Evidence for Reaction at Two Binding Sites in the Oxidation of Parsley Plastocyanin by Tris(1,10-phenanthroline)cobalt(III) †

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The effect of redox inactive complexes of charge 4+, 5+, 6+, and 7+ as competitive inhibitors for the $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline) oxidation of parsley plastocyanin PCu(I) has been studied. The maximum effectiveness of the inhibitors is 53% (at pH 5.8) increasing to 61% (at pH 7.5). This is believed to represent the extent of reaction at the east face binding site close to Tyr 83. Some or all of the remaining reaction is at the His 87 north site.

The single (type 1) blue copper protein plastocyanin (*M* 10 500) is involved in electron transport between cytochrome f and P700 in the chloroplast of higher plants.[1,2] It has an isoelectric point (pI) of 4.2,[3] and from the amino-acid composition[4] the charge at pH 7.5 for parsley plastocyanin is estimated to be −8 for the PCu(I) state and −7 for PCu(II). The study of 1:1 electron transfer reactions with inorganic complexes has provided much information on the behaviour of the protein, and in particular the influence of charge on reactivity.

Based on n.m.r.[5,6] and kinetic studies[7-11] the case has been made for the negatively charged oxidant $[Fe(CN)_6]^{3-}$ reacting at a site on the protein close to His 87 (sometimes described as the north site), which represents the closest possible approach (~6 Å) to the Cu active site.[12] The positively charged oxidant $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline) is believed to react at an alternative site on the east face of the molecule, which is believed to incorporate the negatively charged patch 42—45 (and possibly other negatively charged residues), and is close to Tyr 83, some 10—12 Å from the Cu.[12] No evidence was reported from the n.m.r. experiments for redox inactive $[Cr(phen)_3]^{3+}$ interacting at the His 87 site,[5,6] and interpretations to date have assumed that $[Co(phen)_3]^{3+}$ reacts exclusively at the Tyr 83 site (Figure 1). Results presented here suggest that this is not so, and that such a stance has now to be modified.

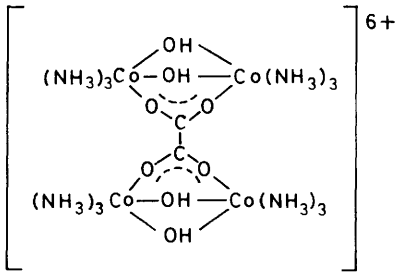
The approach has been to examine blocking effects of redox inactive 4+, 5+, 6+, and 7+ complexes (B) on the $[Co(phen)_3]^{3+}$ oxidation of parsley PCu(I). Experimental details are as already described.[7-11] The effects of 4+ and 5+ complexes, $[Pt(NH_3)_6]^{4+}$ (pH 5.8) and $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ (pH 7.5), have been studied previously.[13] Because protonation of parsley PCu(I) occurs at both the active site and the Tyr 83 binding site,[14] it is essential to study blocking effects at the same pH. A pH of 5.8 has been chosen so as to avoid acid dissociation of $[Pt(NH_3)_6]^{4+}$ (pK_a 7.1),[8] an effect which is not relevant in the case of the Co^{III} ammine complexes. The 6+ and 7+ ions used (see structures below) are the previously characterised complexes μ_4-oxalato-bis{di-μ-hydroxo-bis[triamminecobalt(III)]}, $[(NH_3)_6(OH)_2Co_2(C_2O_4)Co_2(OH)_2(NH_3)_6][ClO_4]_6 \cdot 4H_2O$ [$\lambda_{max.}$ at 525 nm, ε 227 M^{-1} cm^{-1}; 380 nm (sh), 400 M^{-1} cm^{-1}], and μ_4-oxalato-{μ-amido-μ-hydroxo-bis[triamminecobalt(III)]}{μ-amido-bis[tetra-amminecobalt(III)]}, $[(NH_3)_6(OH)(NH_2)Co_2(C_2O_4)Co_2(NH_2)(NH_3)_8][ClO_4]_7$ [$\lambda_{max.}$ at 513 nm, ε 505 M^{-1} cm^{-1}; 360 nm (sh), 1 060 M^{-1} cm^{-1}].[15] Rate constants indicating the effect of increasing amounts of redox inactive blocking complexes are listed in the Table.

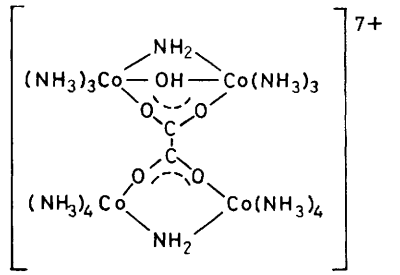
Earlier studies on the inhibition of the $[Co(phen)_3]^{3+}$ oxidation of PCu(I), which included the effect of redox inactive $[Co(NH_3)_6]^{3+}$,[8,13] suggested different degrees of *partial* blocking by the 3+ and 4+ (both extensive extrapolations) and 5+ complexes. Now with the extension of the experiments on the 4+ complexes to higher concentrations of blocker (Table), the study of the 5+ complex at pH 5.8, and inclusion of the 6+ and 7+ complexes, it is apparent, Figure 2, that all attain the same (53 ± 2%) effectiveness at high blocker concentration. Because of the mild effect produced by $[Co(NH_3)_6]^{3+}$ it is not

Table. The effect of redox inactive complexes (B) on second-order rate constants (25 °C) for the $[Co(phen)_3]^{3+}$ (~4 × 10^{-4} M) oxidation of parsley plastocyanin, PCu(I) (~1 × 10^{-5} M) at pH 5.8 (mes–NaOH),* *I* = 0.10 M (NaCl)

B =	$[Pt(NH_3)_6]^{4+}$
10^3[B]/M =	0, 0.30, 0.93, 1.86
$10^{-2}k/M^{-1}$ s^{-1} =	10.4, 7.5, 6.5, 5.7
B =	$[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$
10^3[B]/M =	0, 0.07, 0.16, 0.30, 0.44, 0.90, 1.80
$10^{-2}k/M^{-1}$ s^{-1} =	11.4, 10.2, 9.4, 8.3, 7.8, 6.9, 6.5
B =	$[(NH_3)_6(OH)_2Co_2(C_2O_4)Co_2(OH)_2(NH_3)_6]^{6+}$
10^3[B]/M =	0, 0.08, 0.20, 0.58, 0.76, 1.28
$10^{-2}k/M^{-1}$ s^{-1} =	10.8, 8.7, 7.2, 6.2, 5.8, 5.5
B =	$[(NH_3)_6(OH)(NH_2)Co_2(C_2O_4)Co_2(NH_2)(NH_3)_8]^{7+}$
10^3[B]/M =	0, 0.012, 0.025, 0.049, 0.096, 0.65
$10^{-2}k/M^{-1}$ s^{-1} =	11.0, 10.6, 9.6, 7.9, 5.7

* mes = 2-(*N*-morpholino)ethanesulphonic acid.





† *Non-S.I. unit employed*: M = mol dm^{-3}.

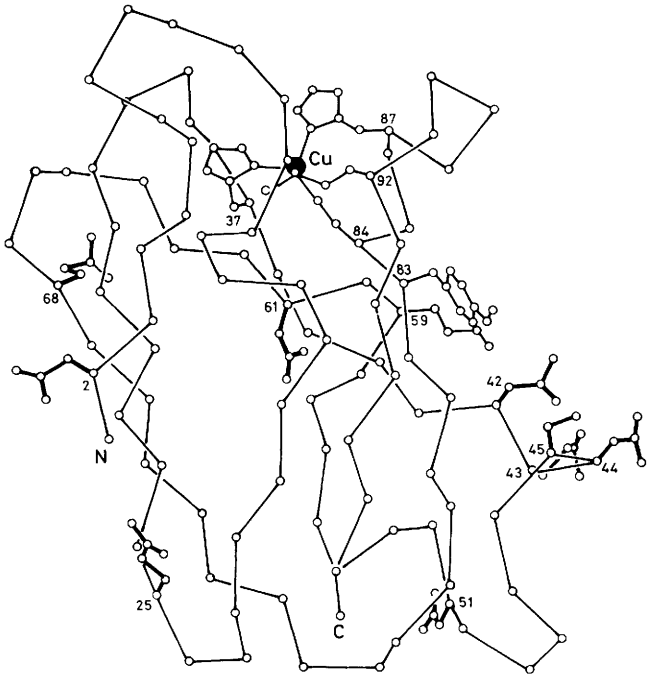


Figure 1. The structure of plastocyanin PCu(II) as reported by Guss and Freeman[12]

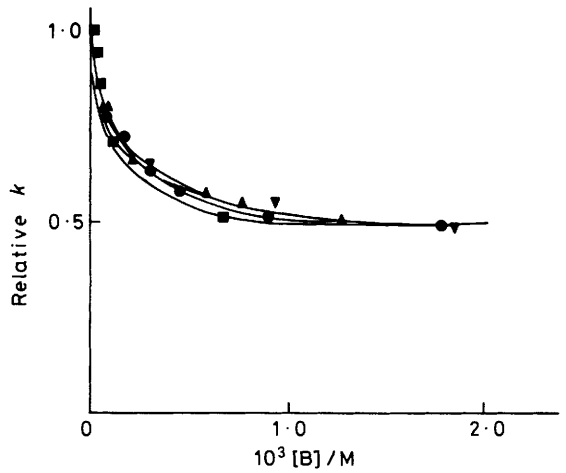


Figure 2. The blocking effect of redox inactive complexes on the $[Co(phen)_3]^{3+}$ oxidation of (parsley) plastocyanin PCu(I). Second-order rate constants (25 °C) shown as relative values were determined at pH 5.8 (mes), I = 0.10 M (NaCl), with $[Pt(NH_3)_6]^{4+}$ (▼), $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ (●), $[(NH_3)_6(OH)_2Co_2(C_2O_4)Co_2(OH)_2(NH_3)_6]^{6+}$ (▲), $[(NH_3)_6(OH)(NH_2)Co_2(C_2O_4)Co_2(NH_2)(NH_3)_8]^{7+}$ (■)

possible further to extend these studies, but we have no reason to believe that this complex behaves any differently to those here described. The variation in charge and size of the complexes (mononuclear to tetranuclear), rules out an explanation in terms of partial blocking of an extensive region centring around Tyr 83 and including the 42—45 and 59—61 negative patches, which could accommodate two complexes at any one time in a partial blocking scheme (see *e.g.* Figure 6 of ref. 8). Clearly for the latter to remain acceptable the larger and more highly charged tetranuclear complexes would have been expected to exhibit a more comprehensive 'blanketing' of the Tyr 83 site. Moreover the parsley plastocyanin sequence has recently been completed,[4] and while the 42—45 negative patch is retained, the 59—61 patch, located close to and on the other side of Tyr 83 is drastically modified and is only 1−. It becomes doubtful therefore whether the east face of parsley plastocyanin is capable of accommodating two positively charged complexes at a time.

An alternative explanation in which the 4+ to 7+ complexes are capable of blocking entirely the 53% reaction (at pH 5.8) occurring at the Tyr 83 site, and the remaining reaction is at some other binding site (or sites) is considered therefore. The reaction sequence proposed is as in equations (1)—(4), where

$$PCu(I) + [Co(phen)_3]^{3+} \overset{K}{\rightleftharpoons} PCu(I), [Co(phen)_3]^{3+} \quad (1)$$

$$PCu(I), [Co(phen)_3]^{3+} \xrightarrow{k_{et}} PCu(II) + [Co(phen)_3]^{2+} \quad (2)$$

$$PCu(I) + B \overset{K_B}{\rightleftharpoons} PCu(I), B \quad (3)$$

$$[PCu(I)]_T + [Co(phen)_3]^{3+} \xrightarrow{k_2} PCu(II) + [Co(phen)_3]^{2+} \quad (4)$$

k_2 is for reaction at a second site on the protein, and takes account of all the protein, $[PCu(I)]_T$, whether B or $[Co(phen)_3]^{3+}$ is associated or not. It is assumed here that association of B or $[Co(phen)_3]^{3+}$ does not effect the reactivity at the second site. The concentration of $[Co(phen)_3]^{3+}$ ($\sim 4 \times 10^{-4}$ M), the reactant present in large excess, was at a sufficiently low level such that its association with protein [equation (1)] was not extensive, and $K[Co(phen)_3{}^{3+}] \ll 1$. Under these conditions the above scheme gives the expression

$$k_{obs.} = \frac{Kk_{et}[Co(phen)_3{}^{3+}]}{1 + K_B[B]} + k_2[Co(phen)_3{}^{3+}] \quad (5)$$

(5). With no B present, the experimentally determined second-order rate constant k is equivalent to $k_{et}K + k_2$. From an unweighted non-linear least-squares fit, association constants K_B (M^{-1}) for the 4+ (16.0×10^3), 5+ (7.9×10^3), 6+ (7.9×10^3), and 7+ (10.1×10^3) complexes (B) at pH 5.8 were obtained, and k_2 values are in the range $(5.1 \pm 0.2) \times 10^2$ M^{-1} s^{-1}. As previously reported, values for PCu(I) are influenced by protonation at the binding site (pK_a') as well as the active site (pK_a).[14] Protonation at the active site gives redox inactive protein,[16] while single protonation at the Tyr 83 site 'switches off' (we assume completely) reaction at this site. No evidence has been obtained for an explanation in terms of the alternative 'dead-end' mechanism, and our stance on this question remains as previously indicated.

An analysis of data for experiments with $[Pt(NH_3)_6]^{4+}$ is consistent with these mechanistic details.[8] It is also apparent that with the 5+ complex there is 61% blocking at pH 7.5,[8,13] decreasing to 53% at pH 5.8. At pH 5.8 therefore both H^+ and the 5+ complex decrease reactivity at the Tyr 83 site, and increase the proportion of reaction taking place at the second site (k_2), which in turn is also affected by the active site protonation. As far as we can tell protonation at the Tyr 83 binding site in effect excludes reaction at this site. Thus it is noted that on decreasing the pH from 7.5 to 5.0 protonation at the binding site gives a net 58% decrease in rate constants for the $[Co(phen)_3]^{3+}$ oxidation of PCu(I),[14] which is very similar to the maximum effectiveness of the 5+ complex at pH 7.5 (61%). After subtraction of the contribution to reaction (39%) at the second site a revised value of K = 340 M^{-1} is obtained for $[Co(phen)_3]^{3+}$ association with PCu(I) at the Tyr 83 site at pH

7.5,* which replaces the value 167 M^{-1} previously obtained.[16] As previously indicated[17] K decreases as the pH decreases due to the process pK_a'.

From studies with negatively charged reactants, *e.g.* the $[Fe(CN)_6]^{4-}$ reduction of PCu(II), it has been concluded that there is no significant protonation at the His 87 binding site,[13,16] consistent with there being no acidic residues in this locality.[12] Since no binding site protonation is observed for k_2, and there are obvious advantages of reaction occurring at the His 87 site (close proximity to the Cu), it would appear that this is the most likely second site for reaction with $[Co(phen)_3]^{3+}$. Indeed it would be surprising under the circumstances if it did not contribute to the reaction (see also ref. 18).

It has previously been shown that binding site protonation is 100% effective in the case of cytochrome c(II) (8+) reduction of PCu(II), and blocking by $[Pt(NH_3)_6]^{4+}$ at pH 5.8 is extensive (extrapolation of data suggests 75%).[11] Similarly with the natural protein partner cytochrome f(II) protonation is 90% effective, and $[Pt(NH_3)_6]^{4+}$ appears to induce complete blocking.[19] These effects clearly suggest that both reactions are predominantly at the Tyr 83 site. Favourable charge distributions at the cytochrome f and plastocyanin binding sites[19] are reflected in the high reactivity, with rate constants at the limit of the stopped-flow range even though the driving force is small. With cytochrome c the positively charged lysine residues around the exposed heme edge are no doubt influential.

In conclusion, while charge is established as a controlling factor in both protein–complex and protein–protein reactions, some differences are to be noted. Protein–protein reactions are capable of much greater specificity because of the distribution of charge and the relatively large potential contact areas involved. Using the n.m.r. proton resonance method Williams and co-workers[20] have demonstrated that complexes such as $[Fe(CN)_6]^{3-}$ associate with cytochrome c at three different binding sites on the protein surface. The contribution of each site to an actual electron-transfer process will be determined by the distance from the Fe active site, the intervening polypeptide, and the strength of binding between the protein and the complex. It is possible that one or two of these sites only contributes to the electron-transfer process. In the present instance with $[Co(phen)_3]^{3+}$ as oxidant for parsley PCu(I) it would appear that at pH 7.5 and in the absence of blocker the Tyr 83 site, at twice the distance from the Cu active site, is about twice as effective as the His 87 site. This apparent contradiction is explained by the more favourable electrostatics at the Tyr 83 site, making association more extensive and sufficient to outweigh the distance factor. Association is not detectable at the His 87 site by kinetic or n.m.r. experiments.

* We have used a set of rate constants $k_{obs.}$ at different $[Co(phen)_3^{3+}]$ (pH 7.5) generated by $K = 167\ M^{-1}$ and $k_{et} = 17.9\ s^{-1}$ (ref. 16), subtracted $k_2[Co(phen)_3^{3+}]$, and refitted to the equation $k_{obs.} = Kk_{et}[Co(phen)_3^{3+}]/\{1 + K[Co(phen)_3^{3+}]\}$ in order to obtain a new value of K. The rate constant k_{et} for reaction at the Tyr 83 site is also modified to $5.7\ s^{-1}$ by this treatement.

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