the other hand, the 8 additional residues in the algal protein all occur in loops at the end of the molecule remote from the redox-active Cu atom.

From an electrostatic point of view, it is important that the amino acid composition of *A. variabilis* plastocyanin leads to an overall positive charge on the molecule at neutral pH, in contrast with the overall negative charge on plant plastocyanins (Table 1). The overall charge on a protein molecule is thought to be one of the factors that determine the conditions for well-defined voltammetry.[12] Thus the plastocyanins afford a unique opportunity for probing the influence of overall charge on the electrode−protein (solution) interface within a single class of metalloprotein.

A related property of many plastocyanins is that the overall negative charge is not distributed uniformly over the molecular surface. In the plastocyanins of higher plants, two groups of highly conserved residues with acidic side chains occur on kinks in the polypeptide backbone at positions 42−45 and 59−61. These residues, which are seen on the right-hand surface of the poplar plastocyanin molecule in the orientation of Figure 1, create a prominent region of negative charge at physiological pH. It has been suggested that the "negative patch" is an electrostatic recognition site for the biological electron donor cytochrome *f*,[13] and also for an electrode surface.[14] However, in *A. variabilis* plastocyanin the residues corresponding to the negative patch are not acidic (Figures 1 and 2),[11] and the kink at residues 59−61 is absent due to the shortening of the polypeptide in that region. The absence of a negative patch, together with the structural differences noted above, creates the possibility that the electron-transfer pathway and/or electron-transfer distance in *A. variabilis* plastocyanin differ from those in plant plastocyanins.

The above considerations are relevant mainly to the *kinetics* of the Cu^{II}/Cu^{I} electron-transfer process.[15] The *thermodynamics* of electron transfer, represented by the reversible potential E°_f, are more likely to reflect differences in the geometry of the Cu site in oxidation states I and II. In the oxidized form of plastocyanin ($Cu^{II}Pc$), the Cu atom has a distorted trigonal-pyramidal geometry, being coordinated by a thiolate (Cys84), a thioether (Met92) and two imidazole (His37, His87) groups (Figure 1).[4] The structure of $Cu^{II}Pc$ is independent of pH.[4b] Reduction of $Cu^{II}Pc$ to the Cu^{I} state produces an equilibrium mixture of a high-pH form ($Cu^{I}Pc$) and a protonated low-pH form ($HCu^{I}Pc$).[16] The dimensions of the Cu site in the oxidized and two reduced forms are shown in Figure 3. In $Cu^{I}Pc$ the coordination of the Cu atom is the same as in $Cu^{II}Pc$, with only slight increases in Cu-ligand bond-lengths. As the pH is decreased, an increasing proportion of molecules switch to the $HCu^{I}Pc$ structure in which the Cu atom has a trigonal-planar coordination geometry.[16] The redox chemistry of plastocyanin is therefore pH-dependent and may be described in terms of the reaction scheme given in eqs 1 and 2. If the electron-transfer

$$Cu^{II}Pc + e^- \overset{E^{\circ}_f}{\rightleftharpoons} Cu^{I}Pc \quad (1)$$

$$Cu^{I}Pc + H^+ \overset{K_a}{\rightleftharpoons} HCu^{I}Pc \quad (2)$$

step (eq 1) and the acid−base equilibrium (eq 2) are coupled reversibly, then the reversible potential E°_f must become more positive with decreasing pH. Any differences between the the structures of plant and algal forms of plastocyanin in oxidation state I or II are likely to modify both E°_f and K_a, and therefore also the dependence of E°_f on pH.[17]

Antecedent Redox Studies on Plastocyanins. Detailed redox studies on plant plastocyanins by chemical and electrochemical techniques have been reported.[18−26] Using rate data derived from chemical redox reactions, Sykes *et al.*[24] concluded

(9) Ambler, R. P. Personal communication cited in ref 4b.
(10) (a) Scawen, M. D.; Ramshaw, J. A. M.; Boulter, D. *Biochem. J.* **1975**, *147*, 343. (b) Rother, C.; Jansen, T.; Tyagi, A.; Tittgen, J.; Herrmann, R. G. *Curr. Genet.* **1986**, *11*, 171.
(11) Aitken, A. *Biochem. J.* **1975**, *149*, 675.
(12) (a) Bond, A. M. *Anal. Proc.* **1992**, *29*, 132. (b) Bond, A. M. *Anal. Proc.* **1993**, *30*, 218 and references cited therein.
(13) Roberts, V. A.; Freeman, H. C.; Olson, A. J.; Tainer, J. A.; Getzoff, E. D. *J. Biol. Chem.* **1991**, *266*, 13431 and references cited therein.
(14) Bond, A. M.; Hill, H. A. O. In *Metal Ions in Biolological Systems*, Vol. 27; Sigel, H., Sigel, A., Eds.; Marcel Dekker: New York, 1991; p 431 and references cited therein.
(15) Bowler, B. E.; Raphael, A. L.; Gray, H. B. *Prog. Inorg. Chem.* **1990**, *38*, 259 and references cited therein.
(16) Guss, J. M.; Harrowell, P. R.; Murata, M.; Norris, V. A.; Freeman, H. C. *J. Mol. Biol.* **1986**, *192*, 361.
(17) We use the symbol E°_f to denote the reversible potential under specified conditions. The distinction between E°_f and the less rigorously defined (but frequently used) biological potential $E^{\circ\prime}$ is to be noted.
(18) Sinclair-Day, J. D.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1986**, 2069 and references cited therein.
(19) Armstrong, F. A.; Hill, H. A. O.; Oliver, B. N.; Whitford, D. *J. Am. Chem. Soc.* **1985**, *107*, 1473.
(20) Armstrong, F. A.; Cox, P. A.; Hill, H. A. O.; Lowe, V. J.; Oliver, B. N. *J. Electroanal. Chem. Interfacial Electrochem.* **1987**, *217*, 331.
(21) Datta, D.; Hill, H. A. O.; Nakayama, H. *J. Electroanal. Chem. Interfacial Electrochem.* **1991**, *297*, 309.
(22) Saloman, Z.; Tollin, G. *Arch. Biochem. Biophys.* **1992**, *294*, 382.

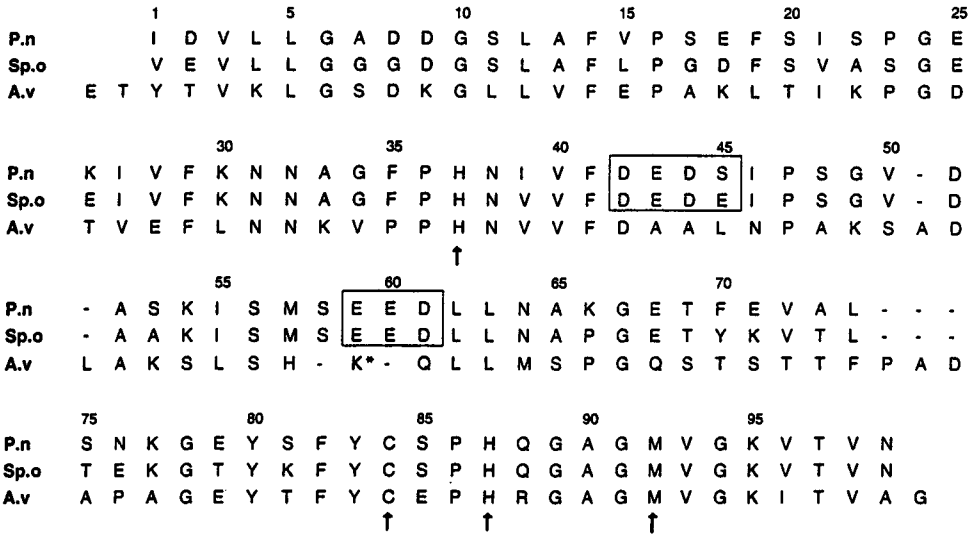

Figure 2. Amino acid sequences of poplar (*Populus nigra* var. *italica*[9]), spinach (*Spinacea oleracea*[10]), and *A. variabilis*[11] plastocyanins. The residue numbers correspond to the amino acid sequence of poplar plastocyanin. Residues Ser58-Glu59-Glu60 in the two higher-plant plastocyanins are replaced by a single Lys residue (labeled Lys59*) in *A. variabilis* plastocyanin.

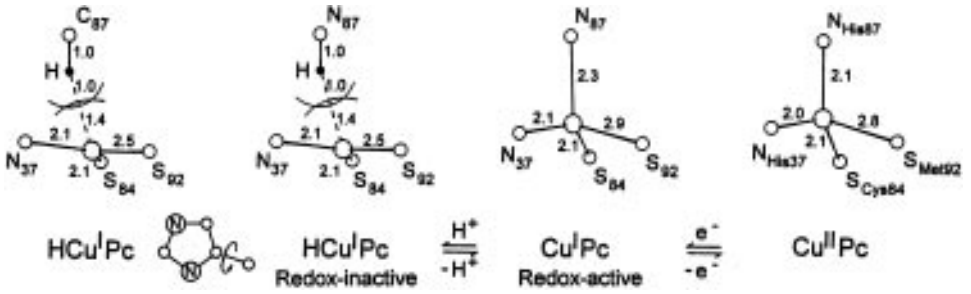

Figure 3. Active site of plastocyanin in (left to right) the protonated reduced form ($HCu^{I}Pc$), the unprotonated reduced form ($Cu^{I}Pc$), and the oxidized form ($Cu^{II}Pc$). The dimensions refer to poplar plastocyanin. (Adapted from refs 4c and 16.

that *A. variabilis* plastocyanin has a less positive $E°_f$ value than the plastocyanins from two plants (parsley and spinach) and another alga (*Scenedesmus obliquus*). Voltammetrically determined $E°_f$ values for four plant plastocyanins (poplar, spinach, cucumber, and parsley) were confined to a narrow range of potentials (389 ± 7 mV vs SHE at 3 °C), although subtle differences among the pH dependences of $E°_f$ and among the electrode−solution interfacial characteristics were found.[23] No voltammetric data for *A. variabilis* plastocyanin have been reported hitherto.

Application of a Microscopic Model to Plastocyanin Electrochemistry. It has been noted previously that the voltammetry of negatively charged plant plastocyanins at graphite electrodes at high pH requires the addition of positively charged surface modifiers, such as Mg^{2+}, if well-defined peak-shaped curves are to be obtained.[19] In the absence of these surface modifiers, small sigmoidal curves are obtained. These observations can be understood in terms of the microscopic model developed by Bond *et al.* for the voltammetry of metalloproteins at graphite electrodes.[12,14,23,31−33] Peak-shaped responses are expected when the entire electrode surface is available for electron transfer. Sigmoidal curves correspond to voltammetry at an electrode surface that is partially blocked. In the latter case, the electron-transfer step occurs at discrete, microscopically small, electroactive sites. When the entire electrode surface is electroactive, mass transport occurs predominantly by linear diffusion to the electrode surface. When the electrode is partially blocked, mass transport occurs by radial diffusion to the residual electroactive sites. The effect of surface modifiers is to unblock a partially blocked surface, i.e., to render the entire surface electroactive. There are concomitant changes from radial to linear diffusion and from sigmoidal to peak-shaped voltammetric responses.

The effects of Mg^{2+} and other positive surface modifiers on the voltammetry of plant plastocyanins at high pH suggest that the mass transport and the electrode−solution interfacial properties are dominated by electrostatic interactions. Contributions to such interactions may result from the large negative overall charge on the plastocyanin molecules and from the presence of a significant number of negative receptor sites on the electrode surface. At low pH, the charge on the electrode surface should

(23) Büchi, F. N.; Bond, A. M.; Codd, R.; Huq, L. N.; Freeman, H. C. *Inorg. Chem.* **1992**, *31*, 5007 and references cited therein.
(24) de Silva, D. G. A. H.; Beoku-Betts, D.; Kyritsis, P.; Govindaraju, K.; Powls, R. P.; Tomkinson, N. P.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1992**, p. 2145.
(25) Conrad, L. S.; Hill, H. A. O.; Hunt, N. I.; Ulstrup, J. *J. Electroanal. Chem. Interfacial Electrochem.* **1994**, *364*, 17.
(26) Sigfridsson, K.; Young, S.; Hansson, Ö. *Biochemistry* **1995**, *35*, 1249.
(27) Armstrong, F. A.; Driscoll, P. C.; Hill, H. A. O. *FEBS Lett.* **1985**, *190*, 242.
(28) Christensen, H. E. M.; Conrad, L. S.; Ulstrup, J. *Photosynth. Res.* **1991**, *28*, 89.
(29) Jackman, M. P.; Sinclair-Day, J. D.; Sisley, M. J.; Sykes, A. G.; Denys, L. A.; Wright, P. E. *J. Am. Chem. Soc.* **1987**, *109*, 6443.
(30) Hananion, G.; Irvine, D.; Eaton, W.; George, P. *J. Phys. Chem.* **1967**, *71*, 2022.
(31) Armstrong, F. A.; Bond, A. M.; Hill, H. A. O.; Psaltis, I. S. M.; Zoski, C. G. *J. Phys. Chem.* **1989**, *93*, 6845 and references cited therein.
(32) Armstrong, F. A.; Bond, A. M.; Hill, H. A. O.; Oliver, B. N.; Psaltis, I. S. M. *J. Am. Chem. Soc.* **1989**, *111*, 9185 and references cited therein.
(33) Büchi, F. M.; Bond, A. M. *J. Electroanal. Chem. Interfacial Electrochem.* **1991**, *314*, 191.

become positive due to the high H^+ concentration, the surface activity should increase, and the positive surface modifiers should be unnecessary. This is the case.

Aims of Present Work. Since *A. variabilis* plastocyanin is distinguished from plant (and other negatively charged) plastocyanins by a reversal of the overall charge and by a different distribution of surface charges, its electrode−solution interfacial properties should be more akin to those of other positively charged metalloproteins, such as horse heart cytochrome *c*.[12,14,31,32] The present voltammetric study of poplar and spinach plastocyanins (negatively charged) and *A. variabilis* plastocyanin and cytochrome *c* (positively charged) was undertaken to probe the influence of electrostatic and/or specific binding terms on the thermodynamics of the electron-transfer process and the electrode−solution interfacial properties. The importance of the electrolyte was studied by varying the pH of the buffer, the concentration of $NaClO_4$, and the concentration of Mg^{2+}. The latter cation was chosen because it is known to bind to plant plastocyanins.[27] The thermodynamic results were compared with results derived independently by Sykes *et al*.[24] The results lead to a more complete understanding of the thermodynamic and kinetic aspects of plant and algal plastocyanin redox chemistry.

Experimental Section

Source, Isolation, and Purification of Metalloproteins. Spinach (*Spinacea oleracea)* and poplar (*Populus nigra*) plastocyanins were purified by the method described by Christensen *et al*.[28] with the following minor modifications. Standard chromatography was used instead of FPLC. Solutions were buffered at pH 6.0 during all stages prior to Q-Sepharose chromatography. Whatman DE-23 and HiLoad Q-Sepharose High Performance resins were replaced by Sephadex A-25 and Q-Sepharose Fast Flow resins, respectively. A final purification step using a Sephacryl S-200 HR gel filtration column was added. It was noted that the two forms of poplar plastocyanin, which display microheterogeneity at 12 amino acid residues,[23] were separated completely on the Q-Sepharose column. The measurements cited in the present paper were made with form I. *A. variabilis* plastocyanin, prepared by a published procedure,[29] was supplied by Professor A. G. Sykes. Horse heart cytochrome *c* was a Boehringer Mannheim product.

The concentration of the plastocyanin used in voltammetric experiments was calculated by measuring the absorbance of the oxidized protein at 597 mm and taking $\epsilon_{597} = 4500\ M^{-1}\ cm^{-1}$. The purity of the protein was confirmed by the ratio A_{280}:A_{597} between the absorbances at 280 and 597 nm. Typical values were 1.13 for spinach and poplar plastocyanins, and 1.15 for *Anabaena* plastocyanin, consistent with the ratio 1.10 reported recently for spinach plastocyanin homogeneous to the resolution of free solution capillary electrophoresis.[26] All plastocyanin samples ran as a single band on SDS polyacrylamide gel-electrophoresis. The molecular weights of the plastocyanins derived from the electrophoresis measurements were 10 500 ± 300.

Materials and Instrumentation for Electrochemical Experiments. All reagents were of analytical grade purity. Water was obtained from a Millipore RO2 system or a Sybron/Barnstadt Nanopure purification system.

Plastocyanin samples (1 mL, 100−200 μM) for the voltammetric measurements were prepared by exhaustive dialysis against the electrolyte solution at the desired pH. The buffers were phosphate/citrate (5 mM) for the range $2.0 \le pH \le 7.5$, and bis-tris propane/HCl for $pH > 7.5$, respectively. $NaClO_4$ (10 mM) was present in all solutions unless otherwise specified. The pH measurements were made with an Orion semi-micro combination pH electrode and a Hanna Instruments pH-meter, which had been calibrated at 3 °C using standard buffers. Stirring and deoxygenation of protein solutions were achieved by bubbling a fine stream of humidified argon or nitrogen (high purity, CIG) through the solution before each experiment, taking care to avoid frothing which may lead to protein denaturation.

A typical electrochemical experiment proceeded as follows: (i) Two identical samples (1 mL) of protein were dialyzed against $NaClO_4$ (10 mM) and $MgCl_2$ (20 mM) at pH ∼6 and pH ∼7, respectively. (ii) The solution at pH ∼7 was placed in the electrochemical cell at 3 °C, its pH was accurately measured, and cyclic voltammograms were recorded. The pH of the sample was then reduced step-wise by adding aliquots of a solution of citrate (50 mM), $NaClO_4$ (10 mM), and $MgCl_2$ (20 mM), and a voltammogram was recorded after each addition. (iii) The solution at pH ∼6 was treated in a similar manner, except that the pH was increased step-wise by adding a solution containing phosphate (50 mM), $NaClO_4$ (10 mM), and $MgCl_2$ (20 mM). (iv) The concentration of $MgCl_2$ or $NaClO_4$ in the electrolyte was then varied as required.

Electrochemical measurements were made in triplicate. The cell for the voltammetric experiments was thermostated at 3 °C rather than at 25 °C, the temperature commonly used for reporting thermodynamic data, in order to minimise the risk of protein deterioration. The edge-plane graphite working electrode used in most experiments was prepared by sealing a disk of pyrolytic graphite (radius 2.4 mm, disk face parallel to the edge plane) in a glass tube. Internal connection to a copper rod was made by means of a silica-bonded epoxy adhesive. The working electrode was polished consecutively with 6-, 1-, 0.1-, and 0.07-μm diamond pastes before initial use. Before each voltammetric experiment the electrode was repolished with an aqueous slurry of 0.5-μm alumina on a clean polishing cloth (Buehler), thoroughly rinsed with water, and sonicated for 30 s. A glassy carbon working electrode (radius 1.5 mm) was used as an alternative to the edge-plane graphite electrode in some experiments. The auxiliary electrode was a platinum wire.

The Ag/AgCl (saturated KCl) reference electrode was calibrated against the $[Fe(CN)_6]^{3-/4-}$ couple. The reference electrode was separated from the test solution by a salt bridge and was thermostated at 25(±0.1) °C. Such a nonisothermal configuration has the advantage that contributions to the potential from the temperature dependence of the reference electrode are eliminated. The calibration was made as follows: (i) Two solutions of $K_3[Fe(CN)_6]$ (200 μM) were prepared: solution A containing KCl (0.1 M) and solution B containing the electrolyte solution to be used for protein electrochemistry. $MgCl_2$ was omitted from the electrolyte solution in order to avoid possible interference due to ion-pairing with $[Fe(CN)_6]^{3-}$. (ii) Both the reference and working compartments of the cell were thermostated at 25(±0.1) °C. The solutions in the reference and working compartments were KCl (0.1 M) and Solution A, respectively. (iii) The potential of $[Fe(CN)_6]^{3-/4-}$ vs Ag/AgCl (saturated KCl) was taken to be the average of the oxidation (E_p^{ox}) and reduction (E_p^{red}) peak potentials in cyclic voltammograms. The literature value for $E°_f$ vs NHE for $[Fe(CN)_6]^{3-/4-}$ in KCl [28] was then used to calibrate the potential of the reference electrode. (iv) The working compartment was emptied and refilled with solution B, which was then thermostated at 3.0(±0.3) °C in order to simulate the working conditions for protein electrochemistry. The reference compartment was filled with the electrolyte solution and maintained at 25(±0.1)°C so as to avoid changing the potential of the reference electrode. (v) The potential of the $[Fe(CN)_6]^{3-/4-}$ couple was measured using cyclic voltammetry. The resulting value, corrected for the potential of the reference electrode, was used in all future experiments as the $E°_f$ of the $[Fe(CN)_6]^{3-/4-}$ couple at 3 °C.

An example of the calculation of the reversible potential $E°_f$ for plastocyanin is given in Figure 4. The $E°_f$ value of the Ag/AgCl reference electrode measured as above was determined to be 168(±1) mV vs NHE, and the $E°_f$ value of the $[Fe(CN)_6]^{3-/4-}$ couple in the phosphate/citrate/$NaClO_4$ electrolyte at 3 °C was 383(±1) mV vs NHE. Drifts in potential were corrected by recalibrating the reference electrode against the $[Fe(CN)_6]^{3-/4-}$ couple before and after each protein electrochemistry experiment.

Cyclic voltammograms were recorded using a Bioanalytical Systems (BAS) Model 100A electrochemical analyzer in conjunction with a Houston Instruments DMP series digital plotter. Typically, voltammograms were recorded at a scan rate of 10 mV s^{-1} over a potential range from +450 to −100 mV vs Ag/AgCl. Reversible potentials were calculated as the average of the reduction (E_p^{red}) and oxidation (E_p^{ox}) peak potentials in cyclic voltammograms. However, if sigmoidal curves were obtained, the reversible potential $E°_f$ was equated with the half-wave potential $E_{1/2}{}^r$.[12,14,17]

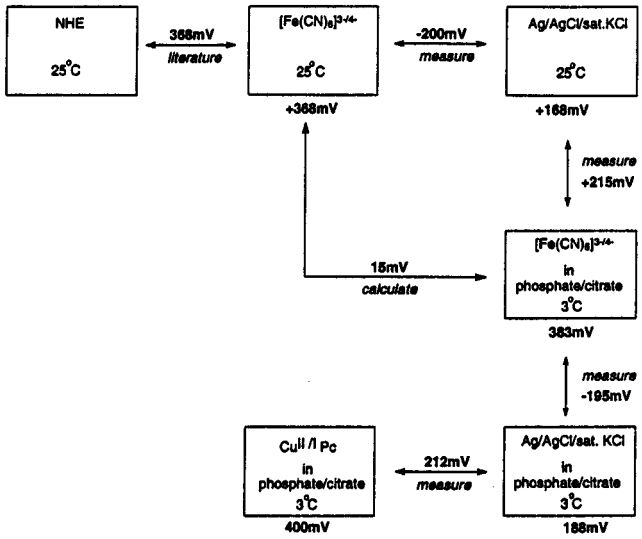

Figure 4. Calibration of the reversible potential of plastocyanin with respect to the normal hydrogen electrode (NHE).

Table 2. Voltammetric Data Recorded at an Edge-Plane Graphite Electrode for Horse Heart Cytochrome *c* (150 μM) at 3 °C in Aqueous Media Containing $NaClO_4$ (10 mM) and Phosphate−Citrate Buffer (5 mM) at pH 7.0

$[Mg^{2+}]$ (mM)	scan rate (mV s^{-1})	E^{o}_f (mV vs NHE)	$E_p^{red} - E_p^{ox}$ (mV)	i_p (μA)	comment
0	10	236	66	0.34	*c*
0	50	235	105	0.40	*d*
0	200	235	(55)[a]	(0.50)[b]	*e*
10	10	233	71	0.19	*c*
10	50	235	105	0.39	*d*
10	200	234	(55)[a]	(0.45)[b]	*e*
20	10	232	78	0.19	*c*
20	50	231	120	0.36	*d*
20	200	233	(55)[a]	(0.42)[b]	*e*

[a] Sigmoidal curve. Value shown is ($E_{3/4} - E_{1/4}$) rather than ($E_p^{red} - E_p^{ox}$). [b] Sigmoidal curve. Value shown is limiting current i_L rather than i_p. [c] Peak-shaped curve corresponding to mass transport predominantly by linear diffusion. [d] Peak detected, but mass transport is by a mixture of linear and radial diffusion. [e] Sigmoidal curve with mass transport predominantly by radial diffusion.

Results

Voltammetry of Cytochrome *c* (Positively Charged) in the Presence of Mg^{2+}. At pH 7, voltammograms for cytochrome *c* at an edge-plane graphite electrode in phosphate/citrate buffer and $NaClO_4$ (10 mM) are well-defined.[31] At low scan rates, the process corresponds to reversible electron transfer with mass transport by linear diffusion. At high scan rates, radial diffusion becomes evident. On addition of Mg^{2+}, the reduction and oxidation currents decrease in magnitude and the radial diffusion component increases. After addition of 30 mM Mg^{2+}, no response above the background current is observed. Relevant data and representative voltammograms are shown in Table 2 and Figure 5, respectively. Despite the dramatic effect of Mg^{2+} on the wave shape, the reversible potential $E°_f$ is essentially independent of Mg^{2+} concentration (Table 2). This implies that the Mg^{2+} cations affect the mass transport rather than the thermodynamics of the electron-transfer process: Mg^{2+} ions become attached to the graphite surface, causing previously active sites to become unfavorable for electron transfer to positively charged cytochrome *c*. The effect of H^+ is similar. If the pH is reduced to 4.5, no $Fe^{III/II}$ couple is observed at the positively charged edge-plane graphite electrode surface. However, while the concentration of Mg^{2+} *cations* has relatively little effect on the reversible potential $E°_f$ of the cytochrome *c* response, the opposite is observed when multiple-charged *anions* are present. It has, for example, been shown elsewhere that changes in the concentration of phosphate anions have a strong influence on the thermodynamics of electron transfer to cytochrome *c* as represented by $E°_f$ (Figure 6).[34]

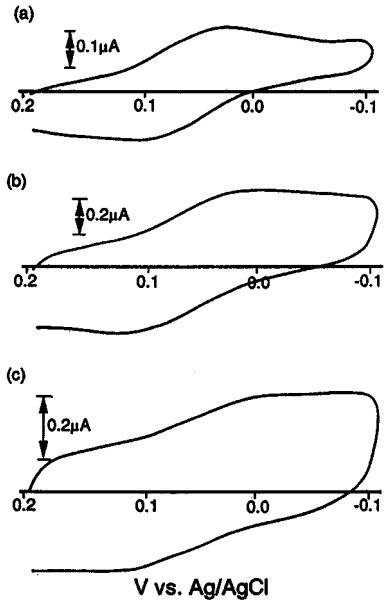

Figure 5. Voltammograms for cytochrome *c* in the presence of Mg^{2+} (20 mM) at an edge-plane pyrolytic graphite electrode. As the scan rate is increased, the voltammograms become more sigmoidal, showing the increasing contribution of radial diffusion (25 °C, pH 7.0, phosphate−citrate buffer, 10 mM $NaClO_4$): (a) 10 mV s^{-1}; (b) 50 mV s^{-1}; (c) 200 mV s^{-1}.

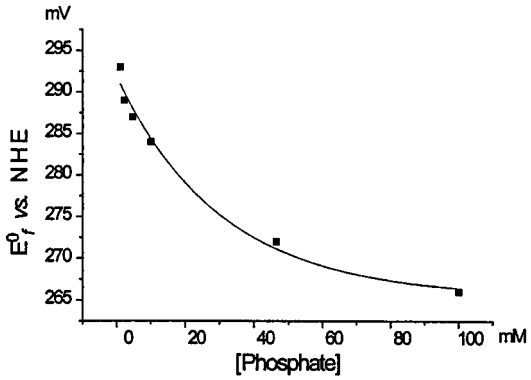

Figure 6. Variation of $E°_f$ for horse heart cytochrome *c* as a function of potassium phosphate buffer concentration at pH 7. The values of $E°_f$ (mV *vs* NHE) were obtained by cyclic voltammetry at a glassy carbon electrode (22 °C, scan rate 10 mV s^{-1}; Data from ref 34).

Voltammetry of *A. variabilis* Plastocyanin (Positively Charged) in the Presence of Mg^{2+}. The overall charge on the *A. variabilis* plastocyanin molecule is small but positive (+1, Table 1), so that electrostatic interaction terms at the electrode−solution interface are predicted to be relatively small. Table 3 provides data from which the influence of Mg^{2+} concentration may be ascertained, while Figure 7 shows some relevant voltammograms. The voltammetry at pH 7.0 in the absence of Mg^{2+} essentially corresponds to mass transport by linear diffusion over the scan-rate range 10−200 mV s^{-1} (Figure 7a). In this regime, i_p^{red} is proportional to the square root of the scan rate. However, if the pH is reduced to 4.5, or if the scan rate is increased, or if Mg^{2+} is added, then sigmoidal curves

(34) Hagen, W. R. *Eur. J. Biochem.* **1989**, *182*, 253.

Table 3. Effect of Mg^{2+} on Voltammetric Data for *A. variabilis* Plastocyanin at an Edge-Plane Pyrolytic Graphite Electrode (3 °C, 100−200 μM Protein, 5 mM Phosphate−Citrate Buffer, 10 mM $NaClO_4$)

$[Mg^{2+}]$ (mM)	pH	scan rate (mV s^{-1})	$E°_f$ (mV vs NHE)	$E_p^{red} - E_p^{ox}$ (mV)	i_p^{red} (μA)
0	7.0	10	323	60	0.53
0	7.0	50	325	72	1.12
0	7.0	200	326	82	2.42
10	7.0	10	326	59	0.30
10	7.0	50	324	72	0.84
10	7.0	200	325	82	1.71
20	7.0	10	321	66	0.42
20	7.0	50	319	72	1.10
20	7.0	200	321	92	2.22
30	7.0	10	326	59	0.30
30	7.0	50	324	72	0.84
30	7.0	200	326	82	1.71
0	4.5	10	370	82	0.21
0	4.5	50	370	95	0.50
0	4.5	200	372	112	0.95
10	4.5	10	366	83	0.18
10	4.5	50	366	114	0.51
10	4.5	200	366	120	0.98
20	4.5	10	350	114	0.19
20	4.5	50	350	95	0.52
20	4.5	200	348	*a*	0.72
30	4.5	10	350	120	0.07
30	4.5	50	350	*a*	*a*
30	4.5	20	350	*a*	*a*

[a] Sigmoidal curve with reversible shape. $(E_{1/4} - E_{3/4}) = 50$ mV.

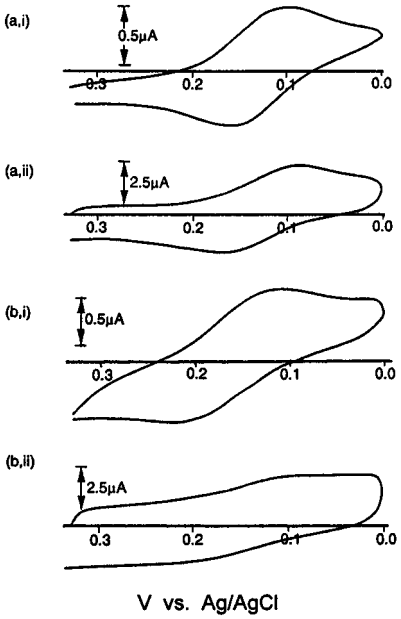

Figure 7. Effects of pH, Mg^{2+} concentration, and scan rate on voltammograms for *A. variabilis* plastocyanin at an edge-plane pyrolytic graphite electrode (3 °C, phosphate−citrate buffer, 10 mM $NaClO_4$): (a) pH 7.0, no Mg^{2+}, scan rate (i) 10 mV s^{-1} and (ii) 200 mV s^{-1}; (b) pH 4.5, 30 mM Mg^{2+}, scan rate (i) 10 mV s^{-1} and (ii) 200 mV s^{-1}.

are obtained (Figure 7b). These observations indicate that radial diffusion becomes dominant, presumably because the presence of positive charges on the electrode surface leads to electrostatic blockage. Consistent with this hypothesis, i_p^{red} shows a reduced dependence on scan rate at pH 4.5 and in the presence of high Mg^{2+} concentrations. The effects of Mg^{2+} are, however, less dramatic than in the case of the more positively charged protein, cytochrome *c*. A further difference from the behavior of cytochrome *c* is that the reversible potential $E°_f$ of *A. variabilis* plastocyanin is affected by the presence of Mg^{2+}, becoming more positive with increasing Mg^{2+}concentration. The details of the pH effects will be considered subsequently.

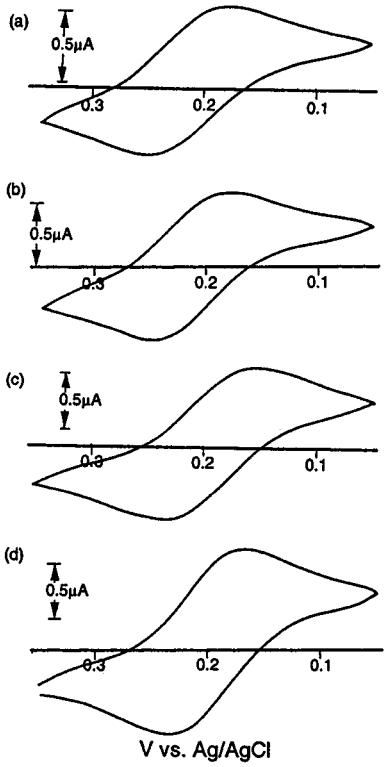

Figure 8. Effects of Mg^{2+} concentration and pH on cyclic voltammograms for poplar plastocyanins at an edge-plane graphite electrode (3 °C, scan rate 10 mV $s^{-1,}$ phosphate−citrate buffer, 10 mM $NaClO_4$): (a) 0 mM Mg^{2+}, pH 7; (b) 0 mM Mg^{2+}, pH 4.5; (c) 30 mM Mg^{2+}, pH 7.0; (d) 30 mM Mg^{2+}, pH 4.5.

Table 4. Effect of Mg^{2+} on Voltammetric Data for Poplar Plastocyanin at an Edge-Plane Pyrolytic Graphite Electrode (scan rate 10 mV s^{-1}, 3 °C, 100−200 μM Protein, 5 mM Phosphate−Citrate Buffer, 10 mM $NaClO_4$)

$[Mg^{2+}]$ (mM)	pH	$E°_f$ (mV vs NHE)	$E_p^{red} - E_p^{ox}$ (mV)
0	7.0	379	82
0	4.5	394	69
10	7.0	380	115
10	4.5	392	80
20	7.0	389	98
20	4.5	401	92
30	7.0	396	72
30	4.5	398	68

In summary, Mg^{2+} appears to block the electrode surface with respect to the voltammetry of positively charged *A. variabilis* plastocyanin. The effect is smaller than for cytochrome *c*, which has a larger positive charge. However, in contrast with cytochrome *c*, the addition of Mg^{2+} to a solution of *A. variabilis* plastocyanin alters the reversible potential, particularly at low pH.

Voltammetry of Spinach and Poplar Plastocyanins (Negatively Charged) in the Presence of Mg^{2+}. As reported previously,[23] and in complete contrast to *A. variabilis* plastocyanin, the voltammetry of negatively charged spinach and poplar plastocyanins at high pH in the absence of Mg^{2+} has a partly sigmoidal character, consistent with significant radial diffusion. Again in contrast with *A. variabilis* plastocyanin, the response is peak-shaped at low pH, irrespective of whether Mg^{2+} is present or not. Voltammograms (Figure 8) and data for poplar plastocyanin at different pH values and Mg^{2+} concentrations (Table 4) illustrate these features. In terms of the influence of electrostatic interactions at the electrode

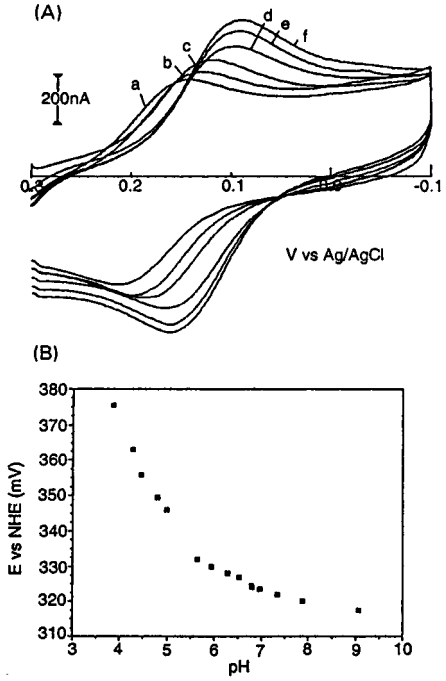

Figure 9. Effect of pH on voltammetric data for *A. variabilis* plastocyanin at an edge-plane pyrolytic graphite electrode. At higher pH's the voltammograms become increasingly peak-shaped, indicating a trend from radial to linear diffusion. The reversible potential reaches an asymptotic value of 318 mV at pH ~9 (3 °C, scan rate 20 mV s^{-1}, phosphate−citrate buffer, 10 mM $NaClO_4$, 20 mM Mg^{2+}): (A) cyclic voltammograms at pH (a) 3.83, (b) 4.27, (c) 4.79, (d) 5.67, (e) 6.95, (f) 7.33; (B) plot of reversible potential versus pH.

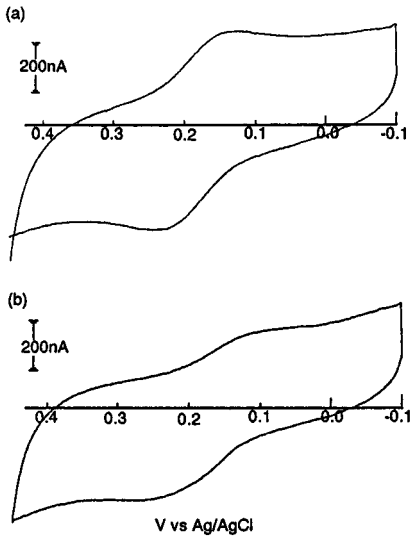

Figure 10. Cyclic voltammograms for poplar plastocyanin at an edge-plane pyrolytic graphite electrode in the presence of 20 mM Mg^{2+} at (a) pH 4.53 and (b) pH 6.21. Radial diffusion makes an increasing contribution as the pH increases, in contrast with *A. variabilis* plastocyanin in Figure 9 (3 °C, scan rate 10 mV s^{-1}, phosphate−citrate buffer, 10 mM $NaClO_4$).

interface, there is an inverse relationship between the negatively charged plant plastocyanins and the positively charged algal *A. variabilis* plastocyanin. For the negatively charged proteins, the contribution of radial diffusion to the voltammetry is decreased by the addition of Mg^{2+}, instead of being enhanced as it is for the positively charged protein. The thermodynamic effects of adding Mg^{2+} to the two classes of plastocyanin, however, differ in magnitude rather than direction. For both negatively and positively charged plastocyanins, the reversible potentials are shifted to more positive values when the concentration of Mg^{2+} is increased, but the shift in potential is considerably larger for the negatively charged proteins. Thus there is a difference between the thermodynamic and electrode−protein interfacial influences of Mg^{2+}: the thermodynamic effects are in the same direction for all plastocyanins, whereas the electrostatic effects at the electrode−protein interface depend on the charge on the protein molecule.

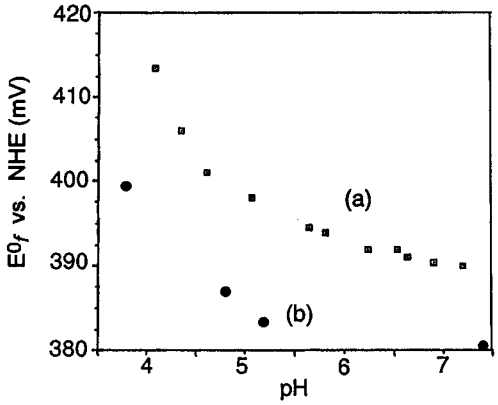

Figure 11. Effect of Mg^{2+} on the value and pH dependence of the reversible potential for spinach plastocyanin at an edge-plane pyrolytic graphite electrode (3 °C, phosphate−citrate buffer, 10 mM $NaClO_4$): (a) 20 mM Mg^{2+}; (b) 5 mM Mg^{2+}.

Voltammetry of Poplar Plastocyanin as a Function of Ionic Strength. In order to study the effect of ionic strength on the electrochemistry of plastocyanin, voltammograms were recorded for poplar plastocyanin in the presence of Mg^{2+} (20 mM) and a range of concentrations of $NaClO_4$ (5−200 mM). All the solutions were at pH 7.0. Within experimental error (±2 mV), the reversible potential was independent of $NaClO_4$ concentration, suggesting that neither the concentration of Na^+ nor the ionic strength are significant variables in the thermodynamics of plastocyanin over the range examined. Parallel voltammetric studies on spinach and *A. variabilis* plastocyanins were less extensive, but sufficient to indicate that the reversible potentials of these proteins are also almost independent of $NaClO_4$ concentration over the range 5−200 mM. The reduction and oxidation peak potentials and other characteristics, such as wave shape, are likewise insensitive to the concentration of $NaClO_4$ for all three plastocyanins. We conclude that the major influence of Mg^{2+} on the reversible potentials arises from specific interactions with the plastocyanin and electrode, rather than from nonspecific ionic-strength effects.

Voltammetry of *A. variabilis*, Spinach, and Poplar Plastocyanins as a Function of pH. The effects of pH on the electrochemistry of *A. variabilis*, spinach, and poplar plastocyanins are illustrated in Figures 9−11. Both the wave shape and the reversible potential of *A. variabilis* plastocyanin in the presence of Mg^{2+} (20 mM) depend on pH (Figure 9). Qualitatively similar observations are shown for the pH dependence of the wave shape for poplar plastocyanin (Figure 10) and for the reversible potential of spinach plastocyanin (Figure 11). The reversible potential $E°_f$ for *A. variabilis* plastocyanin is pH-independent at pH ~8 (318 mV vs NHE), whereas that for spinach plastocyanin is pH-independent at pH ~6.5 (390 mV vs NHE in the presence of 20 mM Mg^{2+} and 380 mV in the presence of 5 mM Mg^{2+}). The behavior of poplar plastocyanin is similar to that of spinach plastocyanin with respect to the pH-independent value, pH dependence, and Mg^{2+} concentration dependence of $E°_f$.

Voltammetry at Glassy Carbon Electrodes. A number of experiments were undertaken to compare the voltammetric behaviors of plastocyanins at glassy carbon and edge-plane

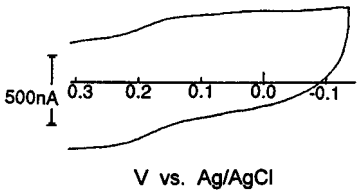

Figure 12. Cyclic voltammogram for spinach plastocyanin at a glassy carbon electrode in the presence of 20 mM Mg^{2+}, showing the predominantly radial diffusion (3 °C, scan rate 10 mV s^{-1}, pH 6.53, phosphate−citrate buffer, 10 mM $NaClO_4$).

graphite electrodes. When glassy carbon electrodes were used, spinach and poplar plastocyanins yielded no detectable response near the reversible potentials obtained at graphite electrodes, unless Mg^{2+} was added. Even then, only very weak sigmoidal responses were observed (Figure 12). We infer that a glassy carbon electrode has very few sites suitable for electron transfer to negatively charged plastocyanin molecules. On the other hand, positively charged *A. variabilis* plastocyanin gave well-defined cyclic voltammograms at a glassy carbon electrode, both in the presence and absence of Mg^{2+}. The reversible potentials were the same as those obtained at the edge-plane pyrolytic graphite electrode. These results confirm those of Sakurai *et al.*,[35] who found that cucumber plastocyanin (negatively charged) gave no response at a glassy carbon electrode without addition of Mg^{2+}, whereas the positively charged blue copper proteins pseudo-azurin, cucumber basic protein and stellacyanin gave well-defined peak-shaped (linear diffusion) voltammograms at a scan rate of 1 mV s^{-1}. The reversible potentials of the positively charged proteins at the bare glassy carbon electrode were identical to those obtained by potentiometric methods. Other positively charged metalloproteins also give well-defined voltammetric responses at unmodified glassy carbon electrodes.[34] In all these cases, the overall charge of the protein molecule seems to be crucial with respect to the interfacial electrode−protein characteristics.

Discussion

The present voltammetric studies demonstrate that addition of Mg^{2+} to plastocyanin solutions affects the measurement of electrochemical data at carbon electrodes in two distinctly different ways. First, Mg^{2+} binds to the electrode surface, or is incorporated into the carbon electrode. This leads to significant electrostatic electrode−protein/solution interactions, which influence the mass transport (radial *versus* linear diffusion). Second, Mg^{2+} binds at specific sites on the plastocyanin molecule, causing changes in the thermodynamics and/or kinetics of electron transfer. We shall discuss these effects in turn.

Electrostatic Electrode−Protein Interactions. Positively charged pyrolytic graphite electrode surfaces provide well-defined and unblocked sites for electron transfer with spinach, poplar, and other negatively charged plastocyanins. This has been recognized previously.[12,14,23,31−33] The addition of Mg^{2+} to the solution creates or enhances positive sites on the electrode surface. This results in well-defined peak-shaped voltammograms (consistent with linear diffusion), as found in the present work for negatively charged plastocyanins over wide pH ranges. Conversely, addition of Mg^{2+} hinders the voltammetry of positively charged proteins, such as *A. variabilis* plastocyanin. In the case of this protein, well-defined voltammograms are obtained directly at edge-plane pyrolytic graphite and glassy carbon electrodes. Similar results are obtained with other positively charged blue copper proteins (see above) and with cytochrome *c*. So far as the metalloproteins are concerned, the dominant parameter in electrostatic electrode−protein/solution interactions is the overall charge, rather than the distribution of charge, on the molecule.

Specific Mg^{2+}−Protein Interactions. The present work shows that the addition of Mg^{2+} also has a significant effect on the reversible potentials and their pH dependence, and that these effects are in the same direction for both negatively and positively charged plastocyanins. On addition of Mg^{2+}, the reversible potentials are shifted to more positive values, and remain insensitive to pH over a much wider pH range. The shift in reversible potential to more positive values means that it is easier to reduce $Cu^{II}Pc$ to $Cu^{I}Pc$ in the presence of Mg^{2+}, i.e., that the Cu^{I} form of the protein is stabilized with respect to the Cu^{II} form by interaction with Mg^{2+}. The pH dependence of the reversible potentials in acid media is attributed to the equilibrium reaction between $HCu^{I}Pc$ and $Cu^{I}Pc$ and is governed by the value of K_a in eq 2. Mg^{2+}-binding therefore stabilizes $Cu^{I}Pc$ not only with respect to $Cu^{II}Pc$ (affecting $E°_f$) but also with respect to $HCu^{I}Pc$ (affecting the pH dependence of $E°_f$).

The changes in reversible potential cannot be due predominantly to ionic strength effects, since it has already been shown that changing the concentration of $NaClO_4$ has no measurable effect. Therefore the effects of Mg^{2+} on the redox and protonation equilibria of plastocyanin imply that there are specific interactions between the cation and the protein.

The hypothesis that there are one or more specific interactions between Mg^{2+} and plastocyanin is supported by proton nuclear magnetic resonance (NMR) studies.[36−41] These have shown, for example, that the rate constant of the normally slow electron self-exchange reaction between spinach $Cu^{I}Pc$ and $Cu^{II}Pc$ can be enhanced dramatically by the presence of Mg^{2+} and other redox-inert multivalent cations.[37] The protein−cation interactions have been investigated by 1- and 2-dimensional proton NMR methods.[38−40] When negatively charged spinach plastocyanin and positively charged horse heart cytochrome *c* interact to form a 1:1 protein−protein complex, binding of cations such as Mg^{2+} occurs at a range of negatively charged sites.[41] The cations may be mobile and diffuse or roll around the protein. Even in the 1:1 plastocyanin−cytochrome *c* complex, the interaction with Mg^{2+} may be multisited. Although NMR data are available only for spinach plastocyanin it may be postulated that interactions with Mg^{2+} are not limited to the negatively charged spinach plastocyanin but may also occur at negative surface sites on the positively charged protein, *A. variabilis* plastocyanin.

Other Examples of Electrostatic Effects in Plastocyanin Voltammetry. The data obtained in the present work imply that the experimental conditions under which the reversible (formal) potentials of metalloproteins are determined must be carefully considered. The presence of cations, anions or other proteins that can bind to a metalloprotein may modify the reversible potential and electrochemical behavior. Plastocyanins appear to be particularly sensitive to this effect. The effect of

(35) Sakurai, T.; Ikeda, O.; Suzuki, S. *Inorg. Chem.* **1990**, *29*, 4715.
(36) Beattie, J. K.; Fensom, D. J.; Freeman, H. C.; Woodcock, E.; Hill, H. A. O.; Stokes, A. M. *Biochim. Biophys. Acta* **1975**, *405*, 109.
(37) Armstrong, F. A.; Driscoll, P. C.; Hill, H. A. O. *FEBS Lett.* **1985**, *190*, 242.
(38) Armstrong, F. A.; Driscoll, P. C.; Hill, H. A. O.; Redfield, F. *Biochem. Soc. Trans.* **1987**, *15*, 767.
(39) Armstrong, F. A.; Driscoll, P. C.; Hill, H. A. O.; Redfield, C. *J. Inorg. Biochem.* **1986**, *28*, 171.
(40) Driscoll, P. C.; Hill, H. A. O.; Redfield, C. *Eur. J. Biochem.* **1987**, *170*, 279.
(41) Bagby, S.; Driscoll, P. C.; Goodall, K. G.; Redfield, C.; Hill, H. A. O. *Eur. J. Biochem.* **1990**, *188*, 413.

positively charged reagents on the electrochemistry of plastocyanin is illustrated by the following examples.

In a recent study, Sykes *et al.* examined the voltammetry of native *Scenedesmus obliquus* plastocyanin and a derivative obtained by attachment of $[-Ru(NH_3)_5]^{3+/2+}$ to the imidazole group of His59.[42,43] The working electrode was edge-plane graphite, and neomycin sulfate was added as an electrode surface modifier. *S. obliquus* plastocyanin has a large negative charge (−8 for $Cu^{II}Pc$), and neomycin is a cation, so that the combination is in principle analogous to spinach or poplar Pc in the presence of Mg^{2+}. When *S. obliquus* plastocyanin was modified by combination with $[-Ru(NH_3)_5]^{3+/2+}$, it gave well-defined voltammetry at pH 7.0 without addition of the so-called cation promoter. Native *S. obliquus* plastocyanin, on the other hand, gave a peak-shaped voltammetric response only when 0.4 mM neomycin was present. During the initial stages of experiments in which neomycin was titrated into the electrochemical cell, the voltammograms were weak, impersistent, and sigmoidal. The presence of 0.4 mM neomycin was required to achieve a stable voltammetric response and a ($E_p^{ox} - E_p^{red}$) peak separation approaching the theoretically expected value of about 60 mV. There is a remarkable similarity between the effects produced by attaching a single Ru complex with a formal 3+ charge at His59, and those produced by adding Mg^{2+} or neomycin to the solution. This strongly suggests that a localized charge as well as the overall charge on the protein can be used to achieve linear diffusion at an electrode and that both specific and nonspecific charge effects are operative.

Data reported by Sykes *et al.*[42,43] also imply that the formal potential of *S. obliquus* plastocyanin is altered by $[-Ru(NH_3)_5]^{3+/2+}$ modification and/or the addition of neomycin. The $E°_f$ values obtained from titrations of native and Ru-modified S. obliquus plastocyanin with $[Fe(CN)_6]^{3-/4-}$ at 25 °C were 363 and 385 mV vs SHE, respectively. In contrast, the $E°_f$ value for the native protein calculated from reaction with $[Co(phen)_3]^{3+/2+}$ was 376(±5) mV vs SHE, and voltammetry in the presence of neomycin gave values 389(±5) and 392-(±5) mV vs SHE for the native and Ru-modified protein, respectively. While there is always some uncertainty in correcting potentials obtained from the experimentally used reference electrode to the NHE or SHE scale, the available data consistently indicate that both the modification of plastocyanins by attaching a positive charge and the addition of neomycin or Mg^{2+} to the solution shift potentials to more positive values. In other words, charged chemical redox reagents and charged voltammetric modifiers are not innocent in the thermodynamic sense.

Figure 13, reproduced from ref 24, illustrates the variation in reversible potentials for several plastocyanins under a range of chemical redox conditions. The voltammetric data obtained in the present work, like the data in Figure 13, also show that positively charged *A. variabilis* plastocyanin has a considerably more positive $E°_f$ value than any of the negatively charged plastocyanins. However, we consistently obtain more positive values in the presence of Mg^{2+} than in the absence of Mg^{2+}, and the pH dependence of the reversible potential is also modified by the addition of Mg^{2+}. These observations and Sykes' $[-Ru(NH_3)_5]^{3+/2+}$ data imply that binding of cations modifies the thermodynamics of the electron-transfer process with respect to the values of both $E°_f$ and K_a. It appears that the binding of cations to plastocyanins stabilizes the Cu^I state

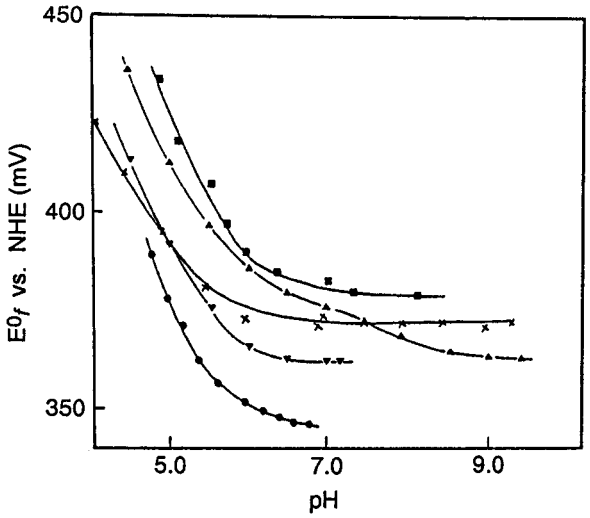

Figure 13. Dependence of the reversible potential on pH for plastocyanins at 25 °C and at ionic strength 0.10 M, obtained from kinetic measurements with $[Co(phen)_3]^{3+/2+}$ or $[Fe(CN)_6]^{3-/4-}$ as redox partner: ▲, *Scenedesmus obliquus* plastocyanin; ×, spinach plastocyanin; ▼, poplar plastocyanin; ●, *A. variabilis* plastocyanin. Reproduced with permission from ref 24. Copyright 1992 Royal Society of Chemistry.

with respect to the Cu^{II} state and also modifies K_a for the equilibrium between the two forms of the Cu^I protein (Figure 3).

Evidence for the importance of the cations in the electrolyte may also be adduced from the work of Hill *et al.*, who have reported voltammetric data for complexes of spinach plastocyanin with cytochrome *c* at pH 7.0.[44] In view of the large positive charge on cytochrome *c*, it is not surprising that well-defined peak-shaped voltammograms are observed for spinach plastocyanin in the presence of cytochrome *c*: if $[-Ru(NH_3)_5]^{3+}$ binding at His59 in a plastocyanin results in well-defined voltammetry, then the formation of a cytochrome *c*−plastocyanin complex should produce a similar result, and this is found to be the case. However, the $E°_f$ value found by Hill *et al.* for the protein−protein complex is 340 mV *vs* NHE, compared with 370 mV vs NHE for spinach Pc in the presence of 2 mM $[Cr(NH_3)_6]^{3+}$. Use of a Zn(II)-cytochrome *c*−plastocyanin combination gave a similar $E°_f$ value, 345 mV vs NHE, so that the Fe atom at the cytochrome *c* redox centre clearly does not influence the plastocyanin electrochemistry. However, voltammetry of a 1:1 cytochrome *c*−plastocyanin mixture at a $(Lys\text{-}Cys\text{-}OMe)_2$-modified gold electrode gives an $E°_f$ value of 370 mV, indicating that even a protein modifier is not necessarily completely innocent in the thermodynamic sense.

NMR studies indicate that the 1:1 complex formed by spinach plastocyanin and cytochrome *c* has an equilibrium constant of 67 mM for the dissociation reaction at pH 7.0.[41] Competitive binding studies show that the plastocyanin molecule is still accessible to $[Cr(NH_3)_6]^{3+}$ in the 1:1 complex and that $[Cr(NH_3)_6]^{3+}$ does not disrupt the formation of the complex in any detectable way. Thus it appears that even though the initial interaction with cytochrome *c* takes place at the "acidic patch" of plastocyanin, the negatively charged surface of plastocyanin may be sufficiently extensive to accommodate both $[Cr(NH_3)_6]^{3+}$ and cytochrome *c* in a dynamic ternary complex. The data in ref 42 are consistent with this concept, since the effects of the positively charged cytochrome and the positively charged $[Cr(NH_3)_6]^{3+}$ on the $E°_f$ value appear to be additive.

(42) Jackman, M. P.; McGinnis, J.; Powls, R.; Salmon, G. A.; Sykes, A. G. *J. Am. Chem. Soc.* **1988**, *110*, 5880.
(43) Armstrong, F. A.; Butt, J. N.; Govindaraju, K.; McGinnis, J.; Powls, R.; Sykes, A. G. *Inorg. Chem.* **1990**, *29*, 4858.
(44) Bagby, S.; Barker, P. D.; Guo, L.-H.; Hill, H. A. O. *Biochemistry* **1990**, *29*, 2313.

Even larger potential shifts are caused by the addition of a positively charged modifier when the electrochemistry is studied at a gold electrode modified with a $(Lys\text{-}Cys)_2$ peptide.[45] At a $(Lys\text{-}Cys)_2$-modified gold electrode and in the absence of $[Cr(NH_3)_6]^{3+}$, the $E°_f$ value for spinach plastocyanin is 85 mV vs SCE at pH 7 (20 mM cacodylate buffer). On addition of 0.5, 1.75, and 5.0 mM $[Cr(NH_3)_6]^{3+}$, the potential changes to 100, 115, and 140 mV vs SCE, respectively, while a well-defined peak-shaped voltammogram is maintained. Again the addition of the cation causes a shift towards positive potentials, and again we conclude that cations that bind are not completely innocent in the redox sense. Finally, if spinach plastocyanin is adsorbed onto a gold electrode modified by $[Cr(NH_3)_5(NCS)]^{2+}$, then at pH 7.50 the average potential $^1/_2(E_p^{red} + E_p^{ox})$ has even more positive, scan-rate-dependent values around 200 mV vs SCE.[21]

Electrostatic effects similar to those found in the present work were recently reported by Rivera *et al.* for cytochrome b_5.[46] The reduction potential of this negatively charged protein may be modulated by physiological concentrations of Ca^{2+} or Mg^{2+} ions and by complex formation with physiological partner proteins of complementary charge. The results for cytochrome b_5 are entirely consistent with the evidence from the present work that Mg^{2+} ions can affect the thermodynamics of plastocyanin redox processes significantly. We conclude that the influence of biologically relevant redox-inactive cations, such as Mg^{2+} or Ca^{2+}, will have to be considered carefully in future studies of the redox chemistry of metalloproteins.

Conclusions

Voltammetric measurements on *A. variabilis* plastocyanin confirm previous results based on chemical redox studies that the $E°_f$ value of this positively charged protein is considerably less positive than the $E°_f$ values of negatively charged plastocyanins such as those from poplar, spinach, and parsley. Further, the overall positive charge on *A. variabilis* plastocyanin leads to a reversal of electrode−protein/solution interfacial properties with respect to negatively charged plastocyanins. In particular, the addition of Mg^{2+} hinders the voltammetry of *A. variabilis* plastocyanin, whereas it promotes linear diffusion and well-defined peak-shaped cyclic voltammograms for the negatively charged proteins.

In addition to modifying the diffusion characteristics of plastocyanins, the binding of cations such as Mg^{2+} has the thermodynamic effects of shifting $E°_f$ to more positive values and modifying the pH dependence of $E°_f$ at low pH's. The effect of Mg^{2+} on the reversible potential is smaller for *A. variabilis* plastocyanin than for negatively charged plastocyanins. That the thermodynamic effects do not originate from changes in ionic strength is shown by the constancy of $E°_f$ in the presence of a wide range of concentrations of $NaClO_4$. Data cited from other studies also lead to the conclusion that the reversible potential of a plastocyanin is shifted to more positive values by the electrostatic binding of cations such as $[Cr(NH_3)_6]^{3+}$ and neomycin, as well as by the formation of a covalent complex with $[-Ru(NH_3)_5]^{3+/2+}$. The cationic complex $[Cr(NH_3)_6]^{3+}$ has a similar effect on the potential of the 1:1 complex of plastocyanin with cytochrome *c*. This result is consistent with NMR data showing that cation binding is not inhibited by protein−protein complex formation.

The present work extends the findings of Armstrong, Sykes, *et al.* on the effects of species variations and chemical modifications on the potential of plastocyanin.[24,43] An interesting new result is that reducing the high negative charge on spinach and poplar plastocyanins by specific electrostatic binding (Mg^{2+}, $[Cr(NH_3)_6]^{3+}$) or by chemical modification ($[-Ru(NH_3)_5]^{3+/2+}$) leads to a positive shift in $E°_f$, whereas reducing the positive charge on *A. variabilis* plastocyanin leads to a negative shift in $E°_f$.

The reasons for the $E°_f$ value for *A. variabilis* plastocyanin being so much lower than the potentials for the cited plant plastocyanins are, however, not well understood. In general terms, increasing the positive charge of a metal center should make that center easier to reduce, i.e., should make $E°_f$ more positive. In poplar plastocyanin, the through-space distance from the Cu atom to the phenolic O atom of Tyr83 at the center of the acidic patch is about 12.5 Å. Interaction with a positively charged ion at the acidic patch should therefore cause a small positive shift in potential.[41] This is the case for the negatively charged plant plastocyanins. However, the same argument also leads to the prediction of a higher $E°_f$ for *A. variabilis* plastocyanin, since the *absence* of an acidic patch corresponds to a far more effective decrease in negative charge (or increase in positive charge) than the binding of a cation. In this sense, the shift of the potential to a *less positive* value in *A. variabilis* plastocyanin is unexpected.

The possibility that the difference in redox behaviors originates from differences in coordination geometry cannot be eliminated. The dimensions of the Cu sites in plant and algal plastocyanins are identical within the limits of precision of the crystallographic structure analyses, but the relevant limits of precision are rather high. The estimated standard deviations of the Cu−ligand bond lengths in poplar plastocyanin (at 1.3 Å resolution) and *A. variabilis* plastocyanins (at 1.7 Å resolution) are 0.04 Å and 0.06 Å, respectively.[4c] A change of 50 mV in the reduction potential corresponds to a change of only 5 kJ mol^{-1}, i.e., only ∼1 kJ mol^{-1} per Cu−ligand bond. The bond-length differences required to produce this change in bond enthalpy would be well within the cited estimated standard deviations.

A general conclusion from the present work and from related studies on other proteins is that, even though biologically relevant cations such as Mg^{2+} or Ca^{2+} are redox-inactive, they may have a significant influence on the redox chemistry of metalloproteins. Their electrochemical effects are not limited to the ionic strength. In some cases such redox-inactive cations may have a small role in modifying the thermodynamic driving force—and hence the rate constant—for biological electron transfer. The major effects of Mg^{2+} binding are likely to be changes in the ion-pairing of natural partners and/or changes in protein conformation. The possibility of charge-dependent conformational changes in plastocyanin, hitherto not detected crystallographically, remains to be explored.

Acknowledgment. This work was supported by the Australian Research Council (Grant A29230677 to H.C.F. and J.M. Guss, and Grant A29030581 to A.M.B.) and by the Dr. Joan R. Clark Research Fund. We thank Mr. Dashuang Shi for providing Figure 1. The generous assistance of Professor A. G. Sykes in supplying *A. variabilis* plastocyanin is gratefully acknowledged.

IC960620U

(45) Barker, P. D.; Di Glera, K.; Hill, H. A. O.; Lowe, V. J. *Eur. J. Biochem.* **1990**, *190*, 171.
(46) Rivera, M.; Wells, M. A.; Walker, F. A. *Biochemistry* **1994**, *33*, 2161.