Proc. Natl. Acad. Sci. USA
Vol. 78, No. 7, pp. 4190–4193, July 1981
Biochemistry

Identification of an electron transfer locus in plastocyanin by chromium(II) affinity labeling

(blue copper protein/π conduction/fluorescence quenching/peptide analysis)

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Communicated by Michael Sela, April 2, 1981

ABSTRACT **Cu(II)–plastocyanin from French beans (*Phaseolus vulgaris*) is reduced quantitatively by $Cr(II)_{aq}$ ions to give a substitution-inert Cr(III) adduct of Cu(I)–plastocyanin. Enzymatic proteolysis of this derivative by thermolysin led to the identification of the Cr(III) binding peptide. This contains four potential ligands for the metal ion: aspartate-42 and -44 and glutamate-43 and -45. In the three-dimensional fold of plastocyanin, this stretch is very close to tyrosine-83. The emission intensity and its pH dependence observed for the tyrosines in this tryptophan-devoid protein differ markedly in the Cr(III) adduct. That difference is interpreted as reflecting proximity and interaction between the latter metal ion and tyrosine-83. The distance between the copper center and the suggested Cr(III) binding site is ≈12 Å. The intervening region contains an array of highly invariant aromatic residues. These are proposed to be involved in the electron transfer process. A mechanism for that process is presented that involves interaction between the *d* electrons of the metal ions with $d\pi$-π^* delocalization through a weakly coupled π^* system. The rationale of this electron transfer pathway for the reactivity of plastocyanin with inorganic redox agents is discussed.**

Plastocyanins (Pc) are blue copper proteins that function as electron carriers in chloroplasts (1). X-ray crystallographic studies of poplar tree (*Populus nigra*) Pc have shown that the single copper ion is inaccessible to solvent, buried ≈6 Å inside the protein in a hydrophobic environment (2). This structural feature raises the fundamental problem of how electrons get to and from the redox center through the intervening protein matrix. Both kinetic (3, 4) and NMR (5–7) studies have brought evidence for the presence of at least two distinct sites on Pc and on azurin for interaction with either inorganic redox complexes or with redox-inactive analogues of such reagents. In a detailed kinetic investigation, redox rate constants have been related to the electron transfer distances for a series of metalloprotein reactions (8). This correlation led to the conclusion that the reactivity is enhanced—corresponding to a smaller electron transfer distance—when inorganic complexes with hydrophobic π-conducting ligands are used. So, identification of electron transfer loci is clearly of great interest. To achieve this goal with Pc, we have made use of a direct approach of labeling such a locus with Cr(III) ions. The chemical basis of this approach lies in the fact that, although the strongly reducing Cr(II) complexes are highly substitution labile, their single-electron oxidation products, Cr(III) complexes, are generally substitution inert. Therefore, the coordination sphere of the metal ion during the transition state of the electron transfer process is expected to be maintained in the substitution-inert Cr(III) reaction product. This approach has been used successfully by Kowalsky (9), who has shown that in the reduction of ferricytochrome *c* by Cr(II), the Cr(III) ions produced remain tightly bound to ferrocytochrome *c*. This finding later led to a study identifying the residues to which the chromium binds (10). We have previously found that Cr(II) reduction of *Pseudomonas* azurin also yields an inert Cr(III) adduct (11). In the present work, we show that Cr(II) reduced the oxidized form of Pc [Pc(II)] stoichiometrically and quantitatively yields Cr(III)–Pc(I), a substitution-inert complex of Cr(III) and the reduced form of Pc [Pc(I)]. Through proteolytic cleavage, we have identified the Cr(III) binding peptide. The known three-dimensional structure of poplar Pc (2), which is assumed to be conserved in that of the French bean protein, shows that the chromium binding site is ≈12 Å from the Cu center. This assignment is corroborated by the emission properties of the tyrosines in the labeled protein. An electron transfer pathway from the Cr(II) at the protein–water interface to the Cu(II) site inside Pc has been proposed. These results illustrate the general applicability of $Cr(II)_{aq}$ for labeling electron transfer loci in proteins.

MATERIALS AND METHODS

Materials. Pc was isolated from French bean (*Phaseolus vulgaris*) leaves that had been frozen immediately after harvest. The juice was extracted from the thawed leaves by pressing according to a procedure described earlier (12). The method for isolation and purification of Pc is given by Milne and Wells (13). The final A_{278}/A_{597} ratio was 1.16. The concentration of native Pc(II) was determined from the absorbance at 597 nm by using $\varepsilon = 4500\ M^{-1}\ cm^{-1}$ (13). Native Pc was reduced with H_2 under anaerobic conditions using a catalytic amount of Pt black (14). ^{51}Cr was an aqueous sodium chromate solution (specific activity 100–400 mCi/mg; 1 Ci = 3.7×10^{10} becquerels) from the Radiochemical Centre (Amersham, England). Thermolysin (3× crystallized) was from Calbiochem. All other chemicals were of analytical grade and used without further purification.

Preparation of Cr(III)-Labeled Pc. Stock solutions of 0.05 M $Cr(ClO_4)_2$ in 0.5 M $HClO_4$ labeled with ^{51}Cr (specific activity ≈10 μCi/mg) were used as reductants (11). Pc solutions (0.1–0.2 mM in 0.1 M Hepes, pH 7.0) were deoxygenated by repeated evacuation and flushing with purified argon. A stoichiometric amount of Cr(II) was then slowly added to the vigorously stirred protein solution cooled in ice. One equivalent of reductant caused complete Cu(II) reduction as judged by the disappearance of the 597-nm absorption. The solution was then dialyzed extensively against 0.1 M Hepes, pH 7.0, for at least 24 hr. The binding stoichiometry of the chromium was established by monitoring the γ-ray activity of ^{51}Cr.

Enzymatic Proteolysis, Peptide Separation, and Analysis. Since Pc is resistant to enzymatic proteolysis, the chromium-labeled protein (typically 5 mg) was denatured with 10% tri-

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Abbreviations: Pc, plastocyanin; Pc(I) and Pc(II), reduced and oxidized forms of Pc, respectively; HPLC, high-pressure liquid chromatography.

chloroacetic acid according to the procedure used for azurin (15). This procedure caused a decrease of ≈15% in the γ activity, which we ascribe to incomplete precipitation of the protein. After the denaturation, the pH was adjusted to 7.0 and 2% (wt/wt) thermolysin in 10 mM $Ca(ClO_4)_2$ was added. The proteolysis was performed at room temperature for 10 hr. The fragments were then separated on a Sephadex G-25 column (2 × 95 cm), eluting with 0.1 M HOAc. The eluate was fractionated in 5-ml portions and analyzed for absorbance at 230 and 280 nm and for ^{51}Cr γ activity. The center of the radioactive peak was concentrated to 1 ml by lyophilization and further purified by high-pressure liquid chromatography (HPLC) using a 4.6 × 250 mm column packed with Lichrosorb C-18, 10 μm from Merck. The elution was performed with a linear gradient of 50 mM aqueous NaOAc, pH 6.5, and 50 mM NaOAc in 50% propanol/water. The elution rate was 60 ml/hr, and the eluate was collected in 1-ml fractions and analyzed for γ activity. The chromium-labeled samples were hydrolyzed in 6 M HCl at 120°C for either 24 or 72 hr. Amino acid analyses were performed with a Durrum D-500 amino acid analyzer.

Other Methods. Fluorescence spectra were measured on a Perkin–Elmer MPF 44 A spectrofluorometer at 25°C. pH titrations were performed by adding microliter quantities of concentrated HOAc or 5 M NaOH to the solution. The iodide quenching was measured after adding aliquots of 10 M NaI to native Pc(I). All solutions were routinely filtered through Millipore filters before measurement. Radioactivity was measured on an automatic Packard scintillation spectrometer.

RESULTS AND DISCUSSION

Pc is rapidly reduced under anaerobic conditions (16) by one equivalent of chromium(II) perchlorate, and the Cr(III) produced remains bound to the protein in a 1:1 ratio. Extensive dialysis of the Cr(III)-labeled protein for 24 hr does not cause any loss of the label, as judged by the ^{51}Cr γ activity. A control experiment in which $Cr(III)_{aq}$ was added to native Pc showed that under these conditions, the metal ion is easily separated on a Sephadex G-25 column. The copper center in Cr(III)-labeled Pc can still undergo redox reactions without loss of label. Thus, the blue color is regained by reoxidation—e.g., by 1 equivalent of $IrCl_6^{2-}$. Also, photooxidation and photoreduction of Cr-labeled Pc with its biological partners has been studied. It was found that although the Cr label did not influence the rate of photoreduction with chloroplasts, the photooxidation of Cr(III)–Pc(I) by isolated photosystem I reaction centers was significantly slower than that of native Pc(I) (unpublished results). To locate the site of Cr(III) binding on Pc, the labeled protein was subjected to proteolytic cleavage. Sequence work on French bean Pc (17) has shown that thermolysin produces reasonably sized fragments suitable for structural identification. An example of the elution pattern of the thermolysin digestion products from a Sephadex G-25 column is shown in Fig. 1. Integration of the radioactive peak consistently shows that >90% of the ^{51}Cr originally bound to the denatured protein is retained in this peak. The three central fractions of this peak were pooled, concentrated to 1 ml by lyophilization, and further purified by fractionation on HPLC column. The radioactivity appeared as a single sharp band in 2 ml of eluate having R_F 0.38 and invariably containing >95% of the γ activity of the pooled sample. The amino acid analysis of this peptide is given in Table 1. Comparison with the known sequence of French bean Pc and the published results of thermolysin digestion of this protein (17) allows the identification of the Cr-containing peptide. The facts that (*i*) HPLC fractionation gives a single sharp symmetrical band containing all the ^{51}Cr, (*ii*) an amino acid composition fitting a single peptide is obtained reproducibly for that peak,

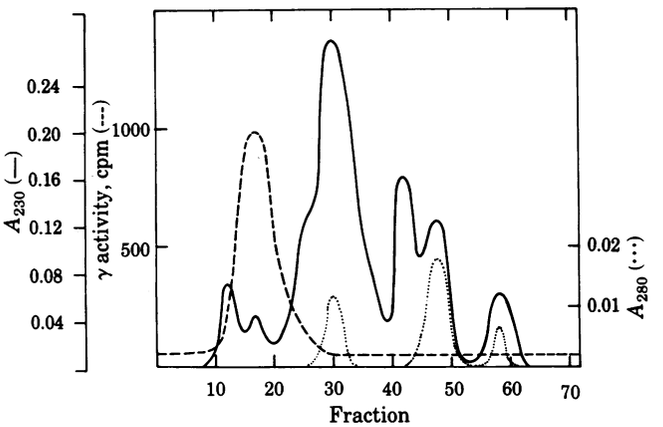

FIG. 1. Elution pattern of the Cr(III)–Pc proteolysis products on a Sephadex G-25 (2.0 × 95 cm) column. Eluent, 0.1 M HOAc; fractions, 5 ml.

and (*iii*) an essentially quantitative yield of ^{51}Cr in the labeled peptide is found, as contrasted with the ease with which a mixture of equimolar amounts of Cr(III) and Pc can be separated, conclusively demonstrate that the chromium label is and remains specifically bound to a unique site. This is most probably the locus to which Cr(II) delivers the electron. An intermolecular electron transfer between Cr(II)–Pc(II) molecules is rather unlikely, as it has been shown (16) that reduction of Pc(II) by Cr(II) is first order in Pc. Migration of the probe after the electron transfer would inevitably lead to a smaller yield of labeled peptide and to a lower specificity. The chromium binding peptide is shown in the footnote to Table 1. It contains four side chains that are potential ligands for chromium(III): aspartate-42 and -44 and glutamate-43 and -45. This stretch is highly invariant in all Pc sequences studied (18). Without detailed structural information about the orientation of these side chains, however, it is not possible to determine the particular carboxylate to which the chromium ion is coordinated. However, the inertness of the Cr–Pc complex suggests that Cr(III) most probably is chelated to at least two of these residues. The binding to this peptide is obviously dominated by electrostatic interactions between the positively charged electron donor and the carboxylate groups. This locus is ≈12–15 Å from the copper center (2). It is also noteworthy that this site coincides with the one proposed for interaction between cationic inorganic complexes and the native reduced protein. Thus proton magnetic resonance studies of the broadening caused by redox-inactive par-

Table 1. Amino acid composition of Cr(III)-labeled peptide

Amino acid	Residues, no.	Residues 40–49*
Aspartate	1.6	2
Glutamate	2.4	2
Proline	1.1	1
Glycine	1.2	1
Alanine	1.1	1
Valine	0.6 (0.9)	1
Isoleucine	0.8	1
Phenylalanine	0.6 (0.9)	1

Results represent the average of eight determinations from four independent digestion experiments. Hydrolysis time was 24 hr except for values in parentheses, which represent a hydrolysis time of 72 hr.
*Corresponds to sequence

Val-Phe-Asp-Glu-Asp-Glu-Ile-Pro-Ala-Gly
40 41 42 43 44 45 46 47 48 49

(17).

amagnetic complexes to proton resonances of certain residues showed two distinct regions of interaction (5–7). Positively charged probes were found to broaden specifically the resonances of the exposed tyrosine-83 (5–7), which lies next to the above mentioned carboxylates and is an evolutionarily conserved residue in all higher plant Pc. Further, the different broadening of the *ortho* and *meta* protons shows that the phenolic side chain is oriented in such a way (7) that the hydroxyl group is pointing toward the proposed Cr binding site.

The proximity of the Cr(III) binding site to tyrosine-83 was also examined by another approach. French bean Pc contains three tyrosines and no tryptophan. Two tyrosines seem to be buried inside the molecule while the third (tyrosine-83) is at the surface (2). An interaction between tyrosine-83 and the chromium label is indicated by the different emission intensities exhibited by native Pc(I) and Cr-labeled Pc(I), as well as by their respective pH dependences (Fig. 2). It is of interest to compare these results with those obtained by Ugurbil and Bersohn (19) for tyrosine emission of the tryptophan-lacking azurin from *Ps. fluorescens*. This protein contains two tyrosines, both of which apparently are buried inside the molecule. Here, the fluorescence intensity is constant in the pH range 3–10 and decreases sharply at both ends (19).

We assign the unique behavior in Pc to the third (solvent-exposed) fluorophore, tyrosine-83. In the native protein at neutral pH, the emission intensity of tyrosine-83 is significantly quenched by its neighboring carboxylate groups. At pH 7.0, the quantum yield of native Pc(I) is 0.0098 [determined relative to L-tyrosine, using $\Phi = 0.14$ (19)]. In the acid pH range, the fluorescence intensity increases, with an apparent pK of ≈4. From the known three-dimensional structure (2), hydrogen bonding between the tyrosine-83 hydroxyl and the carboxylate of glutamate-59 is expected. This residue is invariant in all higher plant Pc. Decreasing the pH leads to protonation of the carboxylates, and therefore the quenching of the latter tyrosine is relieved and an enhancement is observed. The further decrease

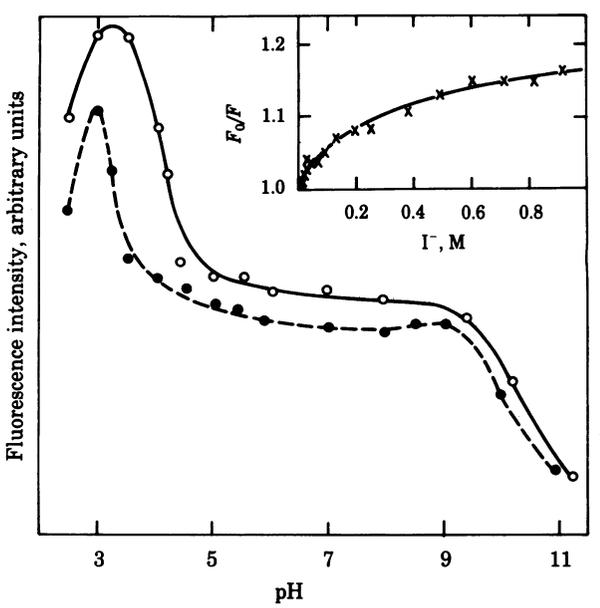

FIG. 2. pH dependence of fluorescence intensity of native Pc(I) (○) and Cr(III)-labeled Pc(I) (●). Excitation λ = 280 nm, emission λ = 300 nm. *A* at 280 nm was 0.069 cm^{-1} for all solutions. The pH was adjusted with microliter additions of concentrated HOAc or 5 M NaOH to 0.1 M protein in Hepes at 25.0°C in a thermostated cell. (*Inset*) Quenching of native Pc(I) by iodide ions at 25°C, pH 7.0, in 0.1 M Hepes. *F*, fluorescence in the presence of quencher (I^-); F_0, fluorescence in the absence of quencher.

in pH results in the unfolding of the protein, which exposes the two internal tyrosines and leads to the inversion and sharp decrease in emission at pH <2.5.

For the Cr(III)-labeled Pc, the pH dependence of the emission differs in several important features from that of the native protein. First, in the pH range 8–4, the quantum yield is 15–20% lower in the former case (quantum yield, 0.0083 at pH 7.0). The difference is assigned to the quenching of the residual emission of tyrosine-83 by its close proximity to Cr(III). This assignment gains further support from measurements of iodide quenching of native Pc(I), which levels off at ≈15% in 0.8 M I^- (Fig. 2 *Inset*). Since tyrosine-83 is the only solvent-exposed fluorophore, its emission is expected to be the one most susceptible to iodide quenching. Second, on lowering the pH, the emission enhancement of the Cr-labeled Pc is shifted ≈0.5 pH unit relative to that of the native protein. In our opinion, this effect is also related to hydrogen bonding between the hydroxyl group of tyrosine-83 and an acceptor. A direct coordination of tyrosine-83 to chromium(III) can be excluded, since this residue is not included in the Cr-labeled peptide. Carboxylates coordinated to Cr(III) can be excluded as acceptors, since their pK values would be much lower. Neighboring carboxylates, on the other hand, are unlikely to be affected by as much as 0.5 pH unit. Therefore, we propose that the acceptor is a hydroxyl group coordinated to Cr(III). A water molecule coordinated to Cr(III) could very well have a pK of ≈3.5. Due to its spatial relationship to Cr(III), it is expected that tyrosine-83 will play a crucial role in the electron transfer process to the copper center.

Examination of the three-dimensional structure of Pc (2) shows that a hydrophobic channel exists between the Cr(III) binding region and the copper center. It contains several highly invariant aromatic residues, together with the sulfur of the Cu-ligating cysteine-84. As has been pointed out (2), these include phenylalanine-14, -41, and -82 and tyrosine-83. We suggest a mechanism that involves interaction between the *d* electrons of the redox centers and πd-π^* delocalization through the extended and weakly coupled π^* system. Such "through space" influence, with direct interaction between the π systems of intervening aromatic rings instead of through a bond system, has been suggested by Taube for mixed valence complexes in certain redox systems (20). Involvement of the cysteine-84 sulfur in the electron transfer path can also be envisaged, considering the polarizability of the thiolate group. It has also been shown that conjugation, apparently broken by sulfur insertion between aromatic groups (i.e., 4,4′-dipyridylsulfide), still provides substantial electronic interaction in mixed valency binuclear metal complexes (21). Similarly, through-space metal-to-metal communication was reported in certain binuclear thioether complexes (20).

This model provides an attractive rationale for the kinetic accessibility scale defined by Gray and his colleagues (22). They have concluded that the extent of orbital overlap and nonelectrostatic interactions determines the electron transfer reactivity. Therefore, agents, such as Tris·(ø-phenanthroline)Co(III) that have hydrophobic π-conducting ligands exhibit much higher reactivities than more hydrophilic ones. This can be explained in terms of the above proposal, since the former residues will provide an excellent overlap with the π-symmetric orbitals of tyrosine-83 in Pc and histidine-35 in azurin (11). Also, the "electron transfer distances" determined on the basis of comparing reactivities of different metalloproteins with inorganic complexes (8) can be rationalized in this context. Thus, the calculated electron transfer distance between the redox site in Pc in the reaction with Tris·(*o*-phenanthroline)Co(III) of 2.9 Å (8), compared with the ≈12-Å distance from the copper center

to the proposed binding site for Cr, is reasonable: With an extensive π overlap between the Cu ligands and other protein residues, the effective redox site can extend well beyond the primary ligand sphere.

Ps. aeruginosa azurin is also reduced by Cr(II), leading to a 1:1 Cr(III)–azurin complex (11). Proteolytic cleavage of this adduct and analysis of the peptides enabled identification of the Cr(III) binding site. Based on the examination of the three-dimensional model of azurin (23), a pathway was proposed that implies an electron transfer through a relay of conjugated systems consisting of two parallel imidazole rings—one the Cu-ligating histidine-46 and the other the noncoordinating surface-proximate histidine-35. This pathway bears conceptual resemblance to the one proposed for Pc—namely, a transfer through a series of conjugated π systems—although the implicated residues differ. Thus, the proposed aromatic π-conduction electron transfer pathway may well have general significance for the mode of operation of redox proteins.

We are grateful to Dr. S. Blumberg for helpful advice and discussions concerning the proteolysis of Pc and for providing us with a sample of thermolysin. We also thank Y. Blatt and Dr. P. Frank for their interest in, and criticism of, this work and Dr. A. Tishbee for carrying out the HPLC separation. O.F. acknowledges a grant from the Danish Natural Science Research Council.

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