Electrochemical Investigations of Poplar, Spinach, Cucumber, and Parsley Plastocyanins at Conventional and Microsized Carbon Electrodes

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Received March 20, 1992

Thermodynamic and kinetic aspects of the electrochemistry of the "blue" copper protein plastocyanin from poplar, spinach, cucumber, and parsley have been studied at both macro- and microsized carbon-disk electrodes. Reversible formal potentials E_F^0 at 3 °C have been determined by cyclic voltammetry in the range $3.6 \leq pH \leq 7.6$. Above pH 6.6 the reversible potentials of all four plastocyanins are independent of pH and have limiting values of 389 ± 7 mV vs NHE. Below pH 6.6 the reversible potentials are functions of pH. Poplar, spinach, and cucumber plastocyanins have similar pH dependences, but the curve for parsley plastocyanin is shifted by ~0.7 pH unit toward higher pH. While the reasons for this difference and for some other details of the pH dependences are not completely understood, the fact that parsley plastocyanin behaves differently from the others is consistent with antecedent observations of redox kinetics. The electrochemical kinetic behavior of the plastocyanin/graphite interface is complex and is influenced by a competing protein adsorption process. Under the experimental conditions employed, only a small fraction (<10%) of the electrode surface is active so that the conventional *linear* diffusion model (in which the entire surface participates) is untenable. Instead, the apparent kinetic parameters (peak separations) of the voltammetric data recorded with normal macrosized electrodes are strongly influenced by *nonlinear* mass transport to micron- to submicron-sized active sites. When *nonlinear* diffusion is taken into account, the experiments are consistent with very fast electron transfer between plastocyanin and the active sites of the carbon electrodes. The detection of nonlinear diffusion, combined with the detection of a voltammetric response for free Cu^{2+}, provides a sensitive probe of the integrity of the protein; the voltammogram of the intact protein is modified in the presence of apoprotein or denatured protein. The data obtained from measurements at conventionally sized electrodes are confirmed by results obtained at carbon microelectrodes. Not only is radial diffusion found to be the predominant mechanism of mass transport (as expected from the small size of the electrodes) but also partial blocking of even these electrodes can be clearly demonstrated.

There is increasing evidence that electron transfer between a metalloprotein and the surface of an electrode mimics electron transfer between a metalloprotein and its biological redox partner, in that both processes involve a high degree of selectivity.[1] In previous papers, the electrochemistry of a heme protein, cytochrome *c*, was reinterpreted in terms of a radial diffusion model in which the *microscopic* properties of the electrode/solution interface play a crucial role.[2] It was shown that earlier interpretations using a conventional *macroscopic* (linear diffusion) model led to incorrect estimates of the standard heterogeneous charge-transfer rate constant at the electrode surface.[1,2] An essential feature of the microscopic model is that redox protein molecules are able to recognize specific sites on an electrode as highly suitable for fast electron transfer and to reject other sites as completely unsuitable. According to this hypothesis, the electrochemistry of metalloproteins at carbon electrodes is strongly influenced by nonlinear (radial) diffusion since only part of the electrode surface is electroactive.[1,2] In the case of cytochrome *c* the hypothesis was confirmed by studies at microelectrodes.[3] We now apply the same concepts to the study of a "blue" copper protein, plastocyanin.

Plastocyanin (Pc) is a component of the photosynthetic electron transport chain in the chloroplasts of higher plants and some algae. It is the subject of three recent reviews.[4] The molecular structure of Pc from poplar leaves, which has been extensively characterized by crystallographic structure analyses,[5-7] is illustrated in Figure 1. In most higher plants the Pc molecule is a single polypeptide of 99 amino acid residues plus a copper atom. There are exceptions such as parsley Pc, where the polypeptide has only 97 amino acid residues. Among the Pc's of higher plants, it is relatively common to find some microheterogeneity (minor differences between the amino acid sequences of the Pc molecules in a single species or even in a single plant);[8] examples which have recently received attention occur in poplar Pc and parsley Pc.[9] The amino acid sequences of the Pc's studied in the present work are shown in Figure 2.

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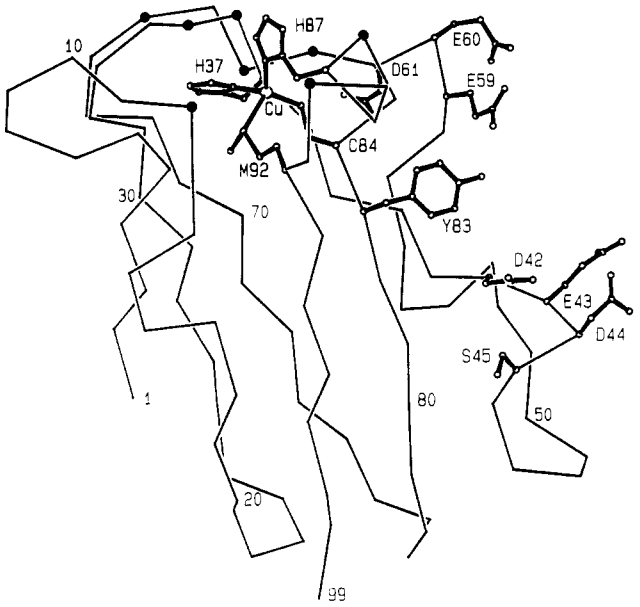

Figure 1. Structure of the poplar plastocyanin (Pc) molecule, emphasizing surface residues which have been implicated in biological or chemical electron-transfer pathways (C^{α} diagram with Cu atom and selected side chains, adapted from ref 6). The side chains at residues 42–45 and 59–61 form an acidic patch above and below the highly conserved residue Tyr83. At the "northern" (top) end of the molecule, the exposed edge of the imidazole ring of a Cu ligand, His87, is surrounded by a patch of conserved or conservatively substituted hydrophobic residues. These are denoted by black C^{α} atoms (Leu12, Phe35, Pro36, Leu62, Leu63, Ala65, Pro86, Ala90). The principal features of the poplar Pc structure are conserved with minor variations in the Pc's of other species. In parsley Pc two of the residues 58–60 are missing: thus the polypeptide backbone cannot have a kink protruding into the solvent in this region, and the negative charge is reduced by 1. In poplar Pc itself, residue 45 is Ser (PcI) or Ala (PcII; see text) instead of Glu.

In the oxidized form of Pc ($Cu^{II}Pc$), the copper atom has a distorted tetrahedral geometry, being coordinated by the $N^{\delta 1}$ atoms of His37 and His87, the S^{γ} atom of Cys84, and the S^{δ} atom of Met92.[6] The coordination of the copper atom is independent of pH.[6] Reduction of the protein produces an equilibrium mixture of two types of molecules: a high-pH form ($Cu^{I}Pc$) and a protonated low-pH form ($HCu^{I}Pc$). In $Cu^{I}Pc$ the copper atom has the same distorted tetrahedral NNSS′ coordination as in $Cu^{II}Pc$, with only minor dimensional changes. In $HCu^{I}Pc$ the copper atom has trigonal NSS′ coordination; the imidazole ring of His87 is protonated, dissociated from the copper atom, and rotated by 180° about C^{β}–C^{γ}.[7] The rearrangement creates a high reorganization barrier which suggests that the low-pH form $HCu^{I}Pc$ should be redox-inactive.[7] This hypothesis is supported by bimolecular kinetic experiments with small inorganic complexes.[14,15] In direct electrochemical measurements, on the other hand, reduced Pc remains redox-active even at low pH. The apparent inconsistency is independent evidence that the equilibrium between the protonated redox-inactive and deprotonated redox-active forms of reduced Pc is rapid on the time scale of voltammetric measurements.[16]

Higher plant Pc's have a charge of about −9 at neutral pH.[4] A number of the acidic residues are highly conserved and are concentrated on one side of the molecule (Figure 1).[5,6] The "acidic patch" comprising residues 42–45 and 59–61 extends along the surface on both sides of another highly conserved residue, Tyr83, and is likely to be an electrostatic recognition site for the biological electron donor to Pc, cytochrome *f*.[17] It has been suggested that the acidic patch is responsible for electrostatic hindrance in in vitro electrochemistry.[18]

In the present study we have used basal-plane pyrolytic graphite electrodes to make new electrochemical measurements of the reversible formal potentials E_F^0 for poplar, spinach, cucumber, and parsley Pc's at several pH values. The fact that direct electrochemistry of Pc can be achieved at carbon electrodes either by mild acidification or by promotion with multivalent inorganic ions and complexes is known from antecedent work.[16,18-20] The promoters may be in solution or surface-bound. The electrochemically determined potentials E_F^0 now reported, as well as their pH dependences at pH <7, exhibit the same trends as values previously obtained by spectroelectrochemical methods or deduced from the rates of homogeneous electron-transfer reactions.[15,21] Parsley Pc, whose amino acid sequence differs from the other three Pc sequences more than they differ among themselves, displays a significantly different dependence of the reversible potential E_F^0 on pH.

In addition to these thermodynamic aspects we have studied the kinetic behavior of the plastocyanin/graphite interface. We find that the electrochemical kinetics of Pc can be simulated by a model in which only a small fraction (<10%) of a pyrolytic graphite electrode surface is electroactive toward intact Pc molecules, and in which the electrochemistry of Pc is strongly influenced by nonlinear (radial) diffusion. Thus our measurements have provided further confirmation of the nonuniform electroactivity of the graphite electrode surface as deduced from the cited studies of cytochrome *c*.

An incidental result of the present work is that the presence of free Cu_{aq}^{2+} can be directly detected, so that the electrochemical experiments provide a sensitive probe for the integrity of the protein.

Experimental Section

Published methods were used for the extraction and purification of $Cu^{II}Pc$'s from poplar (*Populus nigra* var. *italica*), spinach (*Spinacia oleracea*), cucumber (*Cucumis sativus*), and parsley (*Petroselinum sativum*).[22] In the cases of spinach and parsley Pc's, the use of acetone in the extraction procedure[22b] was found to be essential. Poplar Pc was separated chromatographically into two components, PcI and PcII [identical with Pc*a* and Pc*b* in a recent publication[9a]]. The results cited in the present paper were recorded for PcI; no significant differences were observed in measurements using PcII. Samples (~2 mg in 200 μL) were lyophilized after exhaustive dialysis against distilled water. Solutions (50–130 μM) were prepared by dissolving the protein in electrolyte in the electrochemical cell. Ethylenediaminetetraacetic acid (EDTA) was added to suppress free Cu_{aq}^{2+} as required (see below).[16a] Spectrophotometric determinations of the $Cu^{II}Pc$ concentration were only approximate due to the very small sample size and the presence of reduced Pc, apo-Pc and/or denatured Pc in some solutions (see below). Experience showed that autoreduction was decreased by keeping the protein solutions at 3 °C, and all electrochemical measurements were made at this temperature.

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                               10                  20                  30                  40                  50
Poplar I      I D V L L G A D D G S L A F V P S E F S I S P G E K I V F K N N A G F P H N I V F D E D S I P S G V
Poplar II     V · · · · · · · · · · · · · · · · · · · V P A · · · · · · · · · · · · · · · V L · · · · A V · · · ·
Spinach       V F · · · · G G · · · · · · L · G D · · V A S · · E · · · · · · · · · · · · V · · · · · E · · · · ·
Cucumber      I E I · · · G · · · · · · · · · N N · T V A S · · · · T · · · · · · · · · · V · · · · · E · · · · ·
Parsley       A E · K · · S · · · G · · · S · · S · T V A A · · · · T · · · · · · · · · · · · · · · · E V · A · ·
                      ● ●       ● ● ● ●               ● ▽   ▽   ▽ ▽ ● ● ▽ ▽   ● ● ●     ● ● ▽ ▽     ●   ▽ ▽
                                                                          ■
                                                                                    ↑ ↑ ↑ ↑
                                                                                    Acidic
                                                                                    patch I

                               60                  70                  80                  90                99
Poplar I      D A S K I S M S E E D L L N A K G E T F E V A L S N K G E Y S F Y C S P H Q G A G M V G K V T V N
Poplar II     · V · · · · · · · · · · · · · · · · · · · · · · · D · · · · T · · · · · · · · · · · · · · · I · ·
Spinach       · · A · · · · · · · · · · · · P · · · Y K · T · T E · · T · K · · · · · · · · · · · · · · · · · ·
Cucumber      · S G · · · · N · · · · · · · P · · V Y · · Q · T E · · S · · · · · · · · · · · · · · · · · · · ·
Parsley       N · E · · · Q -(P)- E Y · · G A · · · Y · · T · T E · · T · K · · · E · · A · · · · K · E · · · ·
                    ▽ ▽ ●             ●       ● ▽       ▽   ▽       ●   ●   ▽ ▽ ●   ● ●   ● ● ● ●   ▽   ▽   ●
                                                                                ■     ■         ■
                              ↑ ↑ ↑
                              Acidic
                              patch II
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Figure 2. Amino acid sequences of poplar (PcI[10] and PcII[9(a)]), spinach,[11] cucumber,[12] and parsley[13] plastocyanins. Dots (···) indicate identity with poplar PcI sequence. Squares (■) denote Cu-binding residues. Circles (●) denote residues invariant in all known plastocyanin sequences. Triangles (▽) denote residues invariant in all known higher plant plastocyanin sequences. **Bold symbols** denote residues with charged side chains (Asp, Glu, Lys).

The electrolyte solutions for electrochemistry contained acetate buffer (5 mM) for pH ≤5, phosphate buffer (5 mM) for pH ≥5, and $NaClO_4$ (10 mM). The concentration of electrolyte was chosen so as to reduce the solution resistance without increasing the ionic strength to the point where the protein might be precipitated. All reagents for the preparation of electrolyte and buffer solutions were of analytical grade and were used as purchased. Water was obtained from a Millipore 25TS (twin stage) reverse osmosis purification system (Millipore, Bedford, MA) and had a specific conductance of 2 $\mu S \cdot cm^{-1}$. The uncompensated resistance present in measurements with conventionally sized pyrolytic graphite electrodes was determined by the method of He and Faulkner[23] and was found to be 1.4–1.7 kΩ. The effect of uncompensated resistance at microelectrodes was negligible under the conditions of the experiments.[24] It can be shown that the ohmic i_R drop was smaller than 2 mV even at the highest protein concentration (165 μM) and the fastest scan rate (50 $mV \cdot s^{-1}$).

Disks of pyrolytic graphite (radius 2.4 mm, area 18.1 mm^2; graphite from Le Carbone, Lorraine, France) having the disk face parallel to the basal plane were prepared. The disks were sealed in Plexiglas tubes. Internal connection to a copper rod was made by means of silver-loaded epoxy adhesive (RS 555-673, RS Components Ltd.).

Carbon-disk microelectrodes (nominal radius 5.5–6 μm) were made by sealing a single carbon fiber (from Hercules Corp.) into a glass capillary tube with epoxy resin (Araldite HY 951, Ciba-Geigy). The internal connection was made by sealing the fiber to a copper wire with silver epoxy composite.

The radius of each microelectrode disk was determined by measuring the limiting currents for the 1-electron reduction of $K_3Fe(CN)_6$ in a series of aqueous solutions containing $K_3Fe(CN)_6$ (0.1–1.0 mM), H_2SO_4 (1 mM), and K_2SO_4 (50 mM), and using the equation[25]

$$i_L = 4ncrDF \qquad (1)$$

where i_L is the limiting current, n is the number of electrons ($n = 1$), c is the concentration, r is the radius of the microelectrode, D is the diffusion coefficient ($D = 5.0 \times 10^{-6}\ cm^2 \cdot s^{-1}$ for ferricyanide[26]), and F is the Faraday constant.

The electrodes used to record the data reported in this paper were initially polished with aqueous slurries of 5-, 0.3-, and 0.05-μm alumina on polishing cloths (Buehler Ltd.). Before each voltammetric experiment, the electrodes were repolished with the 0.05-μm slurry on a fresh piece of cloth to ensure that the surface was not contaminated with residual protein. After polishing, the electrodes were thoroughly washed with water. To eliminate the possibility that alumina contamination modifies the metalloprotein electrochemistry, some experiments were repeated with electrodes that had been polished with a diamond paste. The results were in all cases identical with those obtained after alumina polishing.

The electrochemical experiments were made in a standard electrochemical cell (Princeton Applied Research (PAR), No. 21960) mounted on a slightly modified cell top (PAR No. 219581). For all experiments with Pc the cell was thermostated to 3.0 (±0.2) °C. The potential of the Ag/AgCl (3 M KCl) reference electrode (PAR No. K0265) versus the normal hydrogen electrode (NHE) was determined from cyclic voltammograms for the $[Fe(CN)_6]^{3-/4-}$ redox couple under conditions identical to those used for the Pc measurements (3 °C, $K_3Fe(CN)_6$ (0.2 mM), $NaClO_4$ (10 mM), acetate buffer (5 mM), pH 4.7, scan rate 50 $mV \cdot s^{-1}$). Measurements at gold and carbon electrodes gave a value of 0.219 ± 0.002 V vs NHE, assuming that $E°\{[Fe(CN)_6]^{3-/4-}\} = 0.410$ V vs NHE. The auxiliary electrode was a platinum wire.

The volumes used in the experiments with Pc were routinely 500–600 μL. Stirring and deoxygenation were achieved by bubbling a fine stream of humidified high-purity N_2 through the solution before each experiment. A flow of N_2 over the solution was maintained during the measurements.

The electrochemical measurements were recorded using a BAS 100A electrochemical analyzer (Bioanalytical Systems Inc.) equipped with a Faraday cage and preamplifier (BAS PA-1) for experiments with microelectrodes. Experimental data were transferred to a Personal Computer via a RS232 interface.

Prior to curve analysis, the experimental cyclic voltammetric curves were corrected for the background current by subtracting the extrapolated prepeak baselines separately for the scans in the negative and positive potential directions. The peak potentials for reduction and oxidation were then identified as the data points of maximal and minimal current, respectively. Noisy sets of data were smoothed before peak determinations using a Blackman type digital filter. This procedure allowed the determination of peak potentials with a precision of ±3 mV.

Results and Discussion

Special Conditions. In the range 3.5 ≤ pH ≤ 5.0, voltammetric measurements at a basal plane oriented pyrolytic graphite

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Table I. E_F^0 Values vs NHE for Plastocyanins at Five pH Values

plastocyanin source	pH 3.6[a,c]	pH 4.7[a,d]	pH 5.4[b,d]	pH 6.6[b,d]	pH 7.6[b,d]
poplar	441	413	399	394	
cucumber	458	413	389	384	
spinach	473	419	395	389	
parsley	497	447	419	391	388

[a] Acetate buffer (5 mM), $NaClO_4$ (10 mM). [b] Phosphate buffer (5 mM), $NaClO_4$ (10 mM), $Mg(ClO_4)_2$ (35 mM). [c] Experimental error ±10 mV. [d] Experimental error ±5 mV.

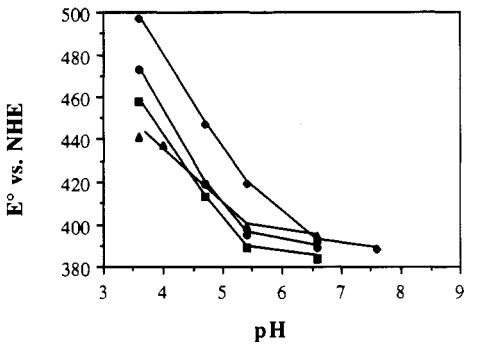

Figure 3. pH dependence of E_F^0 for poplar (▲), cucumber (■), spinach (●), and parsley (♦) plastocyanins at 3 °C. Values were determined by cyclic voltammetry at a basal plane oriented pyrolytic graphite electrode of conventional size. Solutions contained $NaClO_4$ (10 mM) and acetate buffer (5 mM) at pH <5, or $Mg(ClO_4)_2$ (35 mM), $NaClO_4$ (10 mM), and phosphate buffer (5 mM) at pH >5.

electrode yielded direct electrochemistry of Pc. In more basic solutions ($6.0 \leq pH \leq 7.6$), well-defined reduction of $Cu^{II}Pc$ occurred only when $Mg(ClO_4)_2$ (35 mM) was present, as found in earlier measurements at edge-oriented graphite electrodes.[16,27] For some samples an additional reduction peak, ca. 150 mV more negative than the Pc reduction peak, was observed. This was identified as resulting from the reduction of Cu_{aq}^{2+} (see below). To avoid interference from the Cu_{aq}^{2+} response, EDTA (10–30 μM) was added to the Pc solutions.[16a]

It was later realized that the additional reduction peak attributed to Cu_{aq}^{2+} was an artifact of the lyophilization of the protein for transport between the collaborating laboratories. This was confirmed by a set of experiments using a sample of poplar Pc which had been stored as a frozen solution at −20 °C instead of being lyophilized. No free Cu_{aq}^{2+} was detected electrochemically in this sample. The CuPc E_F^0 value was identical with that determined for the lyophilized samples, but the peak currents relative to the mass of protein were 10–50% higher. We now know (see Kinetic Results, below) that these observations were telling us that the peak currents were extremely sensitive to the presence of small amounts of apoprotein or denatured protein in the lyophilized samples.

Thermodynamic Results. The reversible formal electrode potentials E_F^0 for Pc from poplar, spinach, cucumber, and parsley at four pH's in the range $3.6 \leq pH \leq 6.6$ are shown in Table I and Figure 3. The values of E_F^0 have been obtained from cyclic voltammograms recorded at conventional electrodes (not microelectrodes), by averaging the reduction and oxidation peak potentials. There is an implicit assumption that the resulting value is equal to the reversible half-wave potential $E_{1/2}^r$, i.e., $E_F^0 = E_{1/2}^r = [E_p^{red} + E_p^{ox}]/2$. This assumption is justified provided that the diffusion coefficients of oxidized and reduced Pc are equal. Since the oxidized and reduced forms of Pc have effectively identical molecular weights and shapes, it is highly probable that the latter condition is satisfied.

(27) In our experiments, the faradaic responses (i_f) observed at equally polished basal and edge oriented electrodes were identical, but the basal substrate showed smaller charging currents (i_c) and exhibited better i_f/i_c ratios. The basal substrate was therefore used for all experiments.

Inspection of Figure 3 leads to two generalizations, to each of which there is a significant exception. Firstly, the E_F^0's approach an asymptotic limit of 389 ± 7 mV vs NHE. For poplar, spinach, and cucumber Pc's, the limit is reached at pH ~7, but for parsley Pc a higher pH is required. Secondly, below pH ~7 the E_F^0 values are strongly pH-dependent. The limiting slope is $\partial(E_F^0)/\partial(pH) \simeq -50$ mV for spinach, cucumber, and parsley Pc's, but only about −25 mV for poplar Pc. We discuss each of these observations.

Asymptotic E_F^0 Values. The differences between the asymptotic E_F^0 values for the four Pc's are not significant in terms of the experimental errors (footnote to Table I). The values themselves, 389 ± 7 mV, agree well with the most recent values for *S. obliquus* Pc (389 ± 5 mV) and spinach Pc (384–387 mV).[28] Other values, obtained from kinetic and other methods of measurement on higher plant Pc's under a variety of conditions of temperature, ionic strength, buffers, and reference electrode corrections, lie in the range 340–395 mV.[4c,28] As has been noted elsewhere,[28] the scatter among the published values, sometimes even for a single Pc species, is considerable. In the absence of error estimates the precision of many of the measurements is uncertain.

It is to be noted that the present measurements were made at 3 °C whereas most of the cited values of E_F^0 were determined at (or extrapolated to) 25 °C. For *S. obliquus* Pc, the measured variation of E_F^0 with temperature is about −0.5 mV·deg^{-1},[28] so that the present values may have to be reduced by 11 mV to make them comparable with values determined at 25 °C.

pH Dependences. The region where E_F^0 is independent of pH corresponds to the reversible redox process[29]

$$Cu^{II}Pc + e^- \overset{E_F^0}{\rightleftarrows} Cu^{I}Pc \qquad (2)$$

As the pH is decreased, the shift of E_F^0 to more positive potentials is associated with the stabilization of Cu^I by trigonal NSS′ coordination in HCu^IPc compared with distorted tetrahedral NNSS′ coordination in Cu^IPc.[7] The slope −50 mV/pH unit is consistent with (2) in combination with

$$Cu^{I}Pc + H^+ \overset{K_a}{\rightleftarrows} HCu^{I}Pc \qquad (3)$$

Why is the slope for poplar Pc smaller than −50 mV/pH unit (Figure 3)? Our preferred interpretation is that the pH dependence for poplar Pc becomes parallel to the others at a lower pH which, however, is inaccessible due to insolubility of the protein. An alternative hypothesis that the electrochemistry of poplar Pc proceeds by a different mechanism can be rejected on the grounds that none of the sequence differences between poplar Pc and the other three Pc's (Figure 2) appears to be capable of exerting a strong influence on the proton and electron equilibria at the active site. Inspection of a recently published compilation (Figure 8 in ref 4c) suggests that there are differences between the slopes of the E_F^0 versus pH plots of other Pc's as well. It would clearly be of interest to obtain more precise values for these slopes than are currently accessible by kinetic methods.

If allowance is made for a possible off-set in the case of poplar Pc, the E_F^0 versus pH curves for spinach, cucumber, and poplar Pc's are qualitatively similar. The curve for parsley Pc has the same shape as the other three, but is shifted by ~0.7 pH unit toward higher pH. The different behavior of parsley Pc is in excellent agreement with kinetic determinations of the pK_a at the active site.[30] The pH dependences of the rates of homogeneous

(28) Armstrong, F. A.; Butt, J. N.; Govindaraju, K.; McGinnis, J.; Powls, R.; Sykes, A. G. *Inorg. Chem.* **1990**, *29*, 4858–4862.

(29) The symbol E° can be used in (2) instead of E_F^0 because the behavior is almost ideal. The activities of $Cu^{II}Pc$ and Cu^IPc must be very nearly equal, and (3) does not contribute significantly in the pH-independent region.

(30) McGinnis, J.; Sinclair-Day, J. D.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1986**, 2007–2009.

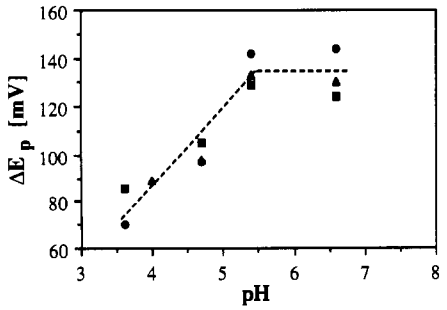

Figure 4. Separation ΔE_p between reduction and oxidation peaks in cyclic voltammograms of poplar (▲), cucumber (●), and parsley (■) plastocyanins (55 μM) at 3 °C. Conditions: basal plane oriented pyrolytic graphite electrode of conventional size; scan rate 2 mV·s^{-1}; solution composition as in Figure 3.

electron transfer between Pc and redox-active inorganic complexes, supported by independent values from ^{1}H NMR measurements, lead to a substantially higher active site pK_a for parsley Pc (pK_a = 5.5) than for spinach Pc (pK_a = 4.8) and poplar Pc (pK_a = 4.7).[4b,30]

The nuances of the pH dependences of the reversible potentials E_F^0 of parsley and the other three Pc's are not yet completely understood. The differences between them must reflect the differences between the respective peptide sequences. Parsley Pc differs from the other three Pc's in the present study by having charged side chains at residues 85 (Glu instead of Ser) and 93 (Lys instead of Val) and by having a shorter polypeptide backbone with the loss of two charged side chains at residues 58–60 (Pro instead of Ser-Glu-Glu) (Figure 2). These are the only sequence differences that appear to occur sufficiently close to the active site to have a significant effect on the reversible potential. Residues 85 and 93 lie on the otherwise conserved loop between residues 82 and 94 and are immediately adjacent to two of the Cu-binding residues, Cys84 and Met92 (Figure 1). The two-residue deletion at residues 58–60 results in the straightening of a prominent kink in the polypeptide backbone; perhaps more importantly, it reduces the concentration of negative charge at the northern end of the acidic patch. The effects of charges in this region are known to be transmitted to the active site. In *S. obliquus* Pc, where residues 58–60 are replaced by a single His59, deprotonation of the His59 side chain at pH ~8 causes a measurable perturbation of the NMR resonance of an imidazole proton of the active site residue His37.[31a] Interactions between the Cu site and titrating nonligating histidine residues have also been invoked to account for variations of the reduction potential as a function of pH in the related blue Cu protein, azurin.[31b]

In agreement with an earlier electrochemical investigation on spinach Pc,[16] the present study of four Pc's yields no evidence that the protonated form of the reduced protein is kinetically inactive on the electrochemical time scale. [The qualification "on the electrochemical time scale" is important, since it would be difficult to reconcile reversible redox activity with the known structure of HCu^{I}Pc. The lifetime of a proton on HCu^{I}Pc is sufficiently short (eq 3) to permit the concentration of the redox-active species Cu^{I}Pc to be restored to its equilibrium value (as given by the Nernst equation) prior to each measurement of the current.] If a kinetically inactive form were not in equilibrium with an active form, then the oxidation peak current on the reverse scan of cyclic voltammograms would be smaller than the reduction peak current on the forward scan. This is not the case (Figure 4). Further, the data in Figure 4 show that the peak-to-peak separation (ΔE_p) in cyclic voltammograms decreases with decreasing pH over the range 3 ≤ pH ≤ 5.4. If it were assumed that mass transport occurs by *linear* diffusion, Figure 4 would

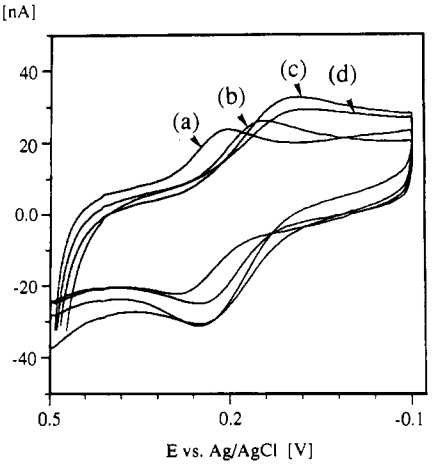

Figure 5. Cyclic voltammograms of cucumber plastocyanin (60 μM) at 3 °C and (a) pH 3.6, (b) pH 4.7, (c) pH 5.4, and (d) pH 6.6. Conditions: basal plane oriented pyrolytic graphite electrode of conventional size; scan rate 2 mV·s^{-1}; solution composition as in Figure 3.

lead to the conclusion that the rate of heterogeneous electron transfer at the electrode surface increases with decreasing pH. However, as discussed in the following section, the real rate of electron transfer can be calculated only after consideration of *nonlinear* mass transport phenomena.

Kinetic Results. Figure 5 shows cyclic voltammograms of cucumber Pc at four pH values, recorded at a scan rate of 2 mV·s^{-1}. Even at such a slow scan rate, the shape of the voltammetric curve depends on the pH. At the higher pH's (5.4, 6.6), where the faradaic response is modified by Mg^{2+}, the separation $\Delta E_p = E_p^{ox} - E_p^{red}$ between the oxidation and reduction peaks is greater than 120 mV. At the lower pH's (3.6, 4.7), the peaks are sharper, and the peak separation ΔE_p is smaller and decreases with decreasing pH. Further, the peak separations in the cyclic voltammograms are functions of pH, as shown for poplar, cucumber, and spinach Pc's in Figure 4. In the absence of additional information, use of a macroscopic model would suggest that peak broadening is associated with slow electron transfer and that the heterogeneous rate constant is a function of pH.

A plausible explanation for the effect of Mg^{2+} and other positively charged species (including H^+) in promoting the voltammetric response of Pc at a graphite electrode is that a reduction of the coulostatic repulsion between the negatively charged protein and the negatively charged electrode results in an increase in the rate of electron transfer.[16] Since there is a concentration of negative charge at the acidic patch (residues 42–45 and 59–61), it follows that this region of the molecular surface makes a major contribution to the overall coulostatic repulsion. Conversely, the binding of Mg^{2+} or protons at the side-chain $-COO^-$ groups of the acidic patch should be particularly effective in reducing the coulostatic repulsion. Glu and Asp residues have a side-chain pK_a ~4, so that the charge of the acidic patch may well change significantly in the range 3.6 ≤ pH ≤ 4.7.

An extrapolation from this hypothesis is that the acidic patch is a part of the molecular surface which must make contact with the electrode if an encounter is to lead to electron transfer. In the case of interactions between Pc and chemical electron donors or acceptors, there is strong evidence for two electron-transfer pathways to and from the Cu atom. One pathway involves Tyr83, in close proximity to the acidic patch.[5,14,30] The other pathway is via His87 at the "north" end of the Pc molecule, and is less relevant to the present discussion. The fact that one pathway used in homogeneous electron-transfer reactions terminates close to the acidic patch does not prove that this pathway is used in heterogeneous electron transfer, but it suggests that the hypothesis

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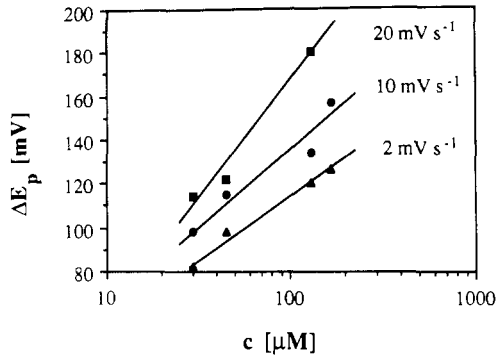

Figure 6. Separation ΔE_p between reduction and oxidation peaks in cyclic voltammograms of poplar plastocyanin (concentrations c, μM) in $NaClO_4$ (10 mM) and acetate buffer (5 mM) at 3 °C and pH 4.7. Conditions: basal plane oriented pyrolytic graphite electrode; scan rates as indicated.

is at least reasonable. Support for the hypothesis comes from kinetic experiments showing that the acidic patch is a weak binding site for Mg^{2+}.[14]

Despite the self-consistency of the preceding description, there are difficulties in relating some features of the voltammetry to coulostatic repulsion terms. For example, due to the amino acid sequence differences shown in Figure 2, the charges at the acidic patch of parsley, poplar, cucumber, and spinach Pc's are −5, −6, −7, and −7, respectively. The structures of cucumber and poplar Pc's are effectively identical, the differences detected by a careful crystallographic refinement being insignificant for the purposes of the present discussion.[32] It is highly probable that spinach Pc has the same structure.[33] In parsley Pc two of the residues at positions 58–60 in other higher plant Pc's are missing (Figure 2) so that the conformation, at least in this region of the molecule, must be different. In the absence of direct structural evidence, we assume that the conformation is similar to that in the known structure of an algal plastocyanin where the same two residues are missing.[34] If this assumption is correct, then the polypeptide backbone of parsley Pc lacks the prominent kink which protrudes into the solvent at residues 59–61 in other higher plant Pc's, but the single acidic side chain remaining in this region (at Glu61) does extend into the solvent.

While a correlation between kinetic effects and molecular charges would have intuitive appeal, Figure 4 shows that there is no clear dependence of the peak separations on the charges of the different Pc molecules. It may be concluded that either the differences arising from the charges are not sufficiently prominent to be seen in the electrochemical experiments or else the changes in voltammetry have another cause. For example, it may be postulated that the differences between the peak separations are caused by factors such as protein–protein repulsion or competitive adsorption of inactive species (including impurities) and that these do not necessarily lead to slow electron transfer. The latter hypothesis is supported by Figure 6, where it is clearly shown that the peak-to-peak separation ΔE_p is concentration-dependent. The coulostatic hypothesis using a simple *linear* diffusion model for mass transport of Pc to an electrode surface can be eliminated since it requires that the peak separation be independent of concentration.

The change in wave shape when the scan rate is changed from 2 to 50 mV·s^{-1} (Figure 7) is another sign that the conventional

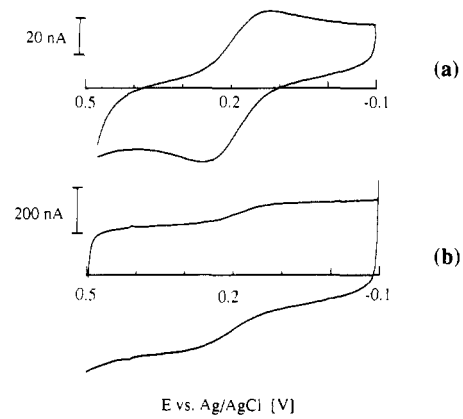

Figure 7. Cyclic voltammograms of poplar plastocyanin (130 μM) in $NaClO_4$ (10 mM) and acetate buffer (5 mM) at 3 °C and pH 4.7. Conditions: basal plane oriented pyrolytic graphite electrode of conventional size; scan rate (a) 2 mV·s^{-1} and (b) 50 mV·s^{-1}.

linear diffusion model is inappropriate. The linear diffusion model predicts a curve with asymmetrically shaped peaks on both the oxidation and reduction components, the peak current being a function of (scan rate)$^{1/2}$ for all scan rates. In contrast with this prediction, Figure 7 shows that the peaked curve observed at low scan rates (2 mV·s^{-1}) is replaced by a sigmoidal curve at high scan rates (50 mV·s^{-1}). Further, the limiting currents observed in the range 50–500 mV·s^{-1} are independent of scan rate.

Evidence for Radial Diffusion. A convincing explanation of the observed phenomena can be obtained if the surface of the electrode is assumed to have the property of localized electroactivity. Armstrong et al. have pointed out that if electron transfer takes place only at specific sites having microscopic dimensions (favored or recognized by the analyte due to charge distribution, chemical heterogeneity, or conductivity), then radial diffusion can become the dominant mode of mass transport and can thus give rise to increased peak separation or—in extreme cases—to sigmoidal-shaped voltammograms.[2,35] Just these effects are observed for Pc experimentally (Figure 7).

The voltammetric behavior of Pc is in fact analogous to that of cytochrome *c* in an earlier study to which we have already referred.[3] The observations on cytochrome *c* were explained quantitatively by a theoretical model based on partially blocked surfaces. The electro*in*active fraction of the electrode surface was found to be closely related to the bulk concentration of cytochrome *c*, and the blocking could be identified as resulting from adsorption of the protein itself or from surface-active trace impurities.

In the case of Pc, the concentration dependence of the peak separation and wave shape can be explained by adsorption of holoprotein, apoprotein, or denatured protein. As the Pc concentration increases, a higher proportion of the active sites on the electrode surface are blocked and unavailable for electron transfer. The lower the density of electroactive sites is, the more important nonlinear (i.e., radial) transport to the remaining active sites becomes. In cyclic voltammetry, increasing nonlinear transport is associated with increasing peak separations and/or a change to a sigmoidal waveform.[35]

The voltammetric behavior of Pc is complicated by its dependence on pH and Mg^{2+} concentration. Although the pH dependence has been interpreted by assigning different heterogeneous charge-transfer rate constants at different pH's,[16] it is possible to combine both the pH dependence and the $[Mg^{2+}]$ dependence into a single model. In this model, the concentrations of H^+, Mg^{2+}, and Pc all influence the access of Pc to the electrode surface, giving rise to a partially blocked surface with limited availability of electroactive sites for the holo-Pc molecules. Each combination of pH, Mg^{2+} concentration, and Pc concentration results in a different proportion of electroactive sites, a different

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(33) Little modification of the poplar Pc structure is required to produce a plausible model for spinach Pc by computer graphics (D. Shi, personal communication).
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Table II. Geometrical Parameters θ and r_i for a Partially Blocked Graphite Electrode Surface, Calculated Using the Model of Matsuda et al.[26,42,43] for Reversible Charge Transfer at the Active Sites

	scan rate, mV·s^{-1}		half-distance between active sites, μm					
	2	20	$r_o = 10$		$r_o = 5$		$r_o = 1$	
[Pc],[b] μM	ΔE_p,[c] mV	ΔE_p,[c] mV	θ	r_i, μm	θ	r_i, μm	θ	r_i, μm
30	82	114	0.90	3.2	0.96	1.00	0.996	0.063
45	98	128	0.96	2.0	0.98	0.71	0.998	0.045
130	120	180	0.98	1.4	0.992	0.45	0.9995	0.022
165	126	*d*	0.985	1.2	0.994	0.39	0.9997	0.017

[a] The values of θ and r_i are calculated for poplar plastocyanin as functions of the plastocyanin concentration, [Pc], and the half-distance between active sites, r_o. Other parameters used in the calculations are diffusion coefficient $D = 5.0 \times 10^{-7}$ cm^2·s^{-1}, transfer coefficient $\alpha = 0.5$, pH 4.7, and temperature 3 °C. [b] Bulk concentration of plastocyanin. [c] Peak separation, experimentally determined for the given scan rate in cyclic voltammetry at a polished basal plane graphite electrode. [d] Cyclic voltammetry curve is sigmoid-shaped.

Table III. Geometrical Parameters θ and r_i for a Partially Blocked Graphite Electrode Surface, Calculated Using the Model of Matsuda et al.[26,42,43] for a Charge-Transfer Rate $k^0 = 0.1$ cm·s^{-1} at the Active Sites (Other Details as in the Title to Table II)

	scan rate, mV·s^{-1}		half-distance between active sites, μm					
	2	20	$r_o = 10$		$r_o = 5$		$r_o = 1$	
[Pc],[a] μM	ΔE_p,[b] mV	ΔE_p,[b] mV	θ	r_i, μm	θ	r_i, μm	θ	r_i, μm
30	82	114	0.90	3.2	0.94	1.22	0.990	0.100
45	98	128	0.94	2.4	0.965	0.94	0.994	0.077
130	120	180	0.975	1.6	0.990	0.50	0.997	0.055
165	126	*c*	0.982	1.3	0.992	0.45	0.998	0.045

[a] Bulk concentration of plastocyanin. [b] Peak separation, experimentally determined for the given scan rate in cyclic voltammetry at a polished basal plane graphite electrode. [c] Cyclic voltammetry curve is sigmoid-shaped.

nonlinear transport pattern, and therefore a different peak separation in the cyclic voltammogram.

The importance of radial diffusion has been demonstrated experimentally and theoretically in a number of electrochemical systems where the electrode surfaces are known to have been partially blocked.[3,36-41] Since a theoretical treatment for randomly blocked electrodes in cyclic voltammetry is not yet available, we have interpreted the present experimental data by means of a model based on a hexagonal blocking pattern.[26,42,43] The model assumes the presence of circular active sites of radius r_i separated by a half-distance r_o (see Tables II and III). Simulations of cyclic voltammograms were made by the procedure of ref 33, using different surface parameters and values $k^0 = \infty$ and $k^0 = 0.1$ cm·s^{-1} for the heterogeneous charge-transfer rate constant. The values used in the simulation correspond to very fast rates of electron transfer and are consistent with values recently reported for other proteins.[1,2] For example, a value $k^0 > 7 \times 10^{-2}$ cm·s^{-1} was reported for conventional linear diffusion models at low concentrations[20] where electrode blockage is minimal, and a value $k^0 > 1$ cm·s^{-1} was proposed for partially active electrode surfaces.[35]

The resulting surface blockage parameters for poplar Pc at pH 4.7 (related to the peak separations in Figure 7) are shown in Tables II and III. The fraction of blocked electrode surface, Θ, lies in the range $0.9 \leq \Theta \leq 0.9997$. Since Θ is the proportion of the surface unavailable for electron transfer to or from Pc molecules, it follows from the microscopic model that blockage of the electrode surface is a major determinant of plastocyanin electrochemistry at graphite electrodes. Tables II and III also show that the effect of varying k^0 in the range $\infty \geq k^0 \geq 0.1$ cm·s^{-1} is small and is not significant in relation to uncertainties in other parameters of the model (such as r_o). In other words, the electron-transfer rate constant is large but the actual value cannot be determined with the present electrochemical method.

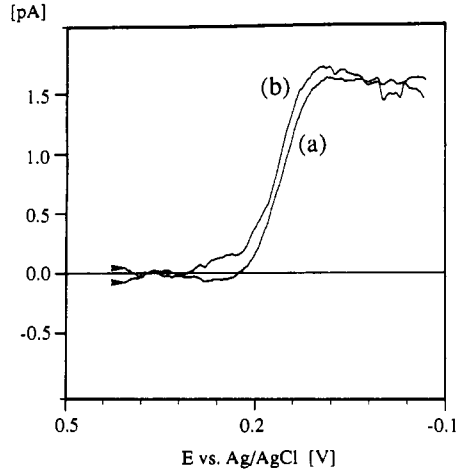

Figure 8. Cyclic voltammograms of poplar plastocyanin (110 μM) at a carbon-microdisk electrode (radius $r = 6.2$ μm). Only negative potential (reduction) scans are shown. Scan rate: (a) 10 mV·s^{-1}; (b) 50 mV·s^{-1}. Other conditions are as in Figure 6.

Microelectrode Measurements. Experiments with a carbon microelectrode (Figure 8) provide a further demonstration that radial diffusion to partially blocked electrode surfaces makes an important contribution to Pc electrochemistry. Because such an electrode is small, diffusion at scan rates such as those used in the present study must be predominantly radial, regardless of whether the electrode is blocked or unblocked. Thus the theory predicts that a sigmoidal voltammogram should be obtained under *all* conditions. This is shown to be the case (Figure 8). Further, the response is seen to be independent of the scan rate over the range 10–50 mV·s^{-1}, as predicted for a steady-state response.

Proof that the microelectrode surface is blocked or unblocked can be obtained by comparing the observed and calculated values of the limiting current. Theoretical curves for the reduction of Pc at microdisk electrodes, calculated by digital simulation using a fast quasi-explicit finite difference method,[44,45] are shown in Figure 9. The observed faradaic currents are consistently smaller than theoretically expected. This fact, together with the steady-state wave shape at all scan rates tested, unambiguously shows that the carbon microelectrode is partially blocked. The same conclusion follows from another result of the calculations (not shown here), namely that the active sites where electron transfer to Pc takes place must have a cumulative area substantially smaller than the $\pi \times 6.2^2$ μm^2 ($=121 \times 10^{-12}$ m^2) area of the carbon microelectrode.

The above results are consistent with the postulate of a very large heterogeneous electron-transfer rate constant.[24] Within the limits of precision, the half-wave potential $E_{1/2}$ derived from the microelectrode measurements is equal to E_F^0, and the Tomes criterion ($E_{3/4} - E_{1/4}$) is ≤70 mV compared with the theoretically predicted value 55 mV for a reversible process.[24] It is, however,

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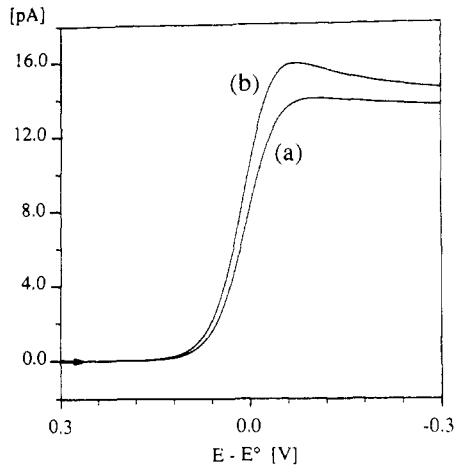

Figure 9. Digital simulations of cyclic voltammograms at a microdisk electrode (radius $r = 6.2\ \mu m$) for the reduction of an electroactive species. Only negative potential (reduction) scans are shown. Conditions: concentration $c_{ox} = 110\ \mu M$; diffusion coefficient $D = 5.0 \times 10^{-7}\ cm^2 \cdot s^{-1}$; heterogeneous rate constant $k^0 = 1.0\ cm \cdot s^{-1}$; transfer coefficient $\alpha = 0.5$; scan rate (a) $10\ mV \cdot s^{-1}$ and (b) $50\ mV \cdot s^{-1}$.

obvious that the voltammetric data recorded with microelectrodes still suffer from considerable noise with a resulting reduction in precision.

Conclusions

The present study clearly demonstrates that the electrochemistry of Pc at carbon electrodes cannot be considered as a simple quasi-reversible system as previously reported and that it is decisively influenced by nonuniformity of the carbon electrode surface. The concentration-dependent peak separations in cyclic voltammograms are symptoms of protein–electrode and/or protein–protein repulsions that are probably derived from a competing adsorption process. Such phenomena may be more common and important than has been supposed.[1] Further investigations by surface-sensitive techniques are now required to identify the mechanism and components responsible for the blocking of the electrode surface.

A strong argument in favor of the radial diffusion hypothesis is that it leads to plausible rate constants for the electron-transfer process. If the present voltammetric results are interpreted by a conventional model assuming linear diffusion to a fully active surface,[46] the calculated first-order rate constants lie in the range $10^{-3} \geq k^0 \geq 10^{-5}\ cm \cdot s^{-1}$ depending on the concentration of the solution. A model that leads to calculated first-order rate constants that are concentration-dependent is obviously unsatisfactory. When the same experimental results are interpreted in terms of partially blocked electrode surfaces, a plausible rate constant $k^0 \geq 0.1\ cm \cdot s^{-1}$ is obtained for all the concentrations and pH values examined.

One of the original objectives of this work was to obtain quantitative data that might be used to seek relationships between the reversible potentials E_F^0 of Pc's and the primary structures and/or charge distributions. The precision of the present measurements is inadequate to achieve this objective. It is highly desirable to develop the electrochemical methodology to achieve higher precision while using, if possible, even smaller protein specimens.

For the time being, the best that can be done is to note that simple electrochemical measurements interpreted via a *microscopic* model lead to thermodynamic parameters that are consistent with published values derived from chemical reaction rate data. Thus we confirm that the reversible potential E_F^0 for parsley Pc has a pH dependence different from those of the other higher plant Pc's studied. This difference must be associated with differences between the respective amino acid sequences, but the precise relationship is not yet understood. A new observation is that the E_F^0 of poplar Pc, too, has a significantly different pH dependence. Since differences may occur among other Pc's as well (Figure 8 of ref 4c), further dissociation should await the availability of additional and more precise data.

Finally, the microscopic model implies an ability of macromolecules to discriminate among sites in an electrode surface. There is not yet any evidence that any orientation of Pc with respect to an electrode active site is more productive than others (in contrast with biological electron transfer where short-range electrostatic interactions lead to families of preferred orientations of Pc with respect to its redox partner[17]). It remains to be established whether specificity in protein–electrode interactions involves a mechanism similar to the recognition of redox partners in biological electron-transfer reactions.

Acknowledgment. We thank Dr. P. A. Lay and Miss D. Niles for helpful comments, Mr. B. A. Fields for providing Figure 1, and Mr. D. Shi for constructing the computer-graphics model of spinach plastocyanin mentioned in the text. The work was supported by the Australian Research Council (Grants A29030581 (A.M.B.) and A28930307 (H.C.F. and J. M. Guss)) and the Swiss National Foundation for Scientific Research.

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