Chemical Modification of Spinach Plastocyanin: Separation and Characterization of Four Different Forms†

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ABSTRACT: Spinach plastocyanin has been chemically modified by using a water-soluble carbodiimide to form an amide bond between a protein carboxyl group and one amino group of ethylenediamine. This reaction produced four distinct species of chemically modified plastocyanin which could be separated either by polyacrylamide gel electrophoresis (in the absence of sodium dodecyl sulfate) or by ion-exchange chromatography on DEAE-Sephadex. The four forms contained 2.1, 3.2, 4.1, and 6.3 mol of ethylenediamine per mol of plastocyanin and were labeled fractions IV, III, II, and I, respectively. All four fractions showed a shift in midpoint redox potential of greater than +40 mV. In contrast, the K_m for $P700^+$ reduction decreased with increasing extents of modification. The addition of Mg^{2+} ions decreased the K_m for the negatively charged fractions II, III, and IV but increased it for the positvely charged fraction I. Fraction III showed an increased incorporation of ethylenediamine into residues 42–45 which are located approximately 18 Å distant from the copper. This fraction also showed a maximal increase in redox potential. Potential calculations showed that the electrostatic effect of incorporating two positive charges at this distance was sufficient to produce a shift in redox potential of +40 mV. However, an electrostatically induced conformational change which would affect the environment of the copper cannot be ruled out.

Plastocyanin is a small copper protein which acts as an electron carrier in photosynthetic electron transport between cytochrome *f* and P700[1] (Boulter et al., 1977; Plesnicar & Bendall, 1973; Gorman & Levine, 1966; Haehnel et al., 1980). Recent studies have shown that cations stimulate the rate of electron transport between plastocyanin and $P700^+$ in both spinach chloroplasts (Lockau, 1979; Tamura et al., 1980; Haehnel et al., 1980) and in isolated photosystem I (PSI) particles (Lien & San Pietro, 1979; Davis et al., 1980; Burkey & Gross, 1981a; Tamura et al., 1981). It was suggested that the cations act by screening the negative charges on both the spinach plastocyanin (Boulter et al., 1977) and on the photosystem I complex (Satoh & Butler, 1978; Siefermann-Harms & Ninnemann, 1979). In addition, it has been shown that abolishing the net negative charge on either the photosystem I complex (Burkey & Gross, 1981a) or the plastocyanin molecule (Davis et al., 1980; Burkey & Gross, 1981b) abolished the cation requirement.

Recently, we used a chemical modification technique to modify the net charge on the plastocyanin molecule (Burkey & Gross, 1981b) and then studied the interactions of the modified plastocyanin with the photosystem I complex. We reacted carboxyl groups on the protein with ethylenediamine in the presence of a water-soluble carbodiimide (Means & Feeney, 1971) (eq 1). This reaction converts a negatively

$$\text{protein–COO}^- + :\text{NH}_2(\text{CH}_2)_2\text{NH}_3^+ \xrightarrow[\text{pH 6.0}]{\text{carbodiimide}} \text{protein–C(=O)NH(CH}_2)_2\text{NH}_3^+ \quad (1)$$

charged carboxyl group into a positvely charged amino group. The modification caused a decrease in the K_m for plastocyanin binding to PSI as well as an increase in the midpoint redox potential from +380 to +420 mV. We observed an average of 4.3 molecules of ethylenediamine $(EDA)^+$ incorporated per plastocyanin molecule. The modified groups included amino acid residues 42–45, 59–61, and 68, which are highly conserved glutamic and aspartic acid residues (Boulter et al., 1977). When we did the peptide mapping, we found more than one spot for several of the tryptic peptides which suggested that the modified plastocyanin was a mixture of several different forms. We have since separated and purified four different forms of modified plastocyanin containing different amounts of ethylenediamine. The properties of these different forms are discussed below.

Experimental Procedures

Materials. Ethylenediamine dihydrochloride, Sephadex G-25, and Sephadex G-10 were obtained from Sigma Chemical Co. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide was obtained from Pierce Chemical Co. DEAE-cellulose and hydroxylapatite were obtained from Bio-Rad Laboratories. TPCK-trypsin was obtained from Worthington Enzymes. [1,2-^{14}C]Ethylenediamine was obtained from New England Nuclear. All other chemicals were of reagent grade.

Isolation of Plastocyanin and Photosystem I Particles from Spinach. Plastocyanin was isolated according to the method of Davis & San Pietro (1979). The purified plastocyanin had a final A_{275}/A_{597ox} ratio of 1.2–1.6 and eluted as one single peak by both Sephadex G-75 gel filtration and DEAE-cellulose ion-exchange chromatography. Plastocyanin concentrations were measured according to Davis & San Pietro (1979) by using an extinction coefficient of 4.9 mM^{-1} cm^{-1} at 597 nm.

PSI particles were isolated according to Shiozawa et al. (1974) as modified by Burkey & Gross (1981b). Chlorophyll concentrations were measured according to the method of Arnon (1949).

Chemical Modification of Plastocyanin. The chemical modification of plastocyanin was carried out by reacting the

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[1] Abbreviations: EDA, ethylenediamine; PSI, photosystem I particles; P700, reaction center chlorophyll of photosystem I which absorbs maximally at 700 nm; $P700^+$, oxidized P700; Chl, chlorophyll; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

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Table I: Effect of Chemical Modification on the Properties of Plastocyanin

fraction	mol of ethylenediamine/ mol of plastocyanin	net charge	redox potential (mV)	K_m (μM)	
				$-MgCl_2$	+5 mM $MgCl_2$
control	0[a]	9–[b]	367[c]	[d]	9.4 ± 0.3
mixture	5.1		399	0.5 ± 0.03	
IV	2.1	5–	405		2.3 ± 0.1
III	3.2	3–	405	5.1 ± 0.2	2.3 ± 0.1
II	4.1	1–	410	3.6 ± 0.4	1.8 ± 0.2
I	6.3	3+	416	0.4 ± 0.03	1.4 ± 0.1

[a] Determined from the incorporation of [^{14}C]EDA into plastocyanin. The incorporation of [^{14}C]EDA was determined according to the method of Burkey & Gross (1981b). [b] Calculated from the amino acid composition of plastocyanin and the incorporation of [^{14}C]EDA. [c] The redox potential was measured as described by Davis & San Pietro (1979). [d] The rate of $P700^+$ was determined as described by Gross (1979). See also Burkey & Gross (1981a,b). The reaction mixture contained PSI (10 μg/mL chl) + 10 mM Tris-HCl (pH 8.2) + 2 mM sodium ascorbate. Other additions were as indicated.

plastocyanin with ethylenediamine (0.2 M) in the presence of 50 mM 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide as previously described by Burkey & Gross (1981b). For the labeling experiments, 5–20 μCi of [1,2-^{14}C]ethylenediamine dihydrochloride (3.5 mCi/mol) was added to the reaction medium containing unlabeled ethylenediamine as previously described (Burkey & Gross, 1981b).

In some experiments, the plastocyanin was modified either in the absence of a nucleophile or in the presence of 0.2 M taurine (β-aminoethanesulfonic acid). In the latter case, the incorporation of taurine was determined by amino acid analysis according to the method of Burkey & Gross (1981b).

Kinetics of $P700^+$ Reduction. The kinetics of $P700^+$ reduction were determined on an Aminco DW-2a spectrophotometer according to the method of Gross (1979). The reaction mixture contained PSI (10 μg/mL chl), 10 mM Tris-HCl, pH 8.2, and 2 mM ascorbate. The reaction mixture was titrated with increasing amounts of control or modified plastocyanin in the presence or absence of $MgCl_2$. The initial rate of $P700^+$ reduction was measured after each addition after which the background ascorbate rate was subtracted. Least-squares analyses were used to analyze the double-reciprocal plots, and the errors are reported to within one standard deviation.

Measurement of the Oxidation–Reduction Midpoint Potential. The midpoint redox potentials were determined as described by Davis & San Pietro (1979) [see also Burkey & Gross (1981b)].

Polyacrylamide Gel Electrophoresis. Discontinuous buffer polyacrylamide gel electrophoresis was performed by adapting the buffer system of Kirchanski & Park (1976) to the method of Brewer (1974). $NaDodSO_4$ was omitted so that the various forms of modified plastocyanin could be separated on the basis of differences in the net charge.

Tryptic Digestion and Peptide Mapping of the Modified Plastocyanin. Tryptic digestion and peptide mapping of the modified plastocyanin were carried out as previously described (Burkey & Gross, 1981b).

Results

Separation of Four Forms of Chemically Modified Plastocyanin. The chemically modified plastocyanin was found to be a heterogeneous mixture of plastocyanin molecules modified to different extents. This was determined by using polyacrylamide gel electrophoresis to separate the molecules on the basis of charge (Figure 1). The modified plastocyanin migrated as four bands (Figure 1A) all of which had a lower mobility than did the native plastocyanin (Figure 1F). This shows that all of the plastocyanin was modified to some extent since there was no peak corresponding to the native plastocyanin. Moreover, the fact that all four forms of modified plastocyanin had smaller mobilities than the native is consistent with replacing negative charges (due to carboxyl groups) with positive charges (due to amino groups).

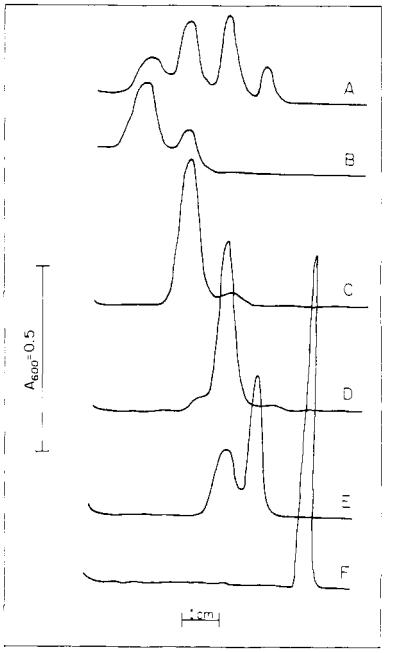

FIGURE 1: Polyacrylamide gel electrophoresis of chemically modified and native plastocyanin. The electrophoresis was carried out as described under Experimental Procedures. $NaDodSO_4$ was omitted so that the various species could be separated on the basis of net charge. The gels were scanned at 600 nm after staining with Coomassie blue. Key: (A) Modified plastocyanin mixture; (B) modified plastocyanin fraction I; (C) modified plastocyanin fraction II; (D) modified plastocyanin fraction III; (E) modified plastocyanin fraction IV; (F) native plastocyanin.

The four forms of modified plastocyanin could also be separated by anion-exchange chromatography on DEAE-Sephadex (Figure 2). The four fractions which were obtained are labeled from I to IV for the order in which they eluted from the DEAE-Sephadex column. Fraction I is the most positive (most modified) and fraction IV the most negative (least modified), as indicated by both their migration on polyacrylamide gels (Figure 1) and the amount of [^{14}C]-ethylenediamine incorporated (Table I). Fractions II and III (traces C and D, Figure 1) consist almost entirely of only one species of modified plastocyanin whereas fractions I and IV (traces A and E, Figure 1) have both a major component and a minor component. Nonetheless, the separation is sufficient to study the properties of the modified plastocyanins as a function of extent of modification.

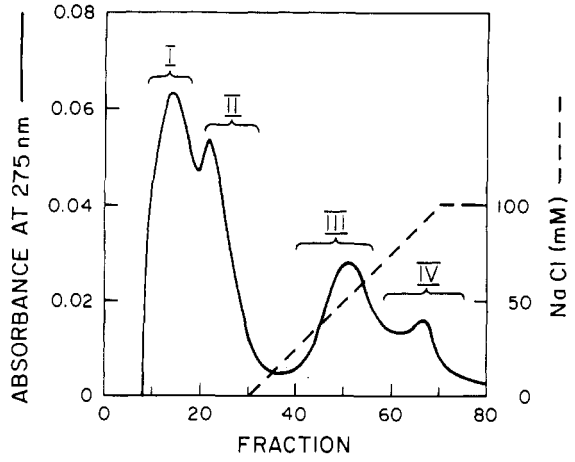

FIGURE 2: Separation of modified plastocyanin species by ion-exchange chromatography on DEAE-Sephadex. A sample of oxidized plastocyanin was applied to a DEAE-Sephadex column equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The column was then eluted with a linear gradient of NaCl (0–100 mM) in the presence of 50 mM Tris-HCl (pH 8.0) after which the column was washed with 0.1 M NaCl containing 50 mM Tris-HCl (pH 8.0) until all of the protein had eluted. The elution of the modified plastocyanin from the column was monitored at 275 nm.

The amount of [^{14}C]ethylenediamine incorporated and the calculated net charge for each fraction are shown in Table I. The amount of [^{14}C]ethylenediamine incorporated varied from 2.1 mol per mol of plastocyanin in fraction IV to 6.3 mol in fraction I. The calculated net charge on the molecules (at pH 8) varied from 5– for fraction IV to 3+ for fraction I.

Effect of the Extent of Chemical Modification on the Redox Potential of the Four Species of Modified Plastocyanin. We also determined the midpoint redox potential for each fraction (Table I). Fraction IV, the least modified fraction, showed a shift in redox potential of +38 mV from +367 to +405 mV. Further modification had shifted the redox potential only another +11 mV. Thus, we can conclude that only a minimal modification is required to produce a large shift in redox potential.

Effect of the Extent of Modification of the K_m for P700$^+$ Reduction. In contrast to these results, the rates of P700$^+$ reduction were dependent on the extent of modification. The K_m's decreased as the extent of modification increased (Table I). The effect is most striking in the absence of $MgCl_2$. In this case, both the control plastocyanin and fraction IV were unable to serve as donor whereas fraction I had a very low K_m of 0.4 μM. The addition of $MgCl_2$ caused a decrease in the K_m's for the negatively charged fractions II and III and increased the K_m for the positively charged fraction I. These results are consistent with those of Davis et al. (1980) who found that cations decreased the K_m's for the negatively charged plastocyanins isolated from higher plants but increased the K_m's for the positvely charged cytochromes and plastocyanins isolated from some cyanobacteria. The effect of Mg^{2+} ions on fractions II and III may be due to the screening of the remaining negative charges. However, chemical modification and the addition of salts are not equivalent processes. Addition of saturating concentrations of $MgCl_2$ (Table I) or other salts (K. O. Burkey and E. L. Gross, unpublished observations) did not decrease the K_m to the same extent as did chemical modification. None of the reactions showed a significant change in the V_{max}.

In conclusion, a large degree of modification was required to cause a significant decrease in the K_m in the absence of Mg^{2+} ions. Thus, there is a differential effect of modification on electron donation to P700$^+$ as compared to the redox potential of the copper.

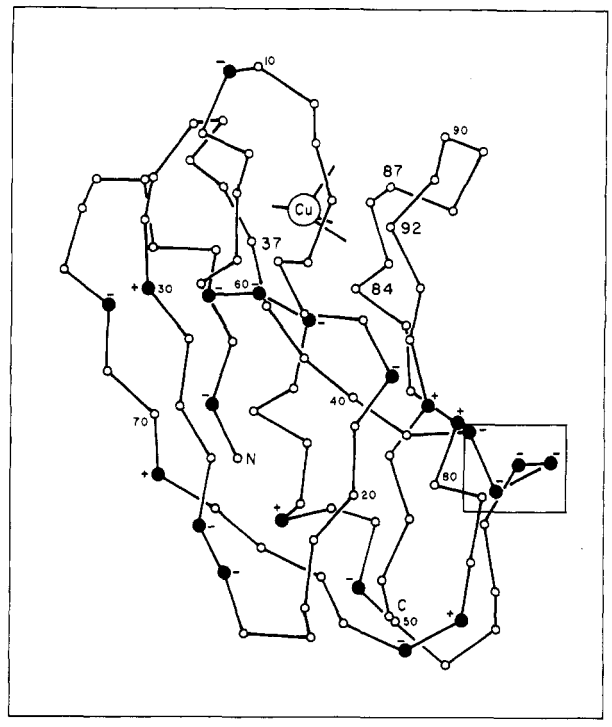

FIGURE 3: Amino acid sequence of spinach plastocyanin projected onto the three-dimensional structure of poplar plastocyanin. The three-dimensional structure of plastocyanin was reproduced from Coleman et al. (1978). The circles represent the α carbons of each amino acid. The dark circles represent the charged amino acids in spinach plastocyanin. The letters N and C denote the amino- and carboxyl-terminal residues, respectively. The boxed region shows residues 42–45 which are highly labeled in fraction III.

Location of the Label in Fraction III of the Modified Plastocyanin. Since only a minimal modification was necessary to produce a maximal shift in redox potential, it was of interest to determine the location of the modified residues in one of the minimally modified fractions. We chose fraction III for these studies because we were able to obtain it in relatively high yield and purity. We found a 2-fold enrichment of the ^{14}C-labeled ethylenediamine in tryptic peptide two (which contains amino acid residues 31–55) compared to the mixture. Peptide two contains the anionic residues 42–45. Thus, the incorporation of ethylenediamine molecules into these residues is mainly responsible for the +40-mV shift in redox potential. These residues are approximately 18 Å distant from the copper (Figure 3; also Coleman et al., 1978). It is of interest to note that further modification in the region of residues 59–61 and 68 which are 10–12 Å from the copper showed very little further shift in the redox potential (Table I). The further modification did, however, significantly decrease the K_m for P700$^+$ reduction (Table I).

The location of the modified residues in fraction III and in the mixture of modified plastocyanins is shown in Figure 3. Figure 3 has been constructed by superimposing the amino acid sequence of spinach plastocyanin (Boulter et al., 1977) on a projection of the three-dimensional structure of poplar plastocyanin (Coleman et al., 1978).

Discussion and Conclusions

Chemical modification is a pwerful tool for studying the nature of electrostatic interactions in proteins because of the possibility of modifying individual amino acid residues. Ferguson-Miller et al. (1978) and Smith et al. (1981) have

used this technique to examine the role of various lysine residues in cytochrome *c* on its ability to interact with cytochrome oxidase and succinate-cytochrome *c* reductase. It has allowed us to generate four forms of modified plastocyanin which differ only in the charge on various parts of the molecule. Using the four forms of modified plastocyanin, we have been able to distinguish between the extent of modification required to shift the redox potential +40 mV and that required to cause a decrease in the K_m for $P700^+$ reduction. Moreover, it has allowed us to tentatively identify residues 42–45 as those responsible for the shift in redox potential.

An interesting question arises as to how the shift in redox potential occurs since residues 42–45 are approximately 18 Å distant from the copper. The shift in redox potential could be due to electrostatic effects of the modification. The substitution of a positive charge for a negative charge will have an effect on the oxidation state of the copper. The positive charge will tend to destabilize the Cu^{2+} form with respect to the Cu^{1+} form, thus shifting the redox potential in a positive direction. The major question concerns whether this effect would be large enough to account for a 40-mV shift in redox potential. To answer this question, we have calculated the electrical potential observed by the copper atom for a positive charge placed at a distance of 18 Å (Frank, 1950). Assuming a dielectric constant of 5 for a protein (Pethig, 1979), we obtain a value of 160 mV. Even if we assume a much larger dielectric constant, we can easily obtain an electrical potential of 40 mV upon substitution of two positive charges for two negative charges.

If this explanation is correct, why does the addition of six additional positive charges at positions still closer to the copper not cause further shifts in redox potential? We do not know the answer, but there are several possibilities. First, there may be differences in the local dielectric constant for various parts of the plastocyanin molecule so that modification at residues 42–45 has a much greater influence on the redox potential than does modification at residues 59–61 and 68. Second, the addition of extra positive charges may affect the ionization state of some of the remaining carboxyl groups. For example, when several carboxyl groups are spaced close together as in regions 42–45 or 59–61, all of the groups may not be ionized even at pH 8. In this case, the addition of positive charges in the region could promote the ionization of unmodified carboxyl groups and cause potential changes in the opposite direction.

Third, there may be an electrostatic effect on protein conformation which would affect the copper environment. For example, there could be charge repulsion between the ethylenediamine molecules incorporated at residues 42–45 and positively charged lysines-81 and -95. These two lysines are at the base of an arm of the protein that contains three ligands to the copper. Charge repulsion between these lysines and the modified carboxyl groups could cause a spacial shift in that whole arm. Changing the spacial orientation of three copper ligands could destabilize the Cu^{2+} form of the protein. If this is true, we should observe changes in the ligands to the copper. Experiments are in progress to test this hypothesis.

To this point, we have been assuming that the modification which produces the shift in redox potential involves the substitution of one positively charged molecule of ethylenediamine for one negatively charged carboxyl group. However, there are other possibilities which must be ruled out. First, there could be cross-linking between the ϵ-amino groups of lysines with neighboring carboxyl groups. To test this possibility, we modified plastocyanin in the absence of an added nucleophile and obtained a shift in redox potential of less than 3 mV. Therefore, this type of cross-linking cannot be responsible for the observed shift in redox potential. There is, however, a second possible type of cross-linking, namely, the linking of two carboxyl groups by a molecule of ethylenediamine. This is statistically unlikely in the presence of high concentrations of ethylenediamine. In addition, this type of cross-linking would be identifiable on the chromatograms as a spot containing two peptides and 1 mol of [^{14}C]ethylenediamine. We identified every spot on the chromatograms with the exception of one and did not observe any dipeptides. The one spot which we could not identify must have contained tryptic peptide one (residues 1–30) since we did not find this peptide anywhere else on the chromatogram. There was not sufficient radioactivity in the spot to account for one molecule of EDA per molecule of plastocyanin. Therefore, any dipeptide which might have been contained in this spot could not have been involved in the redox potential shift because all forms of the modified plastocyanin showed the redox potential shift which would require that every molecule in the mixture be labeled at least once.

Another possibility is that the addition of an extra two carbon atoms rather than the charge change was responsible for the shift in redox potential. To test this possibility, we modified the plastocyanin in the presence of β-aminoethanesulfonic acid which would not cause a charge change. We observed a shift in redox potential of 2 mV upon incorporation of 1.8 mol of β-aminoethanesulfonic acid compared to 38 mV upon incorporation of 2.1 mol of EDA (Table I). These results rule out this possibility.

A fourth possibility is that the carbodiimide reacted with tyrosine groups to form an *O*-arylisourea (Means & Feeney, 1971). This possibility can be ruled out since all three tyrosine residues were recoved unmodified as determined by amino acid analysis.

There is also the interesting question as to whether the progressive decrease in the K_m for $P700^+$ reduction observed with increasing extents of modofication is due to a decrease in the net negative charge on the whole molecule or whether it is due to the modofication of the binding site for photosystem I in the more extensively modified fractions. A decrease in the net negative charge would tend to decrease the extent of charge repulsion between plastocyanin and the negatively charged photosystem I complex. At first glance, both our results and those of Davis et al. (1980) are consistent with this explanation. However, there are some details which are difficult to explain by using a very simple charge-screening mechanism. First, the effects of chemical modification on the K_m cannot be duplicated by the addition of high concentrations of salt. Second, there is some preference for particular cations (Lien & San Pietro, 1979; E. L. Gross and K. O. Burkey, unpublished results). For example, Ca^{2+} ions are more effective than Mg^{2+} ions, and all divalent cations are much more effective than monovalent cations even on an ionic strength basis.

The alternate hypothesis of the modification of the binding site for photosystem I is intriguing in view of what we know concerning electron donation pathways in plastocyanin. Two different pathways of electron donation have been identified on the basis of NMR measurements (Lappin et al., 1979; Cookson et al., 1980; Hanford et al., 1980). One pathway involves histidine-87 in an outer sphere mechanism. The other involves various hydrophobic residues including Tyr-83, Phe-82, and Val-93. These latter residues are closer to residues 42–75 which are modified in fractions III and IV which show

only small decreases in the K_m for $P700^+$ reduction. The existence of two pathways of electron donation has led Haehnel et al. (1980) to suggest the existence of two separate binding sites for cytochrome *f* and P700.

In principle, our experiments should allow us to distinguish between the binding sites for cytochrome *f* and photosystem I. This assumes, of course, that the binding site is close to the site of electron transfer. Since the modification of residues 42–45 causes very little decrease in the K_m for $P700^+$ reduction, this region is probably not the binding site for photosystem I. This conclusion is not supported by the results of Farver & Pecht (1981), who found that the binding of chromium to residues 42–45 inhibited plastocyanin oxidation by photosystem I but not its reduction by cytochrome *f*. Further experiments are necessary to distinguish between these alternatives.

In conclusion, we have obtained four distinct chemically modified forms of plastocyanin which have allowed us to study the effect of the extent of modification on its redox potential and electron donation properties.

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