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Partitioning of Ferrocenium Ions between Multiple Redox Sites on Spinach Plastocyanin

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Kinetic measurements of the oxidation of plastocyanin (spinach) by ferrocenium, (hydroxymethyl)ferrocenium, and (chloromercurio)ferrocenium in the presence of the redox-inactive inhibitors $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$, $[Co(NH_3)_6]^{3+}$, and $[Zr(C_2O_4)_4]^{4-}$ as a function of pH at 25 °C and μ = 0.10 are reported. These results support a mechanism in which electrons are transferred from two locations on plastocyanin's surface: one site being the hydrophobic pocket near His-87 where copper is closest to the surface of the protein; the second being the hydrophilic, negatively charged region surrounding Tyr-83. The oxidizing agent's charge and ligand hydrophobicity and the solution pH are found to control the partitioning of the oxidizing agent between the two active sites, and the relative importance of oxidizing agent charge and hydrophobicity in this partitioning is assessed. Electron transfer from reduced plastocyanin is found to be markedly dependent on pH (pK_a = 5.03 ± 0.06), with the protonated protein being redox inactive.

Plastocyanin is a type 1 blue-copper protein of mol. wt. 10 500 containing one copper atom per molecule.[1,2] It cycles between the copper I/II (PCu^I/PCu^{II}) oxidation states and serves to transport electrons from cytochrome *f* to P-700 in the chloroplasts of higher plants.[3] Plastocyanins from 14 higher plants, including spinach, have been completely sequenced, and many others have been partially sequenced.[4] There is substantial sequence homology among the plant plastocyanins with strict conservation of the copper binding site residues and near conservation of charge—estimated to be between −8 and −10 (−9 for spinach) for PCu^I at pH 7. The isoelectric point for spinach plastocyanin is ca. 4.2.[5] While the crystal structure is not available for spinach plastocyanin, Freeman[2] and co-workers have determined the structure for plastocyanin from poplar leaves to high resolution, and it is presently assumed, on the basis of the extensive sequence homology, that the structures of the higher plant plastocyanins are similar.

The availability of this detailed structural information for the plant plastocyanins coupled with their central role in photosynthetic electron transport makes them especially attractive molecules for electron-transfer studies, and a substantial amount of work on plastocyanin electron transfer has been done.[1,4] Of particular significance to this paper is the recent work of Sykes[4,6,7] and co-workers on parsley and spinach plastocyanin, which indicates that two (or possibly more) sites are used during the oxidation of PCu^I by positively charged oxidizing agents, e.g. $[Co(phen)_3]^{3+}$. The nature of the pH dependence of the rate constant for protein oxidation and the observation that redox-inactive, positively charged coordination compounds can *partially* block electron transfer support this contention.[4] The two principal redox sites (Figure 1) appear to be the negatively charged region, derived

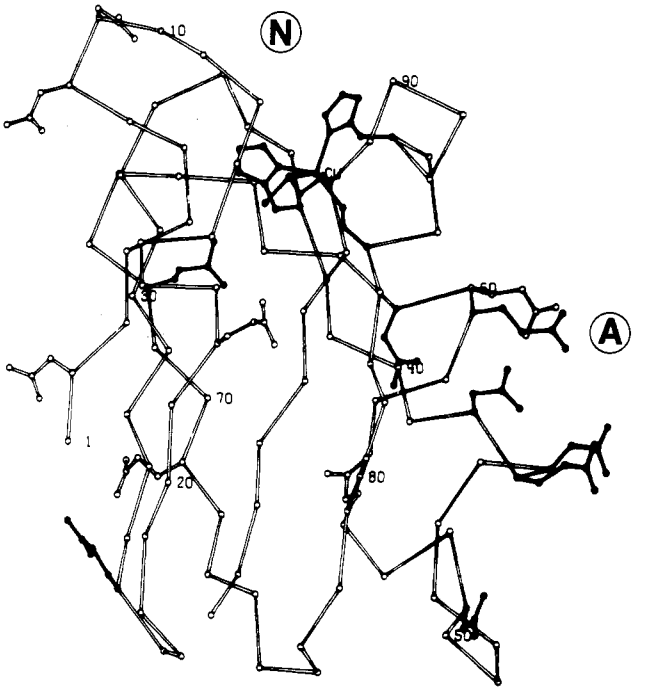

Figure 1. Structure of poplar plastocyanin, slightly modified, as obtained by Freeman.[2]

from glutamic acid and aspartic acid residues at positions 42–45 and 59–61, surrounding Tyr-83 (referred to as the acidic site) and the hydrophobic pocket where copper is closest to the surface of the molecule near His-87 (referred to as the north site).[4] The negatively charged acidic site is implicated as an important site for positively charged oxidants. High-resolution ^1H NMR spectroscopy shows that positively charged complexes, e.g. $[Cr(phen)_3]^{3+}$ and $[Cr(NH_3)_6]^{3+}$, associate with the protein near Tyr-83, supporting this view.[8,9]

We have been examining the reactivity of ferrocenium ($FeCp_2^+$) and its derivatives as redox probes of metalloproteins,[10] including spinach plastocyanin,[11] and have found them to be especially facile oxidants of several metalloproteins. This high reactivity has been

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Table I. Dependence of Rate Constants for the Oxidation of Spinach Plastocyanin by (Chloromercurio)ferrocenium on pH[a]

pH	[FeCpCpHgCl$^+$]/ 10^{-5} M	k_{obsd}/ s^{-1}	k_{expt}/ 10^6 M^{-1} s^{-1}
	Acetate		
4.0	8.42	15.3	0.18
	12.70	21.5	0.17
4.3	5.51	14.4	0.26
	10.31	24.2	0.24
4.5	7.09	24.2	0.34
	12.97	43.2	0.33
4.7	4.27	34.8	0.82
	6.42	40.3	0.63
	7.57	53.8	0.71
	9.02	70.3	0.78
5.0	6.97	49.9	0.72
	10.88	75.6	0.69
	MES[b]		
5.0	6.85	66.8	0.98
	9.53	103.4	1.08
5.2	8.64	119.3	1.38
5.4	4.44	62.6	1.42
	5.33	79.8	1.50
	5.35	81.7	1.53
	6.62	100.3	1.52
	9.06	138.9	1.53
	9.70	133.2	1.37
5.5	5.90	68.0	1.15
	7.50	84.8	1.13
	8.63	99.0	1.15
	10.25	124.2	1.21
	11.44	138.7	1.21
5.6	6.95	104.7	1.50
5.7	3.80	63.5	1.67
	4.04	56.1	1.39
	5.02	87.0	1.73
	6.78	116.6	1.72
	8.08	141.4	1.75
5.8	5.08	84.4	1.65
	7.61	137.0	1.80
6.0	8.63	146.3	1.70
6.2	4.52	92.1	1.87
	7.32	137.2	2.04
6.4	6.74	125.5	1.86
6.5	4.51	82.2	1.82
	6.11	103.0	1.69
6.6	6.30	123.9	1.97
6.8	6.71	132.3	1.97
6.9	3.17	63.6	2.01
	4.90	90.0	1.84
	6.06	123.0	2.03
7.0	7.69	136.5	1.78
	MOPS[b]		
6.8	5.04	102.7	2.04
	6.94	149.7	2.16
7.0	4.75	99.8	2.10
	7.94	162.9	2.05
7.3	5.22	103.8	1.99
	8.31	162.4	1.95
	Tris[b]		
7.3	4.12	109.9	2.67
	6.25	162.9	2.61
7.5	3.21	74.0	2.31
	4.63	107.6	2.32
	4.99	110.4	2.21
	5.31	132.1	2.49
	5.49	137.8	2.51
	6.58	164.6	2.50
	7.03	170.5	2.43
7.7	4.65	119.8	2.58
	7.07	184.0	2.60
8.0	3.92	104.7	2.67
	6.73	181.3	2.69

[a] All data are at 25 °C and μ = 0.10 (0.01 M buffer; NaCl). [b] MES = 2-morpholinoethanesulfonic acid; MOPS = 3-morpholinopropanesulfonic acid; Tris = tris(hydroxymethyl)aminomethane.

attributed in part to the hydrophobic nature of the cyclopentadienide ligands and the related ability of the ferroceniums to access the generally hydrophobic metal environment in redox proteins.[10,11] Since the ferroceniums studied thus far also carry a +1 charge, we were curious as to how the ferroceniums might be partitioned between the north (hydrophobic) site and the acidic (negatively charged) site on plastocyanin. Moreover, since the $[Co(phen)_3]^{3+}$ complex, extensively studied by Sykes,[4,6,7] carries a +3 charge and since the hydrophobicity of the ferroceniums can be varied by derivatization at constant charge (+1), it seemed possible that the relative importance of complex hydrophobicity and charge in partitioning an electron-transfer agent between the two (or more)[12] sites on plastocyanin might be assessed. This paper reports studies of the $FeCp_2^+$ ($E°$ = 0.38 V),[11] (chloromercurio)ferrocenium ($FeCpCpHgCl^+$) ($E°$ = 0.39 V),[11] and (hydroxymethyl)ferrocenium ($FeCpCpCH_2OH^+$) ($E°$ = 0.39 V)[10,11] oxidation of PCu^I in the presence of positively and negatively charged redox-inactive compounds (e.g. $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ and $[Zr(C_2O_4)_4]^{4-}$) designed to address these questions.

Experimental Section

Laboratory-distilled water was further purified by reverse osmosis and ion exchange (Sybron/Barnstead Nanopure). All chemicals were reagent grade or the highest purity available. Argon or nitrogen gas, passed through chromous scrubbing towers to remove traces of oxygen, was used for preparing anaerobic solutions. All UV and visible spectra and spectrophotometric analyses were performed on a Hewlett-Packard 8451 spectrophotometer.

Ferrocene, (chloromercurio)ferrocene, (hydroxymethyl)ferrocene, and their ferrocenium hexafluorophosphate salts were obtained as previously described.[10,11] $FeCp_2^+$ and $FeCpCpHgCl^+$ solutions were prepared by dissolving the hexafluorophosphate salts in buffer and filtering and were analyzed by their visible spectra ($FeCp_2^+$, 618 nm, ϵ = 450 M^{-1} cm^{-1}; $FeCpCpHgCl^+$, 623 nm, ϵ = 504 M^{-1} cm^{-1}). (Hydroxymethyl)ferrocenium decomposes at pH 7.5 and was prepared and analyzed spectrophotometrically (627 nm, ϵ = 400 M^{-1} cm^{-1}) in dilute acid and brought to the correct pH and ionic strength in the stopped-flow apparatus during mixing. Only freshly prepared, filtered, and analyzed solutions were used. Hexaamminecobalt(III) chloride, $[Co(NH_3)_6]Cl_3$ (339 nm, ϵ = 47 M^{-1} cm^{-1}; 473 nm, ϵ = 58 M^{-1} cm^{-1}),[13] and (μ-amido)bis[pentaamminecobalt(III)] bromide, $[(NH_3)_5CoNH_2Co(NH_3)_5]Br_5$ (360 nm, ϵ = 705 M^{-1} cm^{-1}; 505 nm, ϵ = 420 M^{-1} cm^{-1}),[14] were prepared and purified by the literature methods cited. Potassium tetrakis(oxalato)zirconate(IV), $K_4[Zr(C_2O_4)_4]\cdot 5H_2O$,[15] was characterized by oxalate titration.

Buffers were prepared by weighing the neutral form, except for acetic acid, where a standard solution was used, and adjusting the pH with NaOH or HCl by using a Model 611 Orion pH meter.

Plastocyanin was prepared from fresh spinach leaves by a modification[11] of the method of Yocum et al.[16] and purified to an absorbance (A) ratio of A_{278}/A_{597} of 1.7 or less for the oxidized protein. Concentrations of protein were determined on the basis of the absorbance maximum in the visible spectrum (ϵ_{597nm} = 4.9 × 10^3 M^{-1} cm^{-1})[1] of oxidized protein. Buffered protein solutions were stored frozen in liquid nitrogen and were stable therein for several months. Protein from different preparations gave indistinguishable kinetic results. PCu^I was prepared by reduction of PCu^{II} with ascorbic acid followed by extensive dialysis against reaction buffer.

Kinetic measurements were made by observing the formation of PCu^{II} at 597 nm with a Durrum Model D-110 stopped-flow spectrophotometer interfaced to a Nicolet Model 1090 digital oscilloscope and an Apple II computer. Typically, four traces from one drive syringe loading were collected per experiment. Each trace consisted of ca. 500–1000 digitized voltages (V) and times. These digitized data were treated by using a nonlinear least-squares program to fit for the observed pseudo-first-order

(12) All results discussed in terms of two reactive sites on plastocyanin could also be treated equally well in terms of three or more reactive sites. However, since none of the results discussed here would *require* more than two reactive sites for satisfactory data treatment and since there are no data suggesting a third reactive site, all discussions are limited to two sites.

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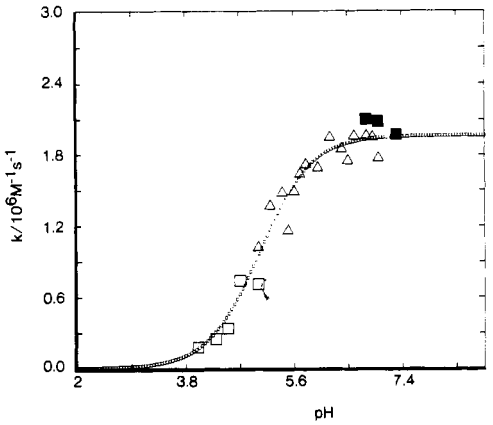

Figure 2. Second-order rate constants for the oxidation of PCu^I by $FeCpCpHgCl^+$ vs. pH: large open squares, acetate; large open triangles, MES; large solid squares, MOPS; small open squares, fitted function using eq 4 and parameters given in the text.

Table II. Rate Constants for the Oxidation of PCu^I by $FeCpCpHgCl^+$ in the Presence of $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ at pH 7.5, 6.5, and 5.5[a]

pH	$[[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}]/10^{-5}$ M	$k_{exptl}/10^6$ M^{-1} s^{-1}
7.5	0.0	2.40
	1.0	2.23
	5.0	1.88
	9.9	1.86
	15.2	1.73
	16.5	1.71
	30.8	1.58
	46.9	1.57
	60.5	1.46
6.5	0.0	1.75
	1.0	1.71
	2.5	1.60
	5.2	1.54
	10.8	1.43
	20.9	1.32
	30.0	1.31
	47.7	1.32
5.5	0.0	1.16
	2.6	1.11
	4.9	1.07
	9.9	1.06
	20.2	1.04
	29.6	1.01
	50.3	0.99

[a] All data are at 25 °C, buffered at pH 7.5 with Tris, at pH 6.5 with MES, and at pH 5.5 with MES, and $[FeCpCpHgCl^+] = (3–9) \times 10^{-5}$ M.

rate constant. The initial and final voltages were fixed at the observed values in this treatment, and a weighting factor that assumed a constant absolute error in the measured voltage was used. In addition, the data acquisition programs provided for the display of $\ln |\Delta V|$ versus time and these plots were linear for more than 3 half-lives. The first-order rate constants, k_{obsd}, were converted to second-order constants, k_{exptl}, by dividing k_{obsd} by the average $FeCp_2^+$, $FeCpCpCH_2OH^+$, or $FeCpCpHgCl^+$ concentration, which in all experiments was in 10-fold or greater excess of PCu^I. A detailed kinetic study of these reactions has been previously reported.[11] Typically, experiments were done by preparing protein and ferrocenium in the appropriate buffer and mixing them on the stopped-flow apparatus. However, in experiments below pH 7.0, PCu^I was prepared in a pH 7.0 buffer of very low concentration and mixed on the stopped-flow apparatus with the ferrocenium solution at a buffer composition such that a solution of desired pH and buffer concentration was achieved after mixing. All experiments were done at 0.010 M buffer and at $\mu = 0.10$ (buffer, NaCl). Experiments with redox-inactive inhibitor complexes present were done by premixing the redox-inactive complex with the ferrocenium prior to mixing with PCu^I on the stopped-flow apparatus. Some experiments were done with redox-inactive complex premixed with the PCu^I solution. The origin of the redox-inactive complex was not observed to be a factor in the reaction rate.

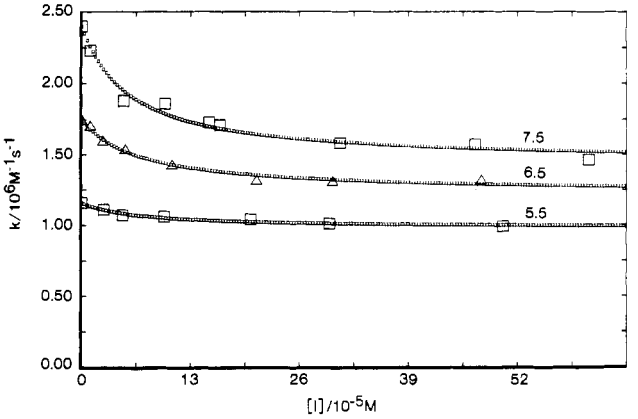

Figure 3. Experimental rate constants, k_{exptl}, vs. the concentration of inhibitor, $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$, for the oxidation of PCu^I by $FeCpCpHgCl^+$ at pH 7.5, 6.5, and 5.5 as indicated. All data are at 25 °C and $\mu = 0.10$ (0.01 M buffer; NaCl).

Results

The oxidation of PCu^I by $FeCpCpHgCl^+$ was studied as a function of pH. Rate constants, k_{exptl} (Table I), are displayed as a function of pH in Figure 2 and conform to the reaction scheme

$$H^+PCu^I \overset{K_a}{\rightleftharpoons} H^+ + PCu^I \quad (1)$$

$$H^+PCu^I + FeCpCpHgCl^+ \xrightarrow{k_H} \text{products} \quad (2)$$

$$PCu^I + FeCpCpHgCl^+ \xrightarrow{k} \text{products} \quad (3)$$

For this reaction k_H makes no detectable contribution to the observed rate and the data have been fit to the expression

$$k_{exptl} = \frac{kK_a}{K_a + [H^+]} \quad (4)$$

The fitted parameters are $k = (1.96 \pm 0.06) \times 10^6$ M^{-1} s^{-1} and $K_a = (9.3 \pm 1.2) \times 10^{-6}$ M ($pK_a = 5.03 \pm 0.06$) at 25 °C and $\mu = 0.10$. Experiments in Tris buffer gave slightly higher k_{exptl} values in buffer overlap regions, and results in Tris (Table I) are not included in Figure 2 or in this fit.

The oxidation of PCu^I by $FeCpCpHgCl^+$ (Table II) at pH 5.5, 6.5, and 7.5 is observed to be inhibited by the presence of the redox-inactive complex $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$. These results are displayed in Figure 3. In addition to the marked pH dependence of the overall rate already described, the extent of inhibition also decreases as the pH decreases. The observed inhibition is consistent with the following reaction scheme, where I represents the positively charged redox-inactive inhibitor:

$$PCu^I + I \overset{K_I}{\rightleftharpoons} PCu^I,I \quad (5)$$

$$PCu^I + FeCpCpHgCl^+ \xrightarrow{k} \text{products} \quad (6)$$

$$PCu^I,I + FeCpCpHgCl^+ \xrightarrow{k_I} \text{products} \quad (7)$$

The following function can be derived from this scheme:

$$k_{exptl} = \frac{k + k_I K_I [I]}{1 + K_I [I]} \quad (8)$$

This is the same scheme previously proposed by Sykes[4,6,7] for reactions of parsley and spinach plastocyanin.

The second-order rate constant in the absence of inhibitor, k, is fixed at the value determined with no inhibitor present, and k_I and K_I are obtained by fitting to eq 8. The values at pH 7.5 are $k = (2.40 \pm 0.10) \times 10^6$ M^{-1} s^{-1}, $k_I = (1.44 \pm 0.05) \times 10^6$ M^{-1} s^{-1}, and $K_I = (1.7 \pm 0.3) \times 10^4$ M^{-1}; at pH 6.5, $k = (1.75 \pm 0.06) \times 10^6$ M^{-1} s^{-1}, $k_I = (1.22 \pm 0.03) \times 10^6$ M^{-1} s^{-1}, and $K_I = (1.4$

Table III. Rate Constants for the Oxidation of PCu^I by $FeCpCpHgCl^+$ in the Presence of $[Co(NH_3)_6]^{3+}$ [a]

$[[Co(NH_3)_6]^{3+}]/10^{-5}$ M	$k_{exptl}/10^6$ M^{-1} s^{-1}	$[[Co(NH_3)_6]^{3+}]/10^{-5}$ M	$k_{exptl}/10^6$ M^{-1} s^{-1}
0.0	2.40	37.5	2.08
5.0	2.37	50.0	2.00
12.5	2.25	75.0	1.85
25.0	2.13	100.0	1.76

[a] All data are at pH 7.5 (Tris) and μ = 0.10 (Tris, NaCl) with $[FeCpCpHgCl^+]$ = (3–7) × 10^{-5} M.

Table IV. Rate Constants for the Oxidation of PCu^I by $FeCp_2^+$ in the Presence of $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ [a]

$[[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}]/10^{-5}$ M	$k_{exptl}/10^5$ M^{-1} s^{-1}	$[[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}]/10^{-5}$ M	$k_{exptl}/10^5$ M^{-1} s^{-1}
0.0	10.6	9.3	9.25
1.0	9.81	20.7	7.95
2.5	9.75	31.4	8.29
4.8	9.38	48.6	7.89

[a] All data are at 25 °C, pH 7.5 (0.01 M Tris), and μ = 0.10 (Tris, NaCl), with $[FeCp_2^+]$ = ca. 1 × 10^{-4} M.

Table V. Rate Constants for the Oxidation of PCu^I by $FeCpCpCH_2OH^+$ in the Presence of $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ [a]

$[[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}]/10^{-4}$ M	$k_{exptl}/10^6$ M^{-1} s^{-1}	$[[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}]/10^{-4}$ M	$k_{exptl}/10^6$ M^{-1} s^{-1}
0.0	4.0	12.0	1.7
6.0	1.9	16.5	1.6
9.0	1.9		

[a] All data are at 25 °C, pH 7.5 (0.01 M Tris), and μ = 0.10 (Tris, NaCl), with $[FeCp_2^+]$ = ca. 5 × 10^{-5} M.

± 0.3) × 10^4 M^{-1}; and at pH 5.5, k = (1.16 ± 0.03) × 10^6 M^{-1} s^{-1}, k_I = (0.98 ± 0.01) × 10^6 M^{-1} s^{-1}, K_I = (1.4 ± 0.4) × 10^4 M^{-1}. The $FeCpCpHgCl^+$ oxidation of PCu^I at pH 7.5 with $[Co(NH_3)_6]^{3+}$ as inhibitor has also been studied (Table III). The values at pH 7.5 are k = (2.40 ± 0.10) × 10^6 M^{-1} s^{-1}, k_I = (1.01 ± 0.17) × 10^6 M^{-1} s^{-1}, and K_I = (8.5 ± 1.6) × 10^2 M^{-1}.

Several experiments on the $FeCpCpHgCl^+$ oxidation of PCu^I were also done with the redox-inactive anionic complex $[Zr(C_2O_4)_4]^{4-}$ present at pH 7.5, and no effect of this complex on the rate was observed.

The complex $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ was also observed to inhibit the $FeCp_2^+$ oxidation of PCu^I at pH 7.5 (Table IV). However, this inhibition is significantly less than that observed for the $FeCpCpHgCl^+$ oxidation at the same pH. The fitted values are k = (1.06 ± 0.05) × 10^6 M^{-1} s^{-1}, k_I = (0.76 ± 0.03) × 10^6 M^{-1} s^{-1}, K_I = (1.4 ± 0.5) × 10^4 M^{-1}. This K_I should have the same value as that obtained from the $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ inhibition of PCu^I oxidation by $FeCpCpHgCl^+$ at pH 7.5, and the two values do agree within the stated uncertainties. However, the greater inhibition in the $FeCpCpHgCl^+$ reaction results in a more precise determination of K_I in that system, and we therefore believe that it is our best determination of K_I.

The $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ inhibition of the oxidation of PCu^I by $FeCpCpCH_2OH^+$ at pH 7.5 was also examined. The instability of $FeCpCpCH_2OH^+$ at pH 7.5 resulted in a limited study for this reagent. In the absence of inhibitor, the second-order rate constant for electron transfer is (4.0 ± 0.3) × 10^6 M^{-1} s^{-1}. In the presence of relatively high concentrations (6.0 × 10^{-4}–1.65 × 10^{-3} M) of $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$, the reaction was inhibited by up to ca. 60%. These data (Table V) have been fit to eq 8 by using K_I = 1.7 × 10^4 M^{-1} and k = 4.0 × 10^6 M^{-1} s^{-1} to yield a value of k_I of (1.6 ± 0.1) × 10^6 M^{-1} s^{-1}.

The results of these inhibition studies at pH 7.5 and the data of Sinclair-Day and Sykes[7] for the $[Co(phen)_3]^{3+}$ and $[Ru(NH_3)_5CH_3CN)]^{3+}$ oxidation of PCu^I at pH 7.5 in the presence of $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ are compared in Figure 4. Relative rate constants, calculated by dividing k_{exptl} at each

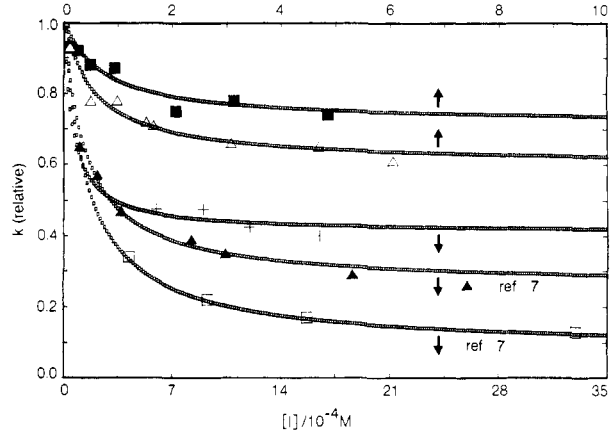

Figure 4. Relative rate constants for the oxidation of spinach PCu^I by $FeCp_2^+$ (large solid squares), $FeCpCpHgCl^+$ (large open triangles), $FeCpCpCH_2OH^+$ (large crosses), $[Co(phen)_3]^{3+}$ (large solid triangles), and $[Ru(NH_3)_5CH_3CN]^{3+}$ (large open squares), all vs. the concentration of inhibitor, $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$. The ↑ arrows indicate data corresponding to the upper inhibitor concentration axis, and the ↓ arrows indicate data corresponding to the lower inhibitor concentration axis. All data are at pH 7.5, μ = 0.10, and 25 °C. The data for $[Co(phen)_3]^{3+}$ and $[Ru(NH_3)_5CH_3CN]^{3+}$ are replotted from data in ref 7. The small open squares are the fitted functions derived from eq 8, fitted parameters given the text, and results reported in ref 7.

$[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ concentration by k_{exptl} in the absence of inhibitor for the given reaction, are plotted vs. the inhibitor concentration to allow comparison of the extent of inhibition in the five systems.

Discussion

The observed pH dependence of the PCu^I oxidation by $FeCpCpHgCl^+$ yields a pK_a value of 5.03 ± 0.06. The pK_a for active-site protonation of 4.9 from NMR studies[17] and the value from kinetic studies[7] with $[Fe(CN)_6]^{3-}$ of 4.8 are close to the value determined here. The value of 5.6 obtained by Sinclair-Day and Sykes from kinetic studies[4,7] using $[Co(phen)_3]^{3+}$ as oxidant is 0.7–0.8 unit higher and has been interpreted as representing the composite of the active-site pK_a and the pK_a for acidic-site protonation. Protonation at the active site is believed[4,7] to make PCu^I inactive to oxidation, and our data support this. The pK_a determined here (5.03) is also likely to be a composite of active-site and acidic-site pK_a's weighted for the relative importance of the two sites to overall reaction. The closeness of the pK_a for PCu^I oxidation obtained here with the NMR and $[Fe(CN)_6]^{3-}$ kinetically determined values suggests that the $FeCpCpHgCl^+$ oxidation is dominated by reaction at the north site (as compared to the $[Co(phen)_3]^{3+}$ reaction) at the pH conditions near this pK_a. A two-pK_a fit of the data has not been applied here because the contribution from the acidic-site pK_a is believed to be too small to be resolved by our data. This point of view is supported by the inhibition studies of the $FeCpCpHgCl^+$ oxidation and the pH dependence of that inhibition as discussed later. Of particular importance is our conclusion that at pH values of 5.5 and below very little of the oxidation of PCu^I by $FeCpCpHgCl^+$ occurs at the acidic site. This is especially evident from Figure 3.

Our results for spinach PCu^I oxidation in the presence of $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ are qualitatively consistent with those of Sykes and co-workers[4,7] for the oxidation of PCu^I from spinach and parsley by various positively charged complexes. The significant but variable inhibition of PCu^I oxidation by various oxidizing agents (e.g., $[Co(phen)_3]^{3+}$, $[Ru(NH_3)_5CH_3CN]^{3+}$, $FeCp_2^+$, $FeCpCpCH_2OH^+$, and $FeCpCpHgCl^+$) observed in the presence of $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$, the nature of the pH dependence of the rate of oxidation and inhibition, and the absence of inhibition of the $[Fe(CN)_6]^{3-}$ oxidation of PCu^I by

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Table VI. Partitioning of Electron Transfer between Multiple Redox Sites on Spinach Plastocyanin[a]

oxidizing agent	% k_N[d]	% k_A[d]
$[Fe(CN)_6]^{3-}$ [b]	100	0
$FeCp_2^+$ [c]	72	28
$FeCpCpHgCl^+$ [c]	60	40
$FeCpCpCH_2OH^+$ [c]	40	60
$[Co(phen)_3]^{3+}$ [b]	25	75
$[Ru(NH_3)_5CH_3CN]^{3+}$ [b]	9	91

[a] All data for oxidation of PCu^I at pH 7.5 and 25 °C. [b] Sinclair-Day, J. D.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1986**, 2069. [c] This work. [d] Computed from the rates at maximum inhibition relative to rates in the absence of inhibitor.

$[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ support an oxidation scheme, eq 9–12, in which reactants are partitioned between two[12] redox-active sites on the surface of PCu^I (Figure 1). This partitioning between

$$PCu^I + I \rightleftharpoons PCu^I,I \quad (9)$$

$$PCu^I + FeCpCpHgCl^+ \xrightarrow{k_A} \text{products} \quad (10)$$

$$PCu^I + FeCpCpHgCl^+ \xrightarrow{k_N} \text{products} \quad (11)$$

$$PCu^I,I + FeCpCpHgCl^+ \xrightarrow{k_N} \text{products} \quad (12)$$

the hydrophilic, negatively charged, acidic site near Tyr-83 and the hydrophobic north site near the partly exposed His-87 is governed by the sign and magnitude of the oxidizing agent's charge as well as its ligand hydrophobicity. In this scheme, binding of a positively charged redox-inactive complex, I, at the acidic site is assumed to completely block electron transfer at this site while not changing reactivity at the north site. From this scheme, one can derive eq 13, which relates k_{exptl} to the rate constants for

$$k_{exptl} = \frac{k_A + k_N + k_N K_I[I]}{1 + K_I[I]} \quad (13)$$

reaction at the acidic, k_A, and north, k_N, sites, and to concentration, [I], and association constant, K_I, for inhibitor. This has the same functional form as given by eq 8, where $k = k_A + k_N$ and $k_I = k_N$. This treatment allows one to compare the partitioning of electron-transfer agents on the surface of PCu^I between the acidic and north sites as a function of oxidant charge and hydrophobicity (Table VI). In this treatment 100% of electron transfer is presumed to be carried by the north site for negatively charged oxidants such as $[Fe(CN)_6]^{3-}$, while positively charged oxidants are partitioned between the two sites depending upon the magnitude of the positive charge and ligand hydrophobicity. $[Fe(CN)_6]^{3-}$ is assumed to react at the north site because the copper is closest to solvent in this region, because the anionic complex $[Cr(CN)_6]^{3-}$ has been shown in ^1H NMR experiments to associate close to His-87 at the north site,[8,9] and because the acidic site is highly disfavored for electrostatic reasons. At pH 7.5, the +3 oxidants $[Co(phen)_3]^{3+}$ and $[Ru(NH_3)_5CH_3CN]^{3+}$ studied by Sinclair-Day and Sykes[7] react 75% and 91% at the acidic site, respectively, as compared to 60%, 40%, and 28%, respectively, for the +1 reagents $FeCpCpCH_2OH^+$, $FeCpCpHgCl^+$, and $FeCp_2^+$ examined here. The increased north-site reactivity of the ferroceniums as compared to the +3 complexes is due, we believe, to the charge differences and to the fact that the very hydrophobic Cp ligands, especially for $FeCp_2^+$, readily shed solvent in accessing the hydrophobic north site. The increased north-site reactivity of $FeCp_2^+$ over $FeCpCpHgCl^+$ and $FeCpCpCH_2OH^+$ and of $[Co(phen)]^{3+}$ over $[Ru(NH_3)_5CH_3CN]^{3+}$ is interpreted as reflecting the greater ligand hydrophobicity of $FeCp_2^+$ and $[Co(phen)_3]^{3+}$ over their like-charged counterparts. These differences are also reflected in Figure 4, where the relative rate at maximum inhibition (interpreted here as percent of reaction at the north site) is seen to be least for $[Ru(NH_3)_5CH_3CN]^{3+}$ and greatest for $FeCp_2^+$. These results are consistent with and supportive of the earlier model[4,6,7] for PCu^I electron transfer, which emphasizes the importance of charge in the distribution of electron-transfer agents between multiple sites, but they extend the model by demonstrating the importance of oxidant hydrophobicity in this partitioning. These results show that hydrophobic effects can be quite important, a feature of plastocyanin reactivity not addressed in previous studies. The pH dependence of $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ inhibition of the $FeCpCpHgCl^+$ oxidation of PCu^I also is consistent with this interpretation. As is evident from data displayed in Figure 3, the fraction of the reaction that can be inhibited is decreased as pH is decreased. This suggests that as pH is decreased, protonation of carboxylate groups at the acidic site lessens the attraction of $FeCpCpHgCl^+$ for the this site and a larger fraction of reaction is consequently carried by the north site.

A number of alternate explanations for the observed rate behavior depicted by eq 8 for the inhibition of the PCu^I oxidation reactions can be offered. For example, the binding of $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ to PCu^I could result in an increase in the $PCu^{II/I}$ redox potential. The expected effect of this would be to decrease the rate of PCu^I oxidation, and this could account for an observed decrease in the rate of oxidation for the PCu^I,I complex. However, this should result in the same relative rate decrease for the $[Fe(CN)_6]^{3-}$, $FeCp_2^+$, and $[Co(phen)_3]^{3+}$ reactions and this is not observed. A decrease in reactivity of a PCu^I,I complex with positively charged oxidants might also be explained on general electrostatic grounds since the complex would have a net charge 5 units higher than uncomplexed protein *regardless* of the site of binding and should react more slowly with a positively charged oxidant than does uncomplexed protein. However, such a general explanation would not account for the significant differences in inhibition for like-charged complexes, such as $FeCp_2^+$ and $FeCpCpCH_2OH^+$. It is also possible that all redox for positively charged complexes occurs at the acidic site and that binding an inhibitor, such as $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$, reduces but does not entirely eliminate reactivity there. This scheme is also disfavored because it could not easily account for the different relative inhibition observed for the oxidants described here. Moreover, McGinnis et al.[6] have recently shown that the same extent of inhibition is observed for the $[Co(phen)_3]^{3+}$ oxidation of PCu^I in the presence of several inhibitors that vary greatly in size. This observation is especially difficult to reconcile with the view that redox in the PCu^I,I complex continues to occur at this site since it seems unlikely that there is room for the large inhibitors and the oxidants to reside at the site simultaneously.[6]

In summary, all data presently available for PCu^I oxidation can be reconciled with an electron-transfer scheme involving two[12] reactive sites on PCu^I with partitioning of electron-transfer agents between the two sites determined by charge and ligand hydrophobicity.

One feature of disagreement between this work and that reported by Sinclair-Day and Sykes is that we determine a value of $K_I = (1.7 \pm 0.3) \times 10^4\ M^{-1}$ for $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ with spinach PCu^I at pH 7.5 and $\mu = 0.10$, while Sinclair-Day and Sykes[7] obtain the value of $(6.8 \pm 1.0) \times 10^3\ M^{-1}$ under the same conditions. We do not know the source of these differences although our value has a large uncertainty because of the high reaction rates, small absorbance changes, and relatively weak inhibition. In some respects, one might have greater confidence in the value obtained from the $[Co(phen)_3]^{3+}$ studies since the reaction is slower and the inhibition is more pronounced. However, comparing this value[7] to the $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ K_I ($K_I = 1.6 \times 10^4\ M^{-1}$) for parsley,[4] which has one less carboxylate group on the acidic site than does the spinach protein, and comparing K_I for $[Co(phen)_3]^{3+}$ for parsley ($340\ M^{-1}$)[6] and spinach ($389\ M^{-1}$)[18] make the lower value for spinach ($6.8 \times 10^3\ M^{-1}$) as compared to that for parsley ($1.6 \times 10^4\ M^{-1}$) obtained from

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the $[Co(phen)_3]^{3+}$ studies[4,7] somewhat surprising. The greater kinetic partitioning[7] of $[Co(phen)_3]^{3+}$ to the acidic site in spinach plastocyanin relative to parsley also suggests greater electrostatic forces in effect here for the spinach protein.

The clear demonstration of multiple electron transfer sites on a protein's surface for a single reacting redox partner gives reason to be cautious in interpreting kinetic parameters for metalloprotein redox processes. In particular, the interpretation of overall activation parameters is hazardous because they may represent averages for very different reactive sites. However, rate studies at several temperatures in the presence of inhibitors at sufficiently high concentration for maximal inhibition combined with similar studies in the absence of inhibitor may allow the correct partitioning of the overall activation parameters between multiple sites since these inhibition studies provide a means of isolating paritcular redox sites. This may provide a better understanding of the solvent and peptide reorganization processes that accompany electron transfer in proteins than has heretofore been possible.

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Registry No. $FeCp_2^+$, 12125-80-3; $(HOCH_2)FeCp_2^+$, 34742-72-8; $(ClHg)FeCp_2^+$, 34742-71-7; $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$, 15771-98-9; $[Co(NH_3)_6]^{3+}$, 14695-95-5; $[Zr(C_2O_4)_4]^{4-}$, 21392-82-5.

Notes

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Synthesis, Characterization, and Reactivity of New Ruthenium and Osmium N_2O_2 Complexes

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The stabilization[1-11] and reactivity[12-19] of transition-metal-oxo complexes have been topics of much current study. In particular, studies involving (polypyridyl)ruthenium-oxo complexes[13,20] and (phosphine)ruthenium(IV)-oxo complexes[12] have demonstrated the utility of ruthenium-oxo centers as organic substrate oxidation agents and catalysts. In addition, N_2O_2 tetraanionic ligands[21-23] and dianionic ligands[24-26] have been combined with first-, second-, and third-row transition metals, in order to generate new transition-metal complexes in which the metal center displays several accessible oxidation states.

In order to further investigate the effects that ligand systems may have on the stabilization of several oxidation states of ruthenium and osmium complexes, we present the synthesis and the characterization of a set of ruthenium and osmium complexes utilizing an N_2O_2 dianionic ligand originally synthesized by Jager,[27] and selected for this study due to its anticipated redox inertness and chemically robust nature. In addition, the redox reactivity of an $N_2O_2Os^{VI}(O)_2$ complex is examined, where the oxidation of benzyl alcohol to benzaldehyde is observed. An unusual reductive activation of the $N_2O_2Os^{VI}(O)_2$ is necessary for substrate oxidation to occur.

Experimental Section

Materials. $Ru^{II}(PPh_3)_3Cl_2$ and $K_2[Os^{VI}(O)_2(OH)_4]$ were prepared as described in the literature.[28,29] The *N,N'*-o-phenylenebis(2-acetyl-1-amino-1-buten-3-one) ligand (H_2phenba) was synthesized by modifications of literature preparations.[27,30] All solvents were reagent grade unless further purification was indicated.

***trans*-[(phenba)$Ru^{II}(PPh_3)_2$].** A 500-mg sample of $RuCl_2(PPh_3)_3$ (0.54 mmol), 177 mg of phenba (0.54 mmol), and 0.15 mL of Et_3N (1.08 mmol) are heated to reflux in 100 mL of degassed THF for 3 h, during which time the solution turns dark red. The solution is cooled and filtered, and then 100 mL of hexanes is added to the filtrate to induce precipitation. A red-brown solid is collected, washed with hexane, and dried; yield 69%. Anal. Calcd for $RuC_{54}H_{48}O_4N_2P_2(H_2O)$: C, 66.79; H, 5.29. Found: C, 66.75; H, 5.54.

***trans*-[(phenba)$Ru^{II}(CO)_2$].** A 250-mg sample of (phenba)$Ru^{II}(PPh_3)_2$ (0.26 mmol) is dissolved in 40 mL of THF. Carbon monoxide is bubbled into this solution until the translucent red-brown solution turns to a transparent orange solution. When the solution is cooled in an ice bath for 15 min, a yellow solid precipitate is formed. Isolation of this

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