Contribution from the Department of Inorganic Chemistry,
The University, Newcastle upon Tyne NE1 7RU, England

Kinetic Studies on 1:1 Electron-Transfer Reactions Involving Blue Copper Proteins. 11. Effects of pH, Competitive Inhibition, and Chromium(III) Modification on the Reaction of Plastocyanin with Cytochrome *f*

DOUGLAS BEOKU-BETTS, STEPHEN K. CHAPMAN, C. VICTOR KNOX, and A. GEOFFREY SYKES*

Received September 24, 1984

Effects (in turn) of pH, competitive inhibition, and Cr(III) modification on rate constants for the plastocyanin, PCu^{II}, oxidation of its natural photosynthetic partner cytochrome *f*, cyt *f*(II), have been studied at 10 °C, I = 0.20 M (NaCl). From effects of pH, an acid dissociation pK_a of 5.1 is obtained. Inhibition is observed with redox-inactive $[Pt(NH_3)_6]^{4+}$ and $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$, and single Cr(III) modification of PCu^{II} produces a retardation effect that is increased by attachment of a second Cr(III). All three effects have been established in previous studies on the oxidation of plastocyanin with inorganic complexes (and cytochrome *c*) as being diagnostic of reaction at the extended negative patch on plastocyanin incorporating Tyr-83 and residues 42–45. It has also been demonstrated that redox-inactive $[Zr(C_2O_4)_4]^{4-}$, which exhibits competitive inhibition for the reaction of negatively charged oxidants with cyt *f*(II), inhibits the reaction with PCu^{II}. From this behavior, it is concluded that cytochrome *f*, although carrying an overall negative charge at pH >5.5, makes use of a positively charged binding site in its reaction with plastocyanin. This site (probably close to an exposed heme edge) is most likely the same as or close to that used by inorganic oxidants. The dependence of rate constants on ionic strength is consistent with a reaction involving oppositely charged centers.

Introduction

The present series of papers[1] has included a consideration of the identification of binding sites on the surface of plastocyanin at which redox partners associate. Effects of pH, competitive inhibition, and chemical modification of the protein have all been shown to be useful in this context.[1-5] Until now, these studies have involved inorganic complexes, or proteins such as cytochrome *c*,[1] that are nonphysiological partners for plastocyanin. Here, we consider the reaction with cytochrome *f*, which is the electron donor to plastocyanin in the photosynthetic electron-transport chain. The two oxidation states of cytochrome *f* containing Fe(II) and Fe(III) are designated as cyt *f*(II) and cyt *f*(III), respectively.

Plastocyanin is a negatively charged, ~8– for PCu^{I}, type I blue copper protein, occurring in all higher plants and green and blue-green algae.[6] The reduction potential for the PCu^{II}/PCu^{I} couple of 370 mV at pH 7.00[7] lies between that previously reported for cyt *f*(III)/cyt *f*(II) (360 mV) and P700 (520 mV), which is the other natural partner to plastocyanin.[8] Cytochrome *f*(III) from charlock has an isoelectric point of p*I* 5.5.[9] The amino acid sequence of pea cytochrome *f* has recently been reported,[11] and compositions of charlock and spinach cytochrome *f* are known.[9] From the sequence, it appears that cytochrome *f* is no longer to be regarded as a hydrophobic protein located completely within the thylakoid membrane. A transmembrane arrangement having a 15 amino acid C-terminal sequence (271–285) in the stroma, a single (probably α helix) stretch of hydrophobic residues (251–270) within the membrane, and a highly charged (22% of residues) N-terminal heme-containing section (1–250) in the intrathylakoid space (where plastocyanin is also located) is now strongly implicated. The reactivity pattern of cyt *f*(II) (the 1–250 section) with a range of inorganic oxidants (5– to 3+) has been investigated.[10]

Having established different approaches,[1-4] it is now appropriate to try and identify the binding site on plastocyanin used by cytochrome *f* and vice versa. Recent proposals concerning the binding site for P700 on plastocyanin are not in agreement. Thus, Farver et al.,[12] using plastocyanin chromium(III) modified at residues 42–45, have indicated that reduction of P700 occurs at the Tyr-83/42–45 site and (by implication) concluded that the His-87 "north" site of plastocyanin is used by cytochrome *f*. However, a study by Burkey and Gross,[13] using ethylenediamine-modified plastocyanin, has led to the suggestion that P700 reduction does not in fact occur at the Tyr-83/42–45 site. From the present studies it has been possible to assign which of these sites is used by cytochrome *f*.[14]

Experimental Section

Proteins. Plastocyanin was isolated from parsley leaves[15] and handled as previously described.[16] The peak ratio A_{278}/A_{597} = 1.7 ± 0.1 was used as a criteria of purity and the absorbance maxima of PCu^{II} at 597 nm (ϵ 4500 M^{-1} cm^{-1}) used to determine concentrations. Cytochrome *f* was isolated from cabbage leaves (*Brassica oleracea*, Durham early variety) by using the method of Gray.[9] The cytochrome *f* obtained (believed to be residues 1–250)[11] was purified to absorbance peak ratios for the reduced form of A_{554}/A_{280} = 0.85 ± 0.10. The absorbance maximum for cyt *f*(II) at 554 nm (ϵ = 32 000 M^{-1} cm^{-1}) was used to determine concentrations.[17] Spectra of cyt *f*(II) (pink) and cyt *f*(III) (orange-pink) are illustrated in a previous paper.[10] To prepare cyt *f*(II), the oxidized protein was reduced with a small amount of D,L-dithiothreitol (Cleland's reagent) and bound onto a short (1.5 cm long, 1 cm wide) DE52 column equilibrated in 10 mM phosphate at pH 7.5. The column behavior is consistent with a negative charge although from the amino acid composition this must be small. Excess reductant was washed out and the reduced protein eluted with the required buffer.

Chromium(III) Modification. Plastocyanin was singly modified to give $[PCu^{I}]Cr^{III}$ and doubly modified to $[PCu^{I}]Cr^{III}_2$ by the method described previously.[5,12] Oxidized PCu^{II} forms were prepared by adding a small crystal of $K_3[Fe(CN)_6]$ to produce $[PCu^{II}]Cr^{III}$ or $[PCu^{II}]Cr^{III}_2$, followed by extensive dialysis against the required buffer.

pH and Buffers. In the pH range 7–8, tris(hydroxymethyl)aminomethane (Tris) (Sigma Chemicals) and HCl buffer were used. Solutions were prepared with 0.010 M HCl and by addition of Tris until the required pH was reached. In the pH range 5–7, the buffer 2-(morpho-

(1) Part 10: Chapman, S. K.; Knox, C. V.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1984**, 2775.
(2) Chapman, S. K.; Davies, D. M.; Watson, A. D.; Sykes, A. G. *ACS Symp. Ser.* **1983**, *No. 211*, 177–197.
(3) Chapman, S. K.; Watson, A. D.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1983**, 2543.
(4) Chapman, S. K.; Sanemasa, I.; Sykes, A. G. *J. Chem. Soc. Dalton Trans.* **1983**, 2549.
(5) Chapman, S. K.; Knox, C. V.; Kathirgamanathan, P.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1984**, 2769.
(6) Crofts, A. R.; Wood, P. M. *Curr. Top. Bioenerg.* **1978**, *7*, 1975–2244.
(7) Lappin, A. G. "Metal Ions In Biological Systems"; Sigel H., Ed.; Marcel Dekker: New York, **1981**; Vol. 13, pp 15–71.
(8) Freeman, H. C. *Coord. Chem.* **1981**, *21*, 29 and references therein.
(9) Gray, J. C. *Eur. J. Biochem.* **1978**, *82*, 133.
(10) Beoku-Betts, D.; Sykes, A. G. *Inorg. Chem.* **1985**, *24*, 1142.
(11) Willey, D. L.; Auffret, A. D.; Gray, J. C. *Cell* **1984**, *36*, 555.
(12) Farver, O.; Shahak, Y.; Pecht, I. *Biochemistry* **1982**, *21*, 1885.
(13) Burkey, K. O.; Gross, E. L. *Biochemistry* **1982**, *21*, 5886.
(14) Beoku-Betts, D.; Chapman, S. K.; Knox, C. V.; Sykes, A. G. *J. Chem. Soc., Chem. Commun.* **1983**, 1150.
(15) Plesničar, M.; Bendal, D. S. *Biochim. Biophys. Acta* **1970**, *216*, 192.
(16) Segal, M. G.; Sykes, A. G. *J. Am. Chem. Soc.* **1978**, *100*, 4585.
(17) Takabe, T.; Niwa, S.; Ishikawa, H.; Takenaka, K. *J. Biochem.* **1980**, *88*, 1167.

0020-1669/85/1324-1677$01.50/0

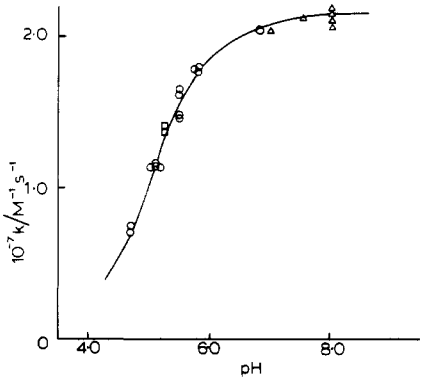

Figure 1. Effect of pH on rate constants (10 °C) for the plastocyanin, PCu^{II}, oxidation of cyt *f*(II) (I = 0.20 M (NaCl)). Buffers: Tris/HCl (△); acetate (□); Mes/NaOH (○).

lino)ethanesulfonic acid (Mes) was used. This was prepared by addition of Mes to 0.010 M NaOH until the required pH was reached. Below pH 5.0 acetate buffer, prepared by addition of acetic acid to 0.010 M sodium acetate, was used. All pHs were checked on a Radiometer (PHM62) pH meter fitted with a combined-glass electrode.

Preparation of Complexes. The following complexes were prepared: hexaammineplatinum(IV) chloride, $[Pt(NH_3)_6]Cl_4 \cdot H_2O$, peak at 260 nm ($\epsilon$ 129 M^{-1} cm^{-1});[18] (μ-amido)bis[pentaamminecobalt(III)] bromide, $[(NH_3)_5CoNH_2Co(NH_3)_5]Br_5$, peaks at 360 (707) and 505 (420);[19] potassium tetrakis(oxalato)zirconate(IV), $K_4[Zr(C_2O_4)_4] \cdot 5H_2O$, the purity of which was confirmed by titration for oxalate.[20]

Stoichiometry. Absorbance changes at 25 °C, pH 7.5 and 5.3, I = 0.20 M (NaCl), for cyt *f*(II) (3.4×10^{-7} M) with PCu^{II} (5.9×10^{-6} M) indicated ≥93% completion consistent with a 1:1 stoichiometry as in (1).

$$\text{cyt } f(\text{II}) + PCu^{II} \rightarrow \text{cyt } f(\text{III}) + PCu^{I} \quad (1)$$

In most runs the PCu^{II} to cyt *f*(II) ratio was ≥10. With the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ couple (E^0 = 410 mV) as mediator, the reduction potential (25 °C) of the cyt *f*(III)/cyt *f*(II) couple was determined as 350 mV at pH 7.5 (Tris/HCl), I = 0.10 M (NaCl), in good agreement with the literature value of 360 mV.[8]

Kinetic Studies. The ionic strength of run solutions was adjusted to 0.20 M, or 0.10 M in the case of the $[Zr(C_2O_4)_4]^{4-}$ blocking experiments, by addition of NaCl (BDH, AnalaR). To retard rates sufficiently and enable rate constants to be determined by the stopped-flow method, the higher ionic strength and/or temperature of 10 °C was used.

A Dionex D-110 stopped-flow spectrophotometer was used to monitor absorbance changes at the cyt *f*(II) peak at 422 nm. For the studies at I = 0.20 M (NaCl) the plastocyanin, PCu^{II}, was in large (≥10-fold) excess. First-order rate constants, k_{obsd}, were obtained on a Datalab DL901 transient recorder interfaced to a Commodore PET 2001-16K desk top computer. Plots of absorbance (A) changes ln ($A_\infty - A_t$) against time were linear to 4 half-lives except in the case of the $[PCu^{II}]Cr^{III}_2$ oxidation where linearity was to 3 half-lives. The kinetics conform to the rate law (2). For the range of $[PCu^{II}]$ investigated, a linear dependence

$$\text{rate} = k_{obsd}[\text{cyt } f(\text{II})] \quad (2)$$

on PCu^{II} was observed, and second-order rate constants k can therefore be defined as in (3). Studies on the effect of $[Zr(C_2O_4)_4]^{4-}$ at I = 0.10

$$k_{obsd} = k[PCu^{II}] \quad (3)$$

M necessitated a different experimental approach. Equimolar concentrations of the two protein reactants (a) were used, and the forward rate constant (k) was obtained from eq 4 for equilibration kinetics,[21] where

$$\ln\left(\frac{x(a - 2x_e) + ax_e}{a(x_e - x)}\right) = \frac{2a(a - x_e)kt}{x_e} \quad (4)$$

x_e pertains to equilibrium conditions. An equilibrium constant of 5.0 at 10 °C determined spectrophotometrically was in satisfactory agreement with the value of 2.2 calculated from reduction potentials at 25 °C for PCu^{II}/PCu^{I} (370 mV) and cyt *f*(III)/cyt *f*(II) (350 mV), I = 0.10 M (NaCl).

Treatment of Data. An unweighted nonlinear least-squares program was used to determine rate constant and equilibrium constant parameters in eq 5 and 12.

Table I. pH Dependence of Rate Constants for the Oxidation of cyt *f*(III) ($\sim 5 \times 10^{-7}$ M) by Plastocyanin, PCu^{II} (10 °C, I = 0.20 M (NaCl))

pH	$10^6 \times [PCu^{II}]$, M	$10^{-2} \times k_{obsd}$, s^{-1}	$10^{-7}k$, M^{-1} s^{-1}
4.7 (OAc)	6.9	0.52	0.75
	8.9	0.63	0.71
5.1 (Mes)	5.1	0.58	1.14
	5.9	0.67	1.14
	6.9	0.77	1.16
	7.9	0.90	1.14
5.2 (OAc)	2.1[a]	0.30	1.41
	3.0[a]	0.42	1.39
5.5 (Mes)	5.4	0.89	1.65
	6.6	1.07	1.62
	8.4	1.23	1.46
	11.0	1.63	1.48
5.8 (Mes)	5.6	1.01	1.80
	6.9	1.22	1.77
	8.5	1.52	1.79
6.8 (Mes)	2.6[a]	0.57	2.19
7.0 (Tris/HCl)	5.0	1.02	2.04
7.5 (Tris/HCl)	9.9	2.12	2.10
8.0 (Tris/HCl)	4.5	0.94	2.09
	5.0	1.10	2.20
	6.3	1.34	2.13
	7.8	1.68	2.15

[a] [cyt *f*(II)] = 1.8×10^{-7} M.

Table II. Effect of Ionic Strength (I), Adjusted with NaCl, on Rate Constants for the Oxidation of cyt *f*(II) ($\sim 5 \times 10^{-7}$ M) with PCu^{II} at 10 °C, pH 7.5 (Tris/HCl)

I, M	$10^6 \times [PCu^{II}]$, M	$10^{-7}k$, M^{-1} s^{-1}	I, M	$10^6 \times [PCu^{II}]$, M	$10^{-7}k$, M^{-1} s^{-1}
0.10	1.0	15[a]	0.20	9.9	2.2
0.15	6.2	4.8	0.35	5.7	0.72
0.20	6.5	2.2	0.50	6.2	0.59

[a] Equimolar concentrations (1×10^{-6} M) of cyt *f*(II) and PCu^{II} used.

Results

Effects of pH. The dependence of k (10 °C) on pH (Table I) fits the expression (5), where the various constants are defined

$$k = \frac{k_o K_a + k_H[H^+]}{K_a + [H^+]} \quad (5)$$

in the sequence (6)–(8). The effect of pH is summarized in Figure

$$H^+PCu^{II} \rightleftharpoons PCu^{II} + H^+ \quad (6)$$

$$\text{cyt } f(\text{II}) + PCu^{II} \xrightarrow{k_o} \text{products} \quad (7)$$

$$\text{cyt } f(\text{II}) + H^+PCu^{II} \xrightarrow{k_H} \text{products} \quad (8)$$

1. From a nonlinear least-squares fit $k_o = (2.13 \pm 0.02) \times 10^7$ M^{-1} s^{-1}, $k_H = (0.22 \pm 0.10) \times 10^7$ M^{-1} s^{-1}, and $K_a = (7.4 \pm 0.89) \times 10^{-6}$ M^{-1}, corresponding to an acid dissociation pK_a of 5.07 ± 0.07.

Effect of Ionic Strength. The effect of ionic strength on rate constants at 10 °C and pH 7.5 (Tris/HCl) is summarized in Table II.

Competitive Inhibition on PCu^{II}. Redox-inactive complexes $[Pt(NH_3)_6]^{4+}$ and $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ give competitive inhibition of cyt *f*(II). This can be explained in terms of the

(18) Essen, L. N. *Inorg. Synth.* **1973**, *15*, 93.
(19) Davies, R.; Mori, M.; Sykes, A. G.; Weil, J. A. *Inorg. Synth.* **1970**, *12*, 212.
(20) Van der Poil, J.; Newmann, H. M. *Inorg. Synth.* **1968**, *11*, 53.
(21) E.g.: Frost, A. A.; Pearson, R. G. "Kinetics and Mechanism", 2nd ed.; Wiley: New York, 1961; p 188.

reaction sequence (9) and (10), where B represents the inhibitor

$$PCu^{II} + B \overset{K_B}{\rightleftharpoons} PCu^{II},B \quad (9)$$

$$\text{cyt } f(II) + PCu^{II} \xrightarrow{k_o} \text{products} \quad (10)$$

(i.e., blocking complex). The data obtained are consistent with complete inhibition and no contribution from (11). The above

$$\text{cyt } f(II) + PCu^{II}, B \rightarrow \text{products} \quad (11)$$

sequence gives rise to (12). The effect of $[Pt(NH_3)_6]^{4+}$ (Table

$$k = k_o/(1 + K_B[B]) \quad (12)$$

III) was studied at pH 5.8 (Mes/NaOH) (Figure 2) to avoid contributions from the 3+ conjugate-base form. Equation 12 can be rearranged to a linear form, the applicability of which is illustrated in Figure 2. The value determined for K_B (10 °C, I = 0.20 M) was 1600 ± 90 M^{-1}. The effect of $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ was studied at pH 7.5 (Tris/HCl) (Table III). The value of K_B (10 °C, I = 0.20 M) obtained was 2200 ± 150 M^{-1}.

Competitive Inhibition on Cyt *f*(II). It has previously been demonstrated that $[Zr(C_2O_4)_4]^{4-}$ inhibits the oxidation of cyt f(II) with the 5- complex $[(CN)_5FeCNCo(CN)_5]^{5-}$, K_B = 530 M^{-1} (25 °C) at pH 7.5, I = 0.10 M (NaCl).[10] When the ionic strength is increased to 0.20 M (NaCl), K_B decreases and inhibition is difficult to detect.[10] Therefore, in the present work it was necessary to investigate the effect of $[Zr(C_2O_4)_4]^{4-}$ at I = 0.10 M (NaCl), which meant working under conditions close to the limit of the stopped-flow range. In order to decrease the rate, a temperature of 10 °C was adopted, and identical protein concentrations (~1.0 × 10^{-6} M) were used. Under these conditions this reaction does not proceed to completion, and only the final ~50% of absorbance changes could be monitored. Apparent rate constants 10^{-8} k (M^{-1} s^{-1}), obtained with $10^3[Zr(C_2O_4)_4^{4-}]$ = 0, 0.25, 1.00, and 2.00 M were 1.5, 1.2, 0.92, and 0.73, respectively (Figure 3). A graph of k^{-1} against $[Zr(C_2O_4)_4^{4-}]$ is linear as required by (12) and gives $K \sim 540$ M^{-1}.

Cr(III) Modification. Effects of Cr(III) modification were studied at three pHs. At pH 8.0 (Tris/HCl) the rate constant for the cyt f(II) reduction of $[PCu^{II}]Cr^{III}$ is ~40% less than that observed for native PCu^{II} (Table IV). At pH 5.8 (Mes/NaOH) rate constants for cyt f(II) reduction of $[PCu^{II}]Cr^{III}$ and $[PCu^{II}]Cr^{III}_2$ are respectively ~60% and 90% less (Figure 4). The effect of single modification at pH 5.1 (Mes/NaOH), which is below pI 5.5 for cytochrome f, is also 60% (Table IV). No biphasic kinetics were detected for the $[PCu^{II}]Cr^{III}$ oxidation of cyt f(II),[5] at any of three pHs investigated.

Discussion

Rate constants (10 °C) for the reduction of PCu^{II} by cyt f(II) (from *B. Oleracea*) show a dependence on pH that gives a pK_a of 5.07 ± 0.07 at I = 0.20 M (NaCl). This is in good agreement with a pK_a of 4.9 (our fit) from data at 20 °C reported by Niwa et al.[22] using cytochrome f from *Brassica Komatsuna*, I = 0.20 M (NaCl). These values are similar to pK_a's obtained for the reduction of PCu^{II} by $[Ru(NH_3)_5py]^{2+}$ and cyt c(II),[4,1] which are 5.0 ± 0.1 and 4.9 ± 0.1, respectively, at 25 °C (I = 0.10 M). Not all reducants exhibit pH dependences of this kind, a notable exception being $[Fe(CN)_6]^{4-}$,[2,4] which has no pronounced dependence in the pH 4.5–7.5 range, consistent with reaction at the uncharged north site. The pK_a's of around 5.0 are believed to correspond to protonation of an acidic residue close to the site at which $[Ru(NH_3)_5py]^{2+}$ and cyt c(II) react.[4] This site has been designated as the extended and highly conserved acidic patch close to Tyr-83, which incorporates acidic residues 42–45. A variety of evidence has been obtained in support of this assignment.[1-5] The fact that the pK_a for the reduction of PCu^{II} by cyt f(II) is close to 5.0 (and k_H is 10% k_o) excludes major contributions from reaction at the north site and favors the Tyr-83 region as binding site.

It has been assumed that the dominant protonation here is occurring on the PCu^{II} and not on the cyt f(II). This is supported by studies on the oxidation of cyt f(II) by negatively charged

(22) Niwa, S.; Ishakawa, H.; Nakai, S.; Takabe, T. *J. Biochem.* **1980**, *88*, 1177.

Table III. Effect of Redox-Inactive Complexes (B) on Rate Constants (10 °C) for the Oxidation of Cytochrome f(II) (5.0×10^{-7} M) by Plastocyanin, PCu^{II} (5.0×10^{-6} M), I = 0.20 M (NaCl)

10^4[B], M	k_{obsd}, s^{-1}	$10^{-7}k$, M^{-1} s^{-1}	10^4[B], M	k_{obsd}, s^{-1}	$10^{-7}k$, M^{-1} s^{-1}
B = $[Pt(NH_3)_6]^{4+}$; pH 5.8 (Mes/NaOH)					
0	96	1.92	4.1	59	1.18
1.38	75	1.50	5.5	51	1.02
2.76	68	1.36			
B = $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$; pH 7.5 (Tris/HCl)					
0	1.02	2.04	3.22	0.61	1.22
0.98	0.88	1.76	5.10	0.45	0.90
1.83	0.72	1.44			

Table IV. Rate Constants (10 °C) for the Oxidation of Cytochrome f (~5 × 10^{-7} M) by Plastocyanin and Modified Plastocyanin (pH As Indicated, I = 0.20 M (NaCl))

	$10^6 \times$ [PCu^{II}], M	k_{obsd}, s^{-1}
PCu^{II}, pH 8.0 (Tris/HCl)	4.5	94
	5.0	110
	6.3	134
	7.8	168
$[PCu^{II}]Cr^{III}$, pH 8.0 (Tris/HCl)	4.7	68
	5.9	84
	7.1	91
	8.9	118
PCu^{II}, pH 5.8 (Mes)	5.55	101
	6.88	122
	8.50	152
$[PCu^{II}]Cr^{III}$, pH 5.8 (Mes)	5.01	35
	8.66	56
	12.2	67
$[PCu^{II}]Cr^{III}_2$, pH 5.8 (Mes)	5.3	9[a]
	8.6	17[a]
	14.1	27
PCu^{II}, pH 5.1 (Mes)	5.1	58
	5.9	67
	6.9	77
	7.9	90
$[PCu^{II}]Cr^{III}$, pH 5.1 (Mes)	5.8	25
	6.6	31
	7.0	32
	8.4	35

[a] Rate constants from unresolved biphasic reaction.

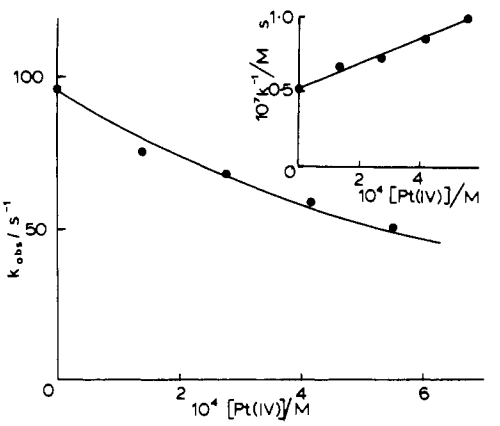

Figure 2. Effect of $[Pt(NH_3)_6]^{4+}$ on first-order rate constants (10 °C) for the plastocyanin, PCu^{II} (5×10^{-6} M), oxidation of cyt f(II) at pH 5.8 (Mes/NaOH, I = 0.20 M (NaCl)). Inset shows the linear plot according to eq 12.

Table V. Summary of Rate Constants for the Oxidation of cyt *f*(II) with Plastocyanin

reductant	oxidant[a]	rate const $\times 10^{-7}$, M^{-1} s^{-1} (temp, °C)	*I*, M	ref
cyt *f*(II) (*Brassica oleracea*)	PCu^{II} (Parsley)	2.1 (10)	0.20	this work
cyt *f*(II) (*Brassica komatsuna*)	PCu^{II}	4.5 (20)[b]	0.20	22
cyt *f*(II) (Japanese radish)	PCu^{II}	6.0 (25)[b]	0.11	25
cyt *f*(II) (Parsley)	PCu^{II}	3.6 (25)[b]	0.10	26

[a] Source indicated if different from that of reductant. [b] Activation parameters reported. Some variations are apparent with $\Delta H^{\ddagger}$ = 8.4–13.4 kcal mol^{-1} and $\Delta S^{\ddagger}$ = 4.9–21.5 cal K^{-1} mol^{-1}.

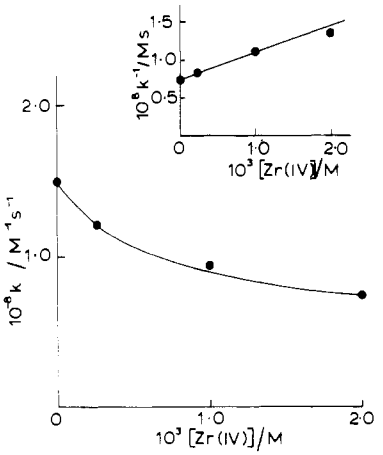

Figure 3. Effect of redox-inactive $[Zr(C_2O_4)_4]^{4-}$ on rate constants for the oxidation of cyt *f*(II) with plastocyanin, PCu^{II} (concentrations ~1.0 × 10^{-6} M) at 10 °C, pH 7.5 (Tris/HCl), *I* = 0.10 M (NaCl). Inset shows the linear plot according to eq 12.

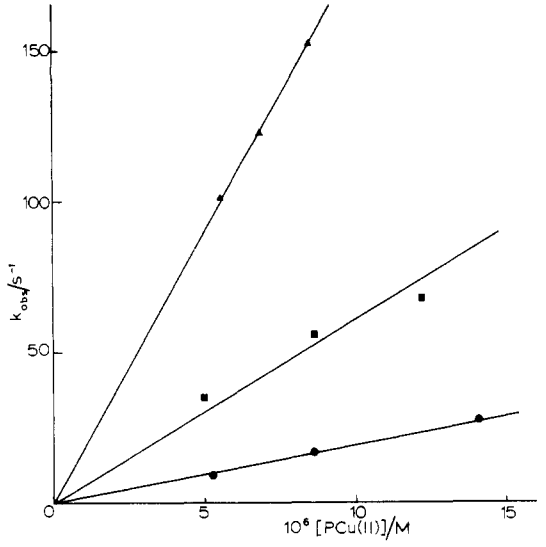

Figure 4. Dependence of first-order rate constants (10 °C) for plastocyanin oxidation of cyt *f*(II) (5 × 10^{-7} M) on oxidant concentration at pH 5.8 (Mes/NaOH), *I* = 0.20 M (NaCl). Key: PCu^{II} unmodified (▲); $[PCu^{II}]Cr^{III}$ (■); $[PCu^{II}]Cr^{III}_2$ (●).

inorganic redox reagents, where an increase in rate is observed with decreasing pH.[10,22] If the effect were originating from a protonation on cyt *f*(II), an increase in rate constants might have been expected with decreasing pH.

Competitive inhibition is observed with redox-inactive $[Pt(NH_3)_6]^{4+}$ and $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$. Since these complexes show specificity for the Tyr-83 site on plastocyanin,[3] this locality is again implicated as being involved in the PCu^{II} oxidation of cyt *f*(II). The possibility that positively charged complexes may associate with cyt *f*(II), which has an overall negative charge at pH >5.5, must also be considered. However, no competitive inhibition effect was observed for either complex on the $[Co(phen)_3]^{3+}$ oxidation of cyt *f*(II).[10] Values of K_B obtained are in reasonable accord with those previously reported,[3] bearing in mind that a higher ionic strength and lower temperature had to be used, both of which are expected[16] to decrease K_B.

It has been shown that redox-inactive $[Zr(C_2O_4)_4]^{4-}$ inhibits the reaction of cyt *f*(II) with $[(CN)_5FeCNCo(CN)_5]^{5-}$, K_B = 530 M^{-1} at 25 °C, *I* = 0.10 M (NaCl),[10] but has no effect on the oxidation of PCu^{II} with $[Fe(CN)_6]^{3-}$.[3] In the present studies it has been demonstrated that $[Zr(C_2O_4)_4]^{4-}$ inhibits the rate of oxidation of cyt *f*(II) by PCu^{II} and that $K \sim 540$ M^{-1} at 10 °C, *I* = 0.10 M. The two values of *K* suggest that the same association of $[Zr(C_2O_4)_4]^{4-}$ with cytochrome *f* is relevant to both reactions and that the same or nearly identical sites for electron transfer on cytochrome *f* are involved in each case.

The Cr(III) modification of PCu^{II} produces a marked inhibition of the reaction with cyt *f*(II). At pH 8.0 there is a 40% effect that increases to ~60% at both pH 5.8 and 5.1. Attachment of two Cr(III)'s to PCu^{II} results in a 60% decrease. Farver and Pecht[23] have concluded that the site of attachment for a single Cr(III) is at acid residues 42–45 on plastocyanin. Since Cr(III) modification of PCu^{II} inhibits the reaction with cyt *f*(II), this suggests that the binding site is close to the 42–45 patch. The large effect caused by attachment of 2 Cr(III)'s to PCu^{II} further supports this assignment, evidence having been obtained for attachment of the second Cr(III) at or near to the 42–45 patch.[5]

The three approaches described, involving the effects of pH, competitive inhibition, and Cr(III) modification of PCu^{II}, all appear to indicate involvement of the Tyr-83 region on PCu^{II} in the reaction with cyt *f*(II). This is in direct contrast to a previous suggestion of Farver et al.[12] The specificity of the Tyr-83 site for positively charged reactants and the conclusion that cyt *f*(II) (overall negative charge) behaves in the same way as cyt *c*(II)[1] (overall positive charge) suggest that there is a positively charged functional region on cyt *f*(II). Since a recurring feature of cytochrome reactions is the use made of an exposed heme edge, this positive region is likely to be in the vicinity of such an exposed edge. It has been observed[10] that the reaction of cyt *f*(II) with $[(CN)_5FeCNCo(CN)_5]^{5-}$ exhibits saturation kinetics, with an association constant, *K*(20 °C), of around 500 M^{-1} at pH 7.5, *I* = 0.10 M (NaCl). Competitive inhibition by $[Zr(Cr_2O_4)_4]^{4-}$ has also been noted.[10] The fact that negatively and not positively charged inorganic complexes associate extensively with cyt *f*(II) further supports the idea of a positively charged region on cyt *f*(II) at or close to the electron-transfer binding site.

To summarize, electrostatic considerations would certainly seem to be important in the reaction of cyt *f*(II) with PCu^{II}. This is supported by the variation of rate constants with ionic strength, which is in agreement with Niwa et al.[22] but is in sharp contrast to observations of Tanaka et al.,[24] who worked under more difficult experimental conditions. The trend observed suggests an involvement of oppositely charged centers on the two proteins. Since cytochrome *f* (mol wt ~30 000) is larger than plastocyanin (mol wt ~10 500), it is possible that a relatively broad contact area on plastocyanin is involved.

Although cytochrome *f* is firmly anchored to the thylakoid membrane, there is now strong evidence to suggest that the highly charged heme-containing section (residues 1–250) is located within the intrathylakoid space where is it able to interact directly with plastocyanin.[11] This is important new information. Moreover, it is clear now that glutamates and glutamines etc. have been

(23) Farver, O.; Pecht, I. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4190.
(24) Tanaka, K.; Takahashi, M.; Asada, K. *Plant Cell Physiol.* **1981**, *22*, 33.

distinguished, that the protein has only a small overall negative charge on the 1-250 section of the protein relevant to this study. Examination of the sequence reveals pairs of positively charged residues at five places in the chain, and Willey et al.[11] speculate that these may well be brought together in the tertiary structure to form a positive patch that can interact with the negative patch on plastocyanin. Our quite independent evidence for a positive patch on cytochrome *f* first indicated in ref 14, is consistent with these observations.

Table V contains a summary of rate constants for the reaction of cyt *f*(II) with plastocyanin PCu^{II},[21,24,25] with proteins isolated from different sources. Assuming $\Delta H^{\ddagger} = 10$ kcal mol^{-1}, a rate constant of 2.1×10^7 M^{-1} s^{-1} at 10 °C increases to 5.1×10^7 M^{-1} s^{-1} at 25 °C. The rate constants listed will also show some variation with ionic strength as has been demonstrated in this study and elsewhere. The observation that PCu^{II} is some 30× more reactive with cytochrome *f* than with other (nonphysiological) cytochromes[25] is consistent with the high specificity of the two reactants for each other. The diffusion-controlled rate constants for such large molecules can be estimated as 10^9-10^{10} M^{-1} s^{-1},[24,25] which however ignores the need for the two proteins to be correctly oriented at the time of encounter. The rate constant observed is probably close to (or at) the limit possible for such a protein-protein reaction.

Structural features of plastocyanin indicated by Freeman[8,28] with the upper (north) surface of the protein hydrophobic may result in association of the protein at the thylakoid membrane surface. This could help in the orientation of the two proteins prior to electron transfer or, alternatively, could be relevant to the subsequent plastocyanin reduction of P700.

Adoption of a "dead-end" type of mechanism as an alternative to eq 9-11 is dependent on association (at the "east" site) completely inactivating the protein. Conformational change(s) that are close to 100% effective rather than an actual physical blocking of the binding site are required for this interpretation to hold. We keep an open mind on such possibilities (e.g., ref 2, p 183) but at present see no compelling evidence[8] for such an interpretation. Certainly we do not exclude small (possibly ~10%) contributions to reaction currently assigned to the Tyr-83 site as occurring at the "north" site. Reaction at the "north" site has the advantage of involving electron transfer to and from the Cu active site over a shorter distance. Reaction at the "east" site benefits from the extensive association due to more favorable electrostatic interactions that outweight the longer (approximately 2×) distance for electron transfer.

Acknowledgment. We thank the U.K. Science and Engineering Research Council for postgraduate (S.K.C.) and postdoctoral (C.V.K.) support.

Registry No. cyt *f*, 9035-46-5; $[Pt(NH_3)_6]Cl_4$, 16893-12-2; $[(NH_3)_5CoNH_2Co(NH_3)_5]Br_5$, 72273-61-1; $K_4[Zr(C_2O_4)_4]$, 12083-35-1; Cr(III), 16065-83-1; hydrogen ion, 12408-02-5.

(25) Wood, P. M. *Biochim. Biophys. Acta* **1974**, *357*, 370.
(26) Bendall, D. S.; Davenport, H. E.; Hill, R. "Methods in Enzymology"; San Pietro, A., Ed.; Academic Press: New York, 1971; Vol. 23, p 327.
(27) Smith, D. D.; Selman, B. R.; Voegeli, K. K.; Johnson, G.; Dilley, R. A. *Biochim. Biophys. Acta* **1977**, *459*, 468.
(28) Guss, G. M.; Freeman, H. C. *J. Mol. Biol.* **1983**, *169*, 521.

Contribution from the Institute of Chemistry, University of Wrocław, 50 383 Wrocław, Poland

Proton Nuclear Magnetic Resonance Characterization of Chloro(*N*-methyl-5,10,15,20-tetraphenylporphyrinato)nickel(II) and Chloro(*N*-methyloctaethylporphyrinato)nickel(II) Complexes

LECHOSŁAW LATOS-GRAŻYŃSKI

Received September 5, 1984

Proton NMR studies of chloro(*N*-methyl-5,10,15,20-tetraphenylporphyrinato)nickel(II) and chloro(*N*-methyloctaethylporphyrinato)nickel(II) complexes have been carried out. The full assignment of resonances has been achieved on the basis of selective deuteration and subsequent chloro and methyl substitution of phenyl rings. The characteristic pattern of pyrrole resonances (three downfield and one upfield) has been found. The temperature dependence of isotropic shifts demonstrated the essential deviation from the Curie law due to dipolar shift contribution arising from zfs. The isotropic shifts of pyrrole and *N*-methyl resonances are dominated by the σ-contact shift mechanism. The direct σ-π overlap mechanism has been proposed to account for the upfield shift of N-methylated pyrrole protons. The delocalization of spin density on phenyl rings via the π mechanism has been determined.

Introduction

N-Alkylporphyrins are formed as products of the interaction of cytochrome P-450 with a variety of substances.[1] This fact has drawn increasing interest toward the chemistry of N-substituted porphyrins and corresponding metal ion complexes. The nitrogen substitution influences to a large extent the structural and electronic properties of these complexes. Structural analysis undertaken via X-ray diffraction has clearly demonstrated a pronounced deviation from planarity, resulting from the nitrogen atom substitution.[2] The *N*-methylporphyrin may serve as a model for the distorted porphyrins that incorporate the metal ion.[3-6] A coordination of some metal ions to *N*-methylporphyrins promotes the demethylation of the pyrrolic nitrogen and transfer of the CH_3^+ fragment to another nitrogen base present in solution.[7-10] This process should be essential in understanding alkyl fragment transfer within the porphyrin molecule.[11] Some complexes are

(1) (a) DeMatteis, F.; Jackson, A. H.; Gibbs, A. H.; Rao, K. R. N.; Atten, A.; Weerasinghe, S.; Hollands, C. *FEBS Lett.* **1982**, *142*, 44. (b) DeMatteis, F.; Hollands, C.; Gibbs, A. H.; De Sa, N.; Rizzardini, M. *FEBS Lett.* **1982**, *145*, 87. (c) DeMatteis, F.; Gibbs, A. H.; Farmer, F. B.; Lamb, J. H. *FEBS Lett.* **1981**, *129*, 328. (d) DeMatteis, F.; Gibbs, A. H.; Jackson, A. H.; Weerasinghe, S. *FEBS Lett.* **1980**, *119*, 109. (e) Oritz de Montellano, P. R.; Beilan, H. S.; Kunze, K. L. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 1490. (f) Oritz de Montellano, P. R.; Kunze, K. L.; Mico, B. A. *Mol. Pharmacol.* **1980**, *18*, 602.
(2) (a) Anderson, O. P.; Lavallee, D. K. *J. Am. Chem. Soc.* **1976**, *98*, 4760. (b) Anderson, O. P.; Lavallee, D. K. *J. Am. Chem. Soc.* **1977**, *99*, 1404. (c) Anderson, O. P.; Lavallee, D. K. *Inorg. Chem.* **1977**, *16*, 1634. (d) Lavallee, D. K.; Kopelove, A. B.; Anderson, O. P. *J. Am. Chem. Soc.* **1978**, *100*, 3025.
(3) Lavallee, D. K. *Bioinorg. Chem.* **1976**, *6*, 219.
(4) Lavallee, D. K.; Bain-Ackerman, M. J. *Bioinorg. Chem.* **1978**, *9*, 311.
(5) Bain-Ackerman, M. J.; Lavallee, D. K. *Inorg. Chem.* **1979**, *18*, 3358.
(6) Lavallee, D. K.; Gebala, A. *Inorg. Chem.* **1974**, *13*, 2004.
(7) Shears, B.; Hambright, P. *Inorg. Nucl. Chem. Lett.* **1970**, *6*, 678.
(8) Lavallee, D. K. *Inorg. Chem.* **1976**, *15*, 691.
(9) Lavallee, D. K. *Inorg. Chem.* **1977**, *16*, 955.
(10) Kuila, D.; Lavallee, D. K. *Inorg. Chem.* **1983**, *22*, 1095.

0020-1669/85/1324-1681$01.50/0