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NMR STUDY OF THE INTERACTION OF PLASTOCYANIN WITH CHROMIUM(III) ANALOGUES OF INORGANIC ELECTRON TRANSFER REAGENTS

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Summary

High resolution nuclear magnetic resonance spectroscopy has been used to examine the interaction of plastocyanins from French bean (*Phaseolus vulgaris*) and cucumber (*Cucumis sativus*) with three complexes—potassium hexacyanochromate(III), hexamminechromium(III) nitrate and tris(1,10-phenanthroline)-chromium(III) perchlorate—which are analogues of inorganic electron transfer reagents. The results indicate a high degree of specificity in the binding of these complexes and two binding sites on the protein are identified. One binding site is situated close to the copper atom and is clearly suited to outer sphere electron transfer through one of the histidine ligands. The other binding site is more distant from the copper atom and this mechanism cannot be operative. Electron transfer via hydrophobic channels or electron tunneling are possible mechanisms of electron transfer.

Introduction

Copper-containing proteins are found in a variety of biological electron transfer systems. Plastocyanin is a 'type 1' or blue copper protein found in plant and algal chloroplasts and is an essential component of the photosynthetic electron transport chain [1,2]. The protein contains one copper atom [2] which is bound in a distorted tetrahedral environment. Spectroscopic studies [3—5] and the X-ray crystal structure [6] show that it is co-ordinated by the thiol group of Cys 84, the thioether group of Met 92 and the δ-nitrogen atoms

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Abbreviations: TSS, trimethylsilylpropane sulphonic acid; 1,10-phen, 1,10-phenanthroline; EDTA, ethylenediaminetetraacetic acid.

of the imidazole groups of His 37 and His 87. The copper atom is inaccessible to the solvent water [5,6].

The low molecular weight (10 500) of plastocyanin makes it ideally suited to study by high resolution NMR spectroscopy. Furthermore, the copper(II) atom may be used as an intrinsic NMR relaxation probe [7]. Particular attention has been paid to identification of the resonances of the amino acids which bind the copper atom [8—12]. In a study of plastocyanins from spinach (*Spinacia oleracea*) and a blue-green alga (*Anabaena variabilis*), Markley et al. [8] showed that oxidation of the protein brought about marked changes in the NMR spectrum. Several peaks were broadened including those assigned to the two histidine side chains. Data obtained from pH titrations of the reduced and apo forms of spinach plastocyanin supported the conclusion that the two histidine residues are directly ligated to the copper [8]. Other NMR studies on plastocyanin from French bean (*Phaseolus vulgaris*) [9—11] have led to the assignment of many resonances due to amino acids near the copper atom. These resonances, which are readily detected in the spectrum of the copper(I) protein, are broadened beyond detection when the protein is in the copper(II) state. This effect diminishes rapidly with increasing distance from the copper site. In addition, assignments of the resonances due to many of the phenylalanine and tyrosine residues have been made using spin decoupling methods [11].

Electron transfer is an essential process in all biological systems. However, the mechanisms by which electrons are transferred between biomolecules are as yet only poorly understood. In one approach to this problem, the kinetics of electron transfer reactions between inorganic complexes and a variety of metalloproteins have been extensively studied [13—18]. The interaction of plastocyanin with both redox active and inactive inorganic complexes has been the subject of several investigations [19—25]. Considerable interest has centred on the mechanism, pathway and specificity of electron transfer [4,26]. It has been suggested that two binding sites on the protein exist [6] and kinetic evidence for the formation of discrete complexes prior to the electron transfer step has been presented [22—25].

In the present paper we report NMR studies of the interactions of plastocyanins from French bean and cucumber (*Cucumis sativus*) with chromium(III) analogues of inorganic electron transfer reagents, viz., potassium hexacyanochromate(III) ($K_3Cr(CN)_6$), hexamminechromium(III) nitrate ($Cr(NH_3)_6(NO_3)_3$) and tris(1,10-phenanthroline)chromium(III) perchlorate ($Cr(1,10\text{-phen})_3(ClO_4)_3$). We are able to identify unequivocally the sites of binding of these complexes to the protein and we propose pathways and mechanisms for electron transfer. The protein structure, particularly in the vicinity of the copper site, is expected to be conserved in French bean and cucumber plastocyanins and those from other higher plant species [6,10,11]. It is thus reasonable to assume that the results of our experiments have rather general validity and can be extrapolated to the plastocyanins from other higher plant species.

Methods

Proteins

Plastocyanin was isolated from French bean and cucumber leaves by a

modification of the method of Ramshaw et al. [27]. Full details will be published elsewhere. Protein with A_{278}/A_{597} ratio of 1.1—1.2 was used throughout. Solutions for NMR measurements were prepared by dissolving the lyophilized protein in ^2H_2O or in deuterated phosphate buffer (50 mM, pH 7.0). The protein was reduced by addition of a dilute solution of sodium dithionite in buffer. The excess sodium dithionite was subsequently removed by dialysis. All operations were carried out under argon. The pH of all unbuffered solutions was adjusted as required by careful addition of NaO^2H or ^2HCl to the protein solution. All pH values quoted are uncorrected meter readings.

Inorganic complexes

$K_3Cr(CN)_6$ [28], $Cr(NH_3)_6(NO_3)_3$ [29] and $Cr(1,10\text{-phen})_3(ClO_4)_3$ [25] were prepared by literature methods. Each sample was dissolved in degassed ^2H_2O and the pH adjusted as required by addition of ^2HCl or NaO^2H.

NMR spectra

NMR spectra were recorded at 40°C using a Bruker HX-270 spectrometer equipped with a Nicolet 1180 computer. The resolution was enhanced by means of the convolution difference technique [30]. Dioxane was used as an internal standard but all peaks are referred to trimethylsilylpropane sulphonic acid (TSS).

Samples were sealed under argon by means of a septum cap. Before spectra were recorded, a trace of ascorbic acid was added to the protein solutions to ensure complete reduction. To prevent air oxidation of the plastocyanin during the experiments, aliquots of the inorganic reagents were injected into the protein solution directly through the septum cap.

Results

(1) NMR spectrum of reduced plastocyanin

Fig. 1 shows the resolution enhanced spectra of fully reduced French bean and cucumber plastocyanins. These spectra have many features in common, consistent with close conservation of tertiary structure. The resonances in the spectrum of French bean plastocyanin which have been assigned previously [11] are shown in Fig. 1a. In the light of the X-ray crystal structure [6], specific assignments can now be made for the two tyrosine residues conserved in all higher plant plastocyanins [2], namely Tyr 80 and Tyr 83. The coupled two-proton doublets at 6.51 and 6.64 ppm are broadened upon oxidation of the protein [11] and are assigned to the ortho and meta protons respectively of Tyr 83 which, from the X-ray structure [6], lies about 10 Å from the copper atom. On the other hand, Tyr 80 lies about 20 Å from the copper atom [6]. The coupled doublets at 6.55 and 7.11 ppm, which are unperturbed by oxidation, are thus assigned to this residue. In cucumber plastocyanin, the corresponding two-proton doublets are at 6.50 and 6.68 ppm (Tyr 83) and 6.55 and 7.08 ppm (Tyr 80). The assignment of the resonances of Tyr 83 by spin decoupling is illustrated in Fig. 2.

The two histidine C-2 proton resonances titrate with pK_a values of 4.9 and <4.5 [8]. The lower field resonance (pK_a 4.9) is more sensitive to changes in

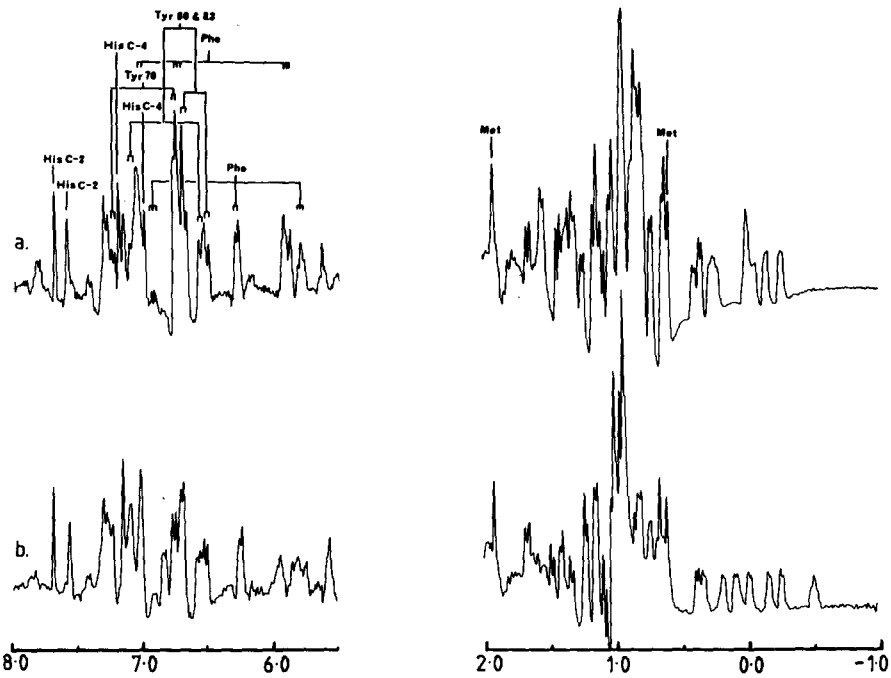

Fig. 1. Aromatic and aliphatic regions of the convolution difference NMR spectra of (a) reduced French bean plastocyanin (pH 7.0, 50 mM phosphate), showing previously assigned resonances [11], (b) reduced cucumber plastocyanin (pH 7.0, 50 mM phosphate). The horizontal scale is measured in parts per million (ppm) from TSS. The vertical scale in the aromatic region (8.0—5.5 ppm) is half that in the aliphatic region (2.0 to —1.0 ppm).

pH and has been assigned to His 87 [5]. This residue lies on the surface of the protein and is exposed to solvent H_2O [6]. His 37, on the other hand, is more buried and protected from the solvent and its resonances should be less dependent upon pH.

In the aliphatic region of the spectrum of French bean plastocyanin, singlet resonances at 1.88 and 0.57 ppm have been assigned to the methyl groups of the two methionine residues [11]. That giving rise to the resonance at 0.57 ppm is close to the copper atom and is conserved in all plastocyanins studied [11], and we thus assign it to the copper ligand, Met 92. The resonance at 1.88 ppm, which is not always conserved [10,11] must therefore arise from Met 57. These assignments are confirmed by measurements on Hg(II) plastocyanin (Cookson, D.J., Hayes, M.T. and Wright, P.E., unpublished). Whereas the resonance at 1.88 ppm (assigned to Met 57) is essentially unperturbed by the replacement of Cu(I) with Hg(II), the resonance of Met 92 is shifted downfield to 0.76 ppm. This effect is consistent with that observed for the methionine complex of mercury(II) [31,32].

(2) Titration of reduced plastocyanin with chromium(III) complexes

$Cr(CN)_6^{3-}$. Small aliquots of a $Cr(CN)_6^{3-}$ solution were added to a solution of reduced plastocyanin at pH 6.3 (50 mM phosphate buffer) and NMR spectra were recorded after each addition. Clear evidence for significant changes was

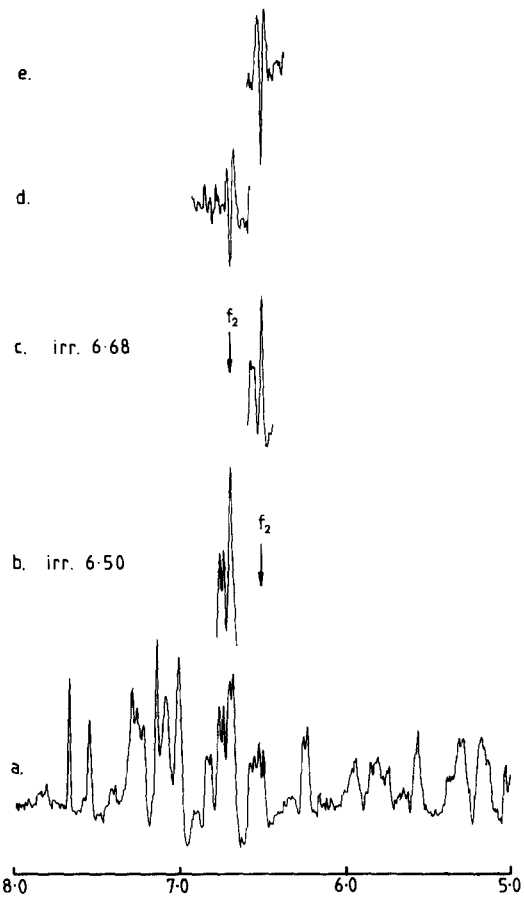

Fig. 2. Decoupling of tyrosine 83 resonances. (a) Aromatic region of the convolution difference NMR spectrum of reduced cucumber plastocyanin (pH 7.0, 50 mM phosphate). (b) Irradiation at 6.50 ppm causes decoupling at 6.68 ppm. (c) Irradiation at 6.68 ppm causes decoupling at 6.50 ppm. (d) Difference (a) — (b). (e) Difference (a) — (c).

obtained by difference spectroscopy (Fig. 3). At low concentrations of $Cr(CN)_6^{3-}$ the resonance at 7.66 ppm, assigned to the C-2 proton of His 87 on the basis of its pH dependence, is observed to broaden. The His C-4 proton resonance [11] at 6.97 ppm is also broadened and is thus assigned to His 87 (Fig. 3d). As the concentration of $Cr(CN)_6^{3-}$ is increased, the resonances of His 37 at 7.57 ppm (C-2) and 7.16 ppm (C-4) begin to broaden. This effect is most readily seen in Fig. 3e.

In addition to the resonances of His 87 and His 37, two resonances at 5.87 and 6.68 ppm are clearly observed in the difference spectrum. They have been assigned to the *para* proton and *meta* protons respectively of a phenylalanine residue [11] (Fig. 1a). The *ortho* proton resonance at 6.99 ppm [11] cannot be detected as it is masked by the C-4 proton resonance of His 87. We can now assign these resonances to Phe 35 which, from the X-ray structure [6], lies near the surface of the protein and very close to both histidine residues. Only minor changes are observed in the aliphatic region of the spectrum upon addi-

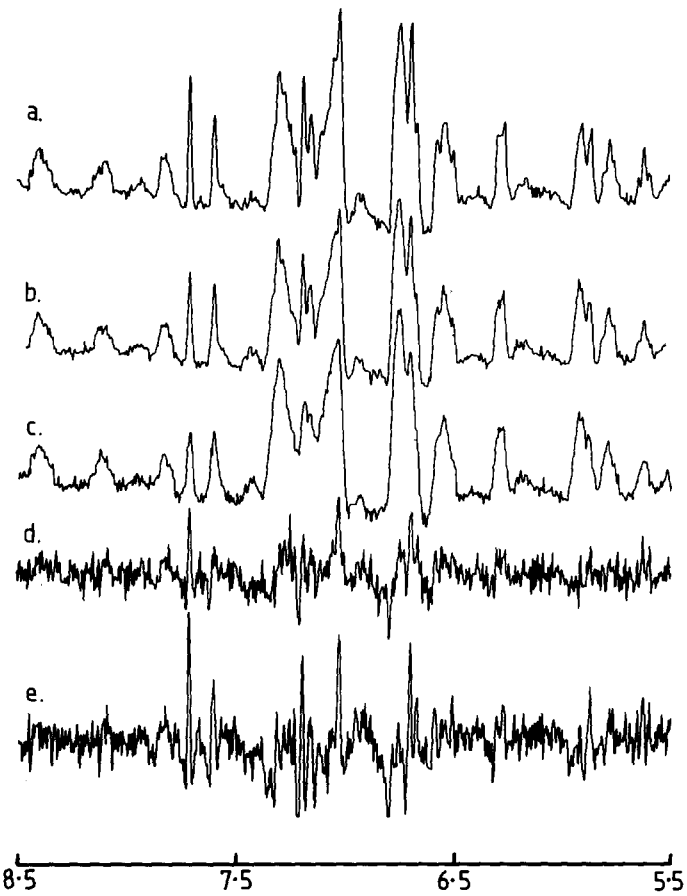

Fig. 3. Titration of reduced plastocyanin with $Cr(CN)_6^{3-}$. Aromatic region of the convolution difference NMR spectrum of reduced French bean plastocyanin (3.5 mM, pH 6.3, 50 mM phosphate). (a) No $Cr(CN)_6^{3-}$ present. (b) $[Cr(CN)_6^{3-}] = 0.2$ mM. (c) $[Cr(CN)_6^{3-}] = 0.3$ mM. (d) Difference spectrum (a) — (b). (e) Difference spectrum (a) — (c). The vertical scale in the difference spectra has been expanded 2-fold.

tion of $Cr(CN)_6^{3-}$. Most notable is a slight broadening of the resonance of the methionine ligand, Met 92, at 0.57 ppm.

It is clear from the specificity of the NMR perturbations induced by $Cr(CN)_6^{3-}$ that this reagent binds to plastocyanin. The broadening of protein proton resonances results from an enhancement in nuclear relaxation brought about by efficient dipolar coupling with the relatively slowly relaxing electron spin of the Cr(III) ion. The broadening effect is dependent entirely upon the inverse sixth power of the distance of the amino acid residue(s) from the paramagnetic centre. Consequently, any resonances broadened by addition of the Cr(III) complex must arise from amino acid residues close to the site of binding to the protein. The perturbations caused by $Cr(CN)_6^{3-}$ cannot arise from partial oxidation of the protein since most of the resonances broadened upon oxidation [11] are unaffected by addition of $Cr(CN)_6^{3-}$.

At pH 7.0 (50 mM phosphate) no changes were observed in the NMR spectrum of reduced plastocyanin when $Cr(CN)_6^{3-}$ was added. We conclude that under these conditions the binding is very weak.

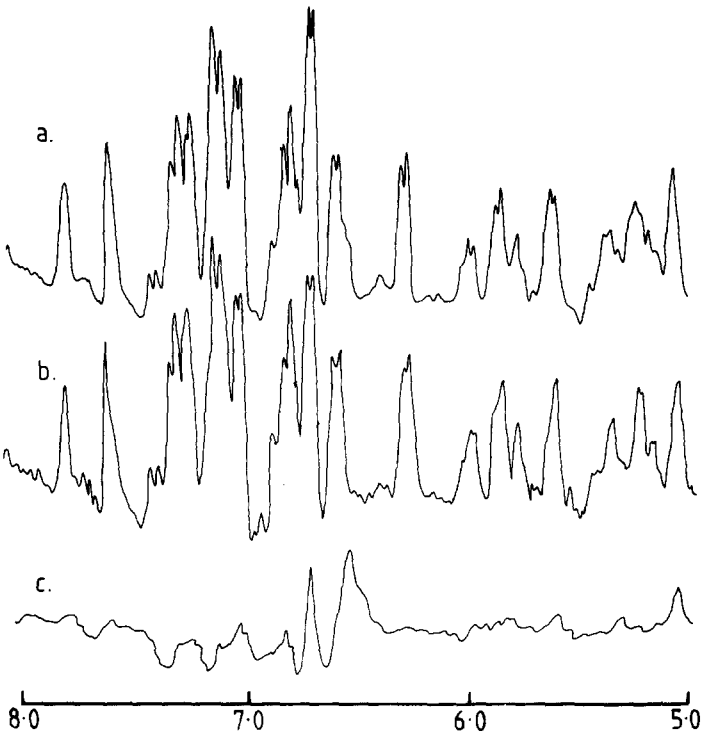

Fig. 4. Titration of reduced plastocyanin with $Cr(NH_3)_6^{3+}$. Aromatic region of the convolution difference NMR spectrum of reduced cucumber plastocyanin (3.5 mM, pH 6.0, no buffer). (a) No $Cr(NH_3)_6^{3+}$ present. (b) $[Cr(NH_3)_6^{3+}] = 1.2$ mM. (c) Difference of direct spectra.

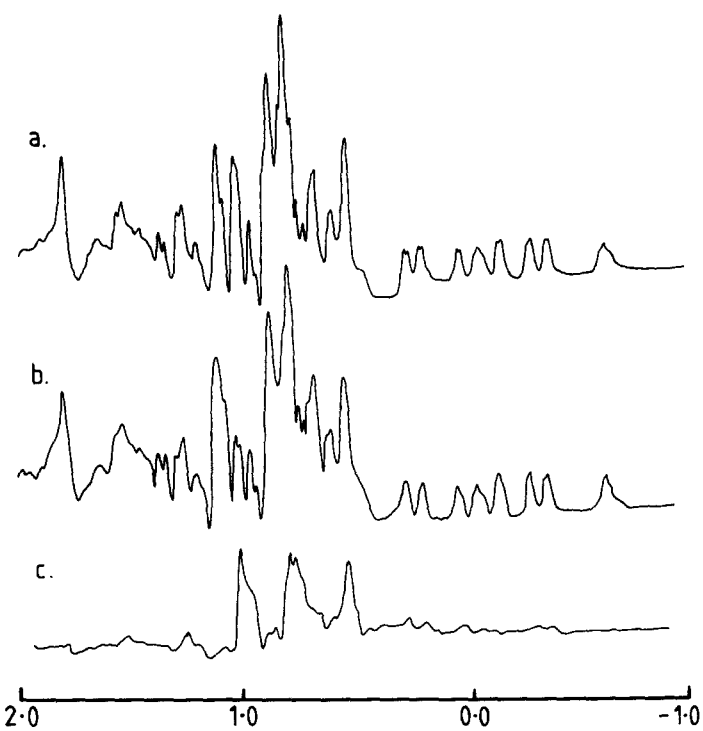

Fig. 5. Titration of reduced plastocyanin with $Cr(NH_3)_6^{3+}$. Aliphatic region of the convolution difference NMR spectrum of reduced cucumber plastocyanin (3.5 mM, pH 6.0, no buffer). Conditions as in Fig. 4.

$Cr(NH_3)_6^{3+}$

Small aliquots of a solution of $Cr(NH_3)_6^{3+}$ were added to an unbuffered solution of reduced plastocyanin at pH 6.0 and NMR spectra recorded. Perturbations are again observed in the spectrum of the protein (Figures 4 and 5), but they are different from those observed with $Cr(CN)_6^{3-}$. The resonances of Tyr 83 are broadened dramatically (Fig. 4), the effect being most noticeable at a $Cr(NH_3)_6^{3+}$ concentration of 1.25 mM (plastocyanin, 3.5 mM). Similar effects are observed in buffered solutions (50 mM phosphate) at pH 6.0. At pH 7.0 comparable broadening of the Tyr 83 resonances is observed at a concentration of $Cr(NH_3)_6^{3+}$ as low as 0.8 mM (plastocyanin, 3.5 mM).

In the aliphatic region, difference spectra show clearly that resonances at 1.08, 0.80 and 0.60 ppm are broadened (Fig. 5). While changes in intensity due to broadening of these resonances are evident in direct and convolution difference spectra, many overlapping peaks are found in this region and it is not yet possible to establish with any certainty to which amino acids they belong.

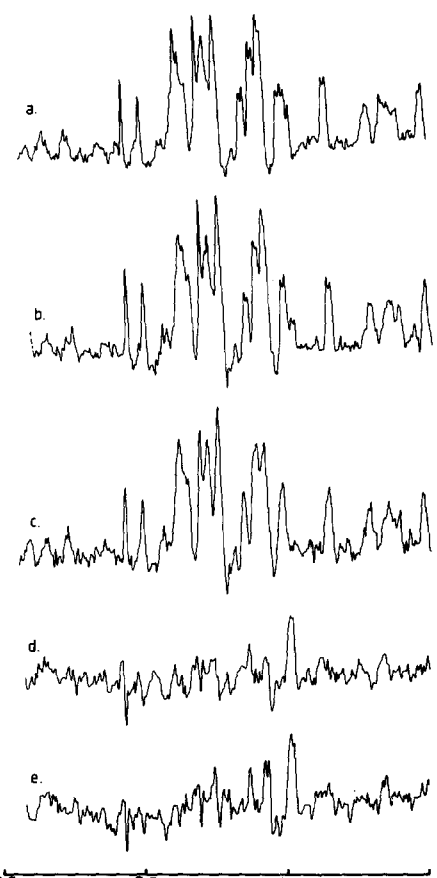

Fig. 6. Titration of reduced plastocyanin with $Cr(1,10\text{-phen})_3^{3+}$. Aromatic region of the convolution difference NMR spectrum of reduced cucumber plastocyanin (3.5 mM, pH 7.0, 50 mM phosphate). (a) No $Cr(1,10\text{-phen})_3^{3+}$ present. (b) $[Cr(1,10\text{-phen})_3^{3+}] = 0.2$ mM. (c) $[Cr(1,10\text{-phen})_3^{3+}] = 0.4$ mM. (d) Difference of direct spectra of (a) and (b). (e) Difference of direct spectra of (a) and (c).

However, from their resonance positions and apparent intensities it is probable that they arise from the methyl groups of two or more aliphatic side chains.

Cr(1,10-phen)$_3^{3+}$

When a buffered solution of reduced plastocyanin is titrated with Cr(1,10-phen)$_3^{3+}$, similar perturbations to those observed with $Cr(NH_3)_6^{3+}$ are evident (Figs. 6 and 7). A strong interaction at pH 7.0 is indicated since even quite low concentrations of Cr(1,10-phen)$_3^{3+}$ (0.2 mM) perturb the NMR spectrum of the reduced plastocyanin. At this concentration, the resonance of the *ortho* (3,5) protons of Tyr 83 at 6.50 ppm is broadened. As the concentration of Cr(1,10-phen)$_3^{3+}$ is increased, the corresponding resonance of the *meta* (2,6) protons at 6.68 ppm begins to broaden. This effect, which can be seen clearly in the difference spectra (Figs. 6d and 6e), gives an indication of the orientation of the side chain of Tyr 83 with respect to the binding site of Cr(1,10-phen)$_3^{3+}$.

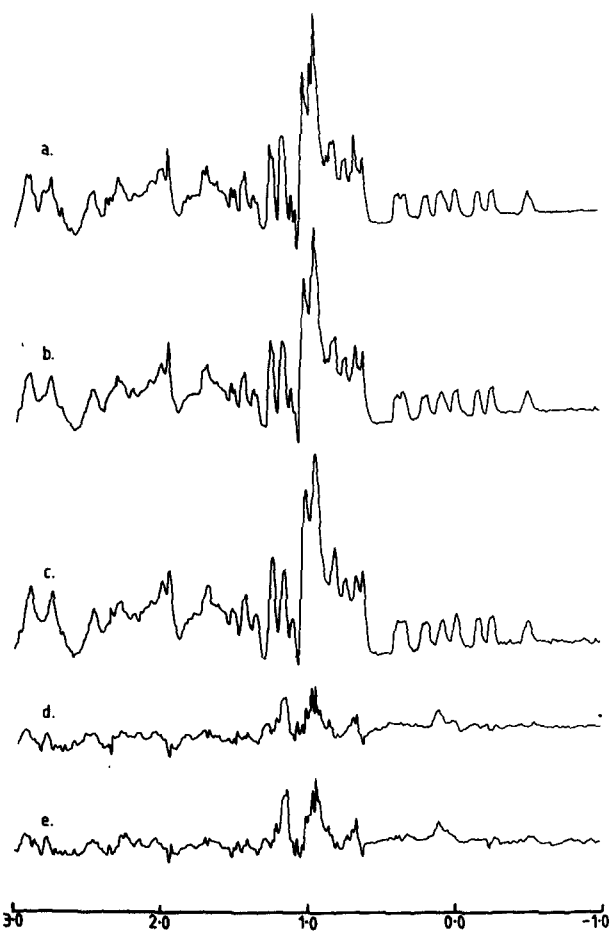

Fig. 7. Titration of reduced plastocyanin with Cr(1,10-phen)$_3^{3+}$. Aliphatic region of the convolution difference NMR spectrum of reduced cucumber plastocyanin (3.5 mM, pH 7.0, 50 mM phosphate). Conditions as in Fig. 6.

In the aliphatic region, the same methyl group resonances that were broadened by $Cr(NH_3)_6^{3+}$ (1.08, 0.80 and 0.60 ppm) are broadened by Cr(1,10-phen)$_3^{3+}$. A methyl group resonance at 0.10 ppm is also broadened. Accordingly these two chromium(III) complexes must bind to the same site on plastocyanin or at least in the same region on the protein surface.

Discussion

The kinetic studies of Sykes and co-workers [22—25] on the oxidation of plastocyanin with $Fe(CN)_6^{3-}$ and Co(1,10-phen)$_3^{3+}$ are consistent with the formation of a productive complex prior to the electron transfer step:

$$\text{PcCu(I)} + \text{M(III) L} \overset{K}{\rightleftharpoons} \text{PcCu(I)} \cdot \text{M(III) L} \quad (1)$$

$$\text{PcCu(I)} \cdot \text{M(III) L} \overset{k_{et}}{\rightarrow} \text{PcCu(II)} + \text{M(II) L} \quad (2)$$

where PcCu(I) and PcCu(II) represent the reduced and oxidized forms of plastocyanin respectively, and M(III)L and M(II)L, the oxidized and reduced metal complexes. The rate constants for the oxidation of parsley plastocyanin with $Fe(CN)_6^{3-}$ and Co(1,10-phen)$_3^{3+}$ are both pH dependent but have different pK_a values (5.7 and 6.1, respectively) [23]. These and other observations have led Sykes and co-workers [24] to the conclusion that at least two binding sites are used in the reactions of plastocyanin with $Fe(CN)_6^{3-}$ and Co(1,10-phen)$_3^{3+}$, although these may be in the same region of the protein [25].

To determine directly the sites of binding of $Fe(CN)_6^{3-}$ and Co(1,10-phen)$_3^{3+}$ to plastocyanin we have used the redox inactive analogues $Cr(CN)_6^{3-}$ and Cr(1,10-phen)$_3^{3+}$ as NMR relaxation probes. Oxidation reactions involving Co(1,10-phen)$_3^{3+}$ are blocked by Cr(1,10-phen)$_3^{3+}$ [25] showing conclusively that these reagents compete for a single binding site on the protein. In view of its similar size and identical charge and ligands, $Cr(CN)_6^{3-}$ is expected to bind to the same site as $Fe(CN)_6^{3-}$. In addition, we have studied the interaction of $Cr(NH_3)_6^{3+}$ with plastocyanin to probe the binding site of the reductant $Ru(NH_3)_6^{2+}$. Reduction by this reagent is too rapid ($k = 2.4 \cdot 10^6\ M^{-1} \cdot s^{-1}$ at 5°C) to allow kinetic detection of complex formation [23].

Our NMR studies clearly indicate specific interactions between reduced plastocyanins from both French bean and cucumber and the chromium(III) complexes $Cr(CN)_6^{3-}$, $Cr(NH_3)_6^{3+}$ and Cr(1,10-phen)$_3^{3+}$. In agreement with Sykes and co-workers [24], we find two binding sites on the protein. One site must lie close to the copper atom itself since the resonances of three of the copper ligands, namely His 87, His 37 and, to a lesser extent, Met 92 are perturbed by $Cr(CN)_6^{3-}$. The copper atom lies at one end of the protein molecule about 6 Å from the surface and is protected from solvent by the imidazole group of His 87 [6]. The resonances at 7.66 and 6.97 ppm, assigned to the C-2 and C-4 protons respectively of His 87, are the first to be broadened by $Cr(CN)_6^{3-}$. Thus both the pH dependence of the chemical shifts and $Cr(CN)_6^{3-}$ broadening are in accord with our assignment.

The second binding site on plastocyanin must lie in the region of Tyr 83, some 10 Å from the copper atom, since the resonances of this residue (6.51

and 6.64 ppm) are broadened by the binding of both $Cr(NH_3)_6^{3+}$ and Cr(1,10-phen)$_3^{3+}$. The two binding sites are shown in Fig. 8.

The existence of two distinct binding sites on plastocyanin, one for the anionic complex, $Cr(CN)_6^{3-}$, and another for the cationic complexes, $Cr(NH_3)_6^{3+}$ and Cr(1,10-phen)$_3^{3+}$, provides some insight into the dominant protein-complex interactions. Although the overall charge on plastocyanin is negative (pI = 4.2) [2], it is conceivable that positive charge from the copper atom is delocalized over the imidazole ring of His 87. This may then be sufficient to bring about an electrostatic interaction with $Cr(CN)_6^{3-}$. Also it is noted that in other proteins, histidine residues are favoured binding sites for $Cr(CN)_6^{3-}$ [30,33].

Sykes et al. [23] have found a pronounced pH dependence for the rate of oxidation of plastocyanin by $Fe(CN)_6^{3-}$. On the basis of the crystal structure of Cu(II) plastocyanin, Colman et al. [6] have suggested that this pH dependence may result from the protonation of His 87 and its dissociation from the metal atom. Markley and co-workers [5,8] have shown that the C-2 proton resonance of His 87 is particularly sensitive to pH. The resonances of His 37 [8] and the

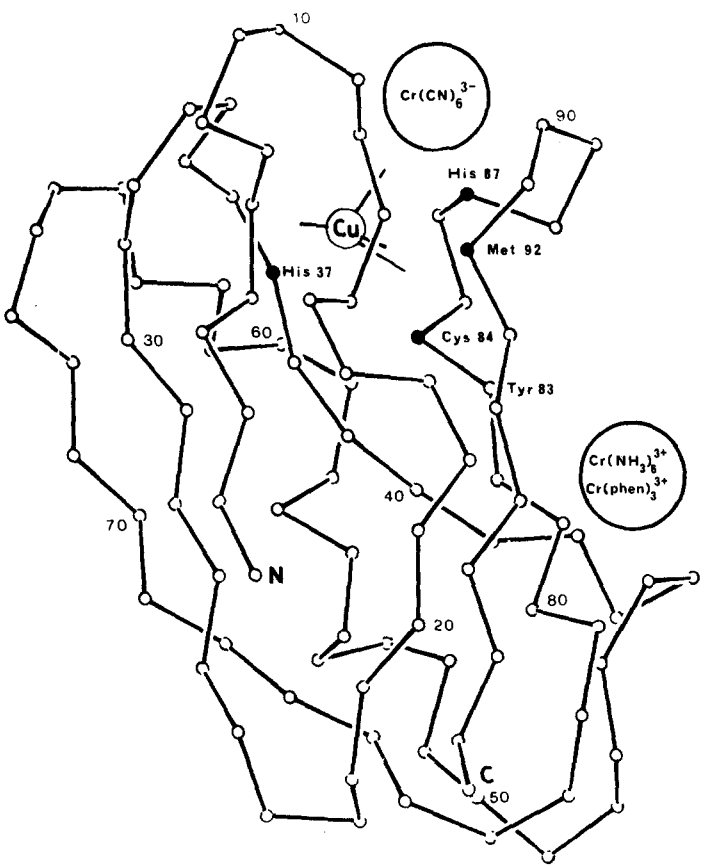

Fig. 8. Outline structure of plastocyanin (from Ref. 6) showing the approximate sites on the molecule where $Cr(CN)_6^{3-}$, $Cr(NH_3)_6^{3+}$ and Cr(1,10-phen)$_3^{3+}$ are expected to bind. The circles represent α-carbon positions.

Met 92 ligand are subject to small shifts at low pH (Cookson, D.J., Hayes, M.T. and Wright, P.E., unpublished data) and clearly reflect a rearrangement of the coordination geometry. The NMR results are thus consistent with protonation of His 87 at low pH (p*K* approx. 4.9 [8]) followed by dissociation from the copper atom to give a stable, 3-coordinate Cu(I) species [6,34]. Oxidation of this 3-coordinate Cu(I) atom would be highly unfavourable, as is indicated by the increase in the reduction potential of plastocyanin as the pH decreases below approx. 6 [35]. Although protonation of His 87 would favour the binding of $Fe(CN)_6^{3-}$, electron transfer to the Cu(I) plastocyanin would be inhibited at low pH [23,25] due to dissociation of this ligand.

The principle of microscopic reversibility indicates that $Fe(CN)_6^{4-}$ should also reduce Cu(II) plastocyanin from the site near His 87. The rate of this reduction is, however, almost independent of pH [23]. The 'hard-soft-acid-base' theory [36,37] predicts that imidazole ligands are favoured by Cu(II) more than by Cu(I) [6]. Thus displacement by a proton of a histidine (imidazole) ligand will be more difficult in Cu(II) plastocyanin. In accord with this prediction, resonance Raman spectroscopy shows no change in intensity or frequency of the metal-ligand vibrations in Cu(II) plastocyanin between pH 7 and pH 5 (Armstrong, R.S. and Cookson, D.J., unpublished data). X-ray diffraction studies also reveal no change in Cu(II) coordination geometry at low pH (Freeman, H.C. and Guss, J.M., personal communication). We thus conclude that in Cu(II) plastocyanin His 87 remains coordinated to the metal, at least above pH 5, and provides a pathway for electron transfer from bound $Fe(CN)_6^{4-}$.

We now consider the interaction of plastocyanin with the positively charged complexes $Cr(NH_3)_6^{3+}$ and $Cr(1,10\text{-phen})_3^{3+}$. The side chain of Tyr 83 lies close to the surface of the protein about 10 Å from the copper atom (Fig. 8) and is surrounded by a cluster of carboxylate groups from Asp 42, Glu 43, Asp 44, Asp 51 and Glu 59 [6]. These residues are conserved in all higher plant plastocyanins [2]. They form a pocket of negative charge which is unique in plastocyanin and clearly provides a favourable binding site for positively charged reagents. The difference in broadening by $Cr(1,10\text{-phen})_3^{3+}$ of the *ortho* (3,5) and *meta* (2,6) proton resonances of Tyr 83 shows that the side chain is aligned in such a way that the *ortho* protons are closer to the binding site.

Oxidation of Cu(I) plastocyanin by $Co(1,10\text{-phen})_3^{3+}$ is also strongly pH-dependent and is 'switched off' at low pH [23]. Sykes and co-workers [23] have shown that there is decreased binding of the complex to the protein at low pH and the present NMR studies support this conclusion. This decreased affinity presumably results from protonation of one or more of the carboxylate groups forming the binding site since, apart from the rearrangement in the copper coordination geometry mentioned above, no pH-induced conformational changes have been detected in the NMR spectrum of Cu(I) plastocyanin (Cookson, D.J., Hayes, M.T. and Wright, P.E., unpublished data).

Our present observation that electrostatic factors dominate the interactions between inorganic electron transfer reagents and the protein plastocyanin is not without precedent. The rates of self-exchange reactions of cytochrome *c* are strongly dependent on pH and ionic strength and clearly demonstrate the importance of electrostatic interactions [38]. Furthermore, the binding of

ferredoxin to cytochrome c_3 is affected by the presence of phosphate ions which presumably bind to the protein, thereby inhibiting the interaction [39]. Similar considerations apply to reactions of proteins with small inorganic complexes. Negatively charged oxidants such as $Co(EDTA)^-$ have been shown to bind to parsley ferredoxin at a different site from that used by positively charged oxidants [40].

It appears then that the overall charge of the protein may be less important in the formation of a complex than is the charge in specific localized regions of the protein surface. For plastocyanin, electrostatic forces appear to be important in the binding of both inorganic complexes and other proteins. Preliminary experiments using the basic protein cytochrome *c* (p*I* = 10.5) indicate binding to plastocyanin at the negatively charged region near Tyr 83 (Cookson, D.J. and Wright, P.E., unpublished observation). It is likely that electrostatic forces also dominate the interactions of plastocyanin with its physiological electron transfer partners (generally assumed to be cytochrome *f* and *P*-700, the primary donor of Photosystem I [19]).

The fact that inorganic electron transfer reagents bind to plastocyanin at two different sites, one of which is close to the copper atom and the other about 15 Å away, means that we must consider at least two electron transfer pathways to and from the copper atom. In recent years there has been considerable interest in the mechanisms of electron transfer involving metalloproteins. The distorted tetrahedral geometry and 2-sulphur, 2-nitrogen coordination of the copper atom in plastocyanin favour an outer sphere mechanism since only minor inner sphere re-organization should be required for fast electron transfer [6,16]. Three possible mechanisms of outer sphere electron transfer include direct orbital overlap with conjugated ligands [16], quantum mechanical tunneling [41] and electron transfer along hydrophobic channels [42]. Electron transfer by the first mechanism could certainly occur through the coordinated imidazole ring of His 87 which we have shown to be directly involved in binding $Cr(CN)_6^{3-}$ and hence, by inference, the oxidant $Fe(CN)_6^{3-}$.

A different mechanism must be employed for electron transfer processes involving the site near Tyr 83 and quantum mechanical tunneling or passage through a hydrophobic channel seem most likely. The present experiments provide no basis for distinguishing between the electron tunneling or hydrophobic channel hypotheses. We do note, however, that, regardless of the mechanism, long range electron transfer along this pathway of approx. 10 Å can be very fast, as in the case of the reduction of copper(II) plastocyanin by $Ru(NH_3)_6^{2+}$ [23].

The existence of at least two electron transfer pathways in plastocyanin 'in vitro' is not surprising in view of its biological role [6,24]. The relevance of these pathways to 'in vivo' electron transport processes cannot be further evaluated at this time until more detailed experimental evidence is available on the location of plastocyanin in the chloroplast and on the exact nature and structure of its physiological electron transfer partners. It is very clear, however, that multiple electron transfer pathways and rapid, relatively long range electron transfer processes over distances of 10—15 Å through proteins must not be discounted in discussions of mitochondrial and chloroplast electron transport.

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