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Effect of Carboxyl Group Modification on Redox Properties and Electron Donation Capability of Spinach Plastocyanin†

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ABSTRACT: Spinach plastocyanin was chemically modified by using a water-soluble carbodiimide to form an amide bond between a protein carboxyl group and one amino group of ethylenediamine. On the average, four plastocyanin carboxyl groups were replaced with positively charged amino groups. Modified plastocyanin facilitated high rates of electron donation to the oxidized P700 reaction center of photosystem I particles in the absence of cations. Control plastocyanin was totally inactive in the absence of divalent cations due to charge repulsion between the negatively charged plastocyanin and photosystem I proteins. The K_m for the binding of modified plastocyanin to photosystem I particles was 2.1 μM compared to 36.5 μM for control plastocyanin in the presence of $MgCl_2$. Therefore, chemical modification was more effective than charge shielding by cations in facilitating the binding of plastocyanin to photosystem I. Chemical modification also increased the midpoint redox potential of plastocyanin from +380 to +420 mV, which indicated an alteration of the copper environment. Ethylenediamine was covalently attached to tryptic peptides from plastocyanin that contained amino acid residues 42–45, 59–61, and 68 which are highly conserved glutamic acid and aspartic acid residues. Chemically altering the charge on these residues changed the activity of plastocyanin which indicates that these amino acids are involved in the ionic regulation of the plastocyanin–PSI interaction. Because chemical modification also altered the environment of the chromophore, certain of these amino acids must be located near the copper site.

Plastocyanin is a small copper protein which acts as an electron carrier in photosynthetic electron transport (Boulter et al., 1977). Reconstitution experiments (Plesnicar & Bendall, 1973; Gorman & Levine, 1966) as well as kinetic evidence (Haehnel et al., 1980) indicate that plastocyanin accepts electrons from cytochrome *f* and donates electrons to $P700^+$.[1]

Recent studies (Tamura et al., 1980; Haehnel et al., 1980) with broken spinach chloroplasts have shown that salts stimulate the rate of plastocyanin electron donation to $P700^+$. Their conclusion was that salts increase the local concentration or accessibility of plastocyanin to the site of P700 on the membrane by causing a shift in the negative surface potential toward neutrality. Both spinach plastocyanin (Boulter et al., 1977) and the thylakoid membrane (Nakatani & Barber, 1980) are negatively charged at neutral pH. By screening the negative charges on these entities, salts reduce electrostatic repulsion between them which allows plastocyanin to donate electrons to $P700^+$ more readily.

This type of electrostatic interaction also exists between spinach plastocyanin and isolated PSI[1] which is also a negatively charged protein complex (Satoh & Butler, 1978; Sieferman-Harms & ninnemann, 1979). Lien & San Pietro (1979) observed a cation stimulation of $P700^+$ reduction by plastocyanin when isolated PSI particles were examined. This stimulation was later found to be the result of lowering the K_m for plastocyanin binding (Davis et al., 1980). Therefore, salts increase the accessibility of plastocyanin to P700 when the isolated components are recombined.

Recently, Burkey & Gross (1981) studied the electrostatic interaction between spinach plastocyanin and isolated PSI particles by altering the net charge of PSI through chemical modification. The modification reaction involved reacting the PSI complex with a water-soluble carbodiimide in the presence of ethylenediamine (see eq 1)

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

(Means & Feeney, 1971). The reaction produced a large number of modified carboxyl groups on PSI which resulted in a positively charged PSI complex with the P700 reaction center intact. The modified PSI was capable of high rates of $P700^+$ reduction by plastocyanin in the absence of salts. Salts were no longer required because the negatively charged plastocyanin was electrostatically attracted to the positively charged modified PSI. This demonstrated that protein charge

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[1] Abbreviations used: chl, chlorophyll; PSI, photosystem I particles; P700, reaction center chlorophyll of photosystem I which absorbs maximally at 700 nm; $P700^+$, oxidized P700; $NaDodSO_4$, sodium dodecyl sulfate; Temed, *N,N,N′,N′*-tetramethylethylenediamine; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; TPCK, tosylphenylalanine chloromethyl ketone; CM, carboxymethyl.

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regulates the interaction of these two components. To examine this electrostatic interaction further, we have chemically modified spinach plastocyanin by using the reaction described in eq 1 and examined the electrostatic interaction of the modified plastocyanin with the native PSI complex.

Experimental Procedures

Materials. Ethylenediamine dihydrochloride, Sephadex G-25, and Sephadex G-10 were obtained from Sigma Chemical Co. 1-Ethyl-3-(3-dimethylaminopropyl)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 dihydrochloride was obtained from New England Nuclear. All other chemicals were reagent grade. Double-distilled deionized water was used in all experiments.

Preparation 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 a single peak by both Sephadex G-75 gel filtration and DEAE-cellulose ion-exchange chromatography using linear NaCl gradients. 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) with two exceptions. First, while on the hydroxylapatite column, the PSI was washed with 1% Triton X-100 and 50 mM Tris-HCl, pH 8.2, until the eluate was colorless, which was a more extensive washing than previously described. Second, the PSI was removed from the hydroxylapatite column with 300 mM sodium phosphate, pH 7.0, containing 0.05% Triton X-100. Chlorophyll concentrations were determined according to the method of Arnon (1949). The final PSI preparations had chl/P700 ratios of 100–150 and chl *a*/*b* ratios of 6–8.

Chemical Modification of Plastocyanin. The plastocyanin was dialyzed against 50 mM sodium borate, pH 8.0, and 50 mM NaCl to remove Tris buffer present from the isolation. Ultrafiltration was used to concentrate the plastocyanin to a concentration of 0.3–1.0 mM. Because the kinetic and redox properties of the modified plastocyanin did not depend on whether the protein was modified in the oxidized or reduced form, no attempt was made to standardize the redox state of plastocyanin before modification. The plastocyanin was then diluted with a known volume of a stock solution of ethylenediamine, pH 6.0, to give a final concentration of 0.2 M ethylenediamine. The pH of this mixture was adjusted to 6.0 ± 0.1 with 0.1 M NaOH or 0.1 M HCl as required. Solid 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was added to a final concentration of 50 mM, and the reaction was allowed to proceed for 30 min at room temperature. The reaction was stopped by passing the reaction mixture through a Sephadex G-25 column equilibrated in 50 mM sodium borate, pH 8.2, to remove unreacted ethylenediamine and carbodiimide from the modified plastocyanin. Control plastocyanin was treated identically except that the carbodiimide was not added. This procedure minimized exposure of plastocyanin to 0.2 M ethylenediamine which was found to remove the copper from the protein over several hours (i.e., if ethylenediamine was dialyzed into the plastocyanin solution before modification).

For the labeling experiments, 5–20 μCi of [1,2-^{14}C]-ethylenediamine dihydrochloride (3.5 mCi/mmol) was added to the reaction mixture containing cold ethylenediamine, before addition of carbodiimide. During the reaction, aliquots of the reaction mixture were removed and used to determine the specific activity (dpm/μmol of ethylenediamine) of the mixture. After elution of the plastocyanin from the Sephadex G-25 column, known amounts of plastocyanin were placed in liquid scintillation vials in a total aqueous volume of 1 mL. Scintillation cocktail (10 mL) (Patterson & Green, 1965) was added to each vial. After mixing, the vials were placed in a Beckman LS-320 liquid scintillation counter to measure levels of radioactivity, after which time the moles of ethylenediamine incorporated per mole of plastocyanin were determined.

Kinetics of P700$^+$ Reduction. The kinetics of P700$^+$ reduction were determined by using an Aminco DW-2a spectrophotometer according to the method of Gross (1979). The reaction mixture contained PSI (10 μg of chl/mL), 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 5 mM $MgCl_2$. The initial rate of P700$^+$ reduction was measured after each addition, and the background ascorbate rate was subtracted from the plastocyanin rate (Burkey & Gross, 1981). Least-squares analyses were used to analyze the double-reciprocal plots of the data, and the errors are reported to within one standard deviation.

Polyacrylamide Gel Electrophoresis. Discontinuous buffer $NaDodSO_4$–polyacrylamide gel electrophoresis was performed by a method adopted from Kirchanski & Park (1976). Denatured samples containing 10 μg of plastocyanin were applied to the gels. After electrophoresis, gels were removed from their tubes, fixed for 12 h in 50% methanol/10% acetic acid (v/v), followed by two 12-h incubations in 20% methanol/7% acetic acid to remove $NaDodSO_4$ from the gels. The gels were stained and destained according to Holbrook & Leaver (1976).

Discontinuous buffer polyacrylamide gel electrophoresis was performed by adopting the buffer system described by Kirchanski & Park (1976) to the method of Brewer (1974). The 1-cm stacking gel (2.5% w/v acrylamide) contained 0.06 M Tris-HCl, pH 6.1, 0.058% Temed, and 0.03% ammonium persulfate. The 8-cm resolving gel (7.5% w/v acrylamide) contained 0.375 M Tris-HCl, pH 9.8, 0.058% Temed, and 0.07% ammonium persulfate. The electrode buffer was 0.03 M glycine/0.005 M Tris (pH 8.3). The protein samples were dialyzed against the electrode buffer, and solid sucrose was added to a concentration of 10% (w/v) before electrophoresis. After electrophoresis, the gels were stained and destained according to Holbrook & Leaver (1976).

Measurement of Oxidation–Reduction Midpoint Potential. The redox midpoint potential of control and modified plastocyanin was measured as described by Davis & San Pietro (1979).

Tryptic Digestion and Peptide Mapping of Modified Plastocyanin. Modified plastocyanin labeled with [^{14}C]-ethylenediamine was digested with trypsin in the presence of 2 M urea by a procedure adapted from the method of Smyth (1967). Modified plastocyanin in 0.01 M NH_4HCO_3 was lyophillized to a powder and denatured in 8 M urea. Ammonium bicarbonate (pH 8.0) was added to give final concentrations of 1% (w/v) plastocyanin, 50 mM NH_4HCO_3, and 2 M urea. TPCK-trypsin was added in a small volume (0.02 mL) of 0.001 M HCl to the stirred solution and the hydrolysis allowed to proceed at room temperature for 4 h. After 4 h a second aliquot of TPCK-trypsin was added which gave a total trypsin concentration of 0.01% (w/v), and the hydrolysis was continued for 16 h. Urea was removed from the peptides by gel filtration on Sephadex G-10 in the presence of 0.01 M NH_4HCO_3 and the peptide elution monitored at 225 nm.

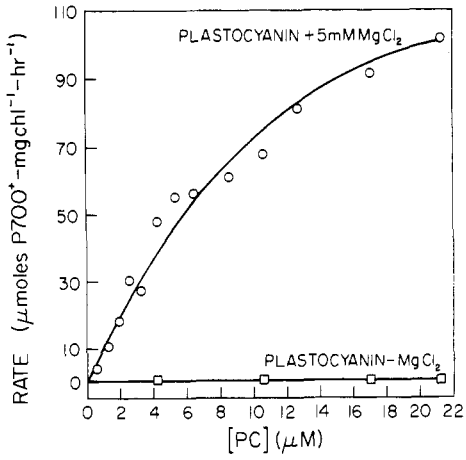

FIGURE 1: Effect of $MgCl_2$ on electron donation by plastocyanin to $P700^+$. The initial rate of $P700^+$ reduction was measured as described under Experimental Procedures.

Table I: Effect of $MgCl_2$ and Chemical Modification on Plastocyanin Donation to $P700^+$ [a]

	control plastocyanin		modified plastocyanin	
	$-MgCl_2$	+5 mM $MgCl_2$	$-MgCl_2$	+5 mM $MgCl_2$
K_m [b]	d	36.5 ± 2.0	2.1 ± 0.08	5.7 ± 0.3
V_{max} [c]	d	176 ± 65	153 ± 38	100 ± 52

[a] See Experimental Procedures for assay conditions. [b] μM. [c] μmol of $P700^+$ (mg of Chl)$^{-1}$ h^{-1}. [d] No reaction.

The lyophilized peptides were redissolved in water and then separated by using the two-dimensional peptide mapping system of Bennett (1967). Descending chromatography in the first dimension was performed by using three repetitive solvent elutions to achieve better separation. Electrophoresis in the second dimension was performed at 10 °C for 1.5 h at a potential of 3200 V. The peptides were located by lightly spraying the chromatogram with a ninhydrin solution (Bennett, 1967).

The peptides were eluted from the paper with 0.001 M HCl and then hydrolyzed under vacuum at 110 °C for 24 h in the presence of 6 N constant boiling HCl and 0.2% phenol. Hydrolysates were analyzed for amino acid content by using a Beckman Model 119 CL analyzer and for [^{14}C]ethylenediamine content by the scintillation counting system described above.

Results and Discussion

Kinetics of $P700^+$ Reduction by Plastocyanin. The reduction of $P700^+$ in PSI particles by isolated plastocyanin required the presence of divalent cations. Figure 1 shows that high rates of $P700^+$ reduction were observed in the presence of $MgCl_2$ while the rate in the absence of this salt was negligible. Salt stimulation of $P700^+$ reduction by plastocyanin has been previously observed for isolated PSI particles (Lien & San Pietro, 1979) and broken chloroplast membranes (Tamura et al., 1980; Haehnel et al., 1980). This demonstrates the existence of electrostatic regulation of the interaction between plastocyanin and $P700^+$.

Chemically modified plastocyanin no longer required Mg^{2+} for electron donation to $P700^+$. Analysis of the initial rate of $P700^+$ reduction by Michealis–Menton kinetics [see Gross (1979)] was used to compare the effects of Mg^{2+} and chemical modification on plastocyanin electron donation to $P700^+$ (see Table I). Control plastocyanin required the presence of Mg^{2+} to donate electrons to $P700^+$. Chemical modification of plastocyanin affected the binding (K_m) of plastocyanin to PSI particles and not the rate of electron transfer (V_{max}) from reduced plastocyanin to $P700^+$. Modified plastocyanin had a much lower K_m than control plastocyanin which indicated that replacing negative charges on the protein with positive charges was much more effective in allowing plastocyanin to interact with PSI than adding Mg^{2+} ions alone. Adding Mg^{2+} to modified plastocyanin caused an increase in the K_m which indicated an inhibition of binding. However, this Mg^{2+} effect on modified plastocyanin was variable in that some preparations showed little or no inhibition. The reason for this variability is unknown at present. Chemically changing the protein charge on plastocyanin in these experiments or on PSI particles as described elsewhere (Burkey & Gross, 1981) substitutes completely for cations in facilitating the plastocyanin–PSI interaction.

Table II: Effect of Chemical Modification on the Oxidation–Reduction Potential of Plastocyanin

	midpoint potential (mV)	
	–NaCl	100 mM NaCl
control plastocyanin	383	380
modified plastocyanin	423	401

Other investigators have studied the plastocyanin–PSI interaction by examining plastocyanin and plastocyanin analogues from several sources, which have different charges. Many algae have replaced plastocyanin with a small *c*-type cytochrome (*c*-533) which has an isoelectric point that can vary from acidic to basic depending on the source. In addition, the alga *Anabaena variabilis* produces a very basic form of plastocyanin. Davis et al. (1980) used plastocyanin and cytochrome *c*-553 from various sources as electron donors to $P700^+$ in the PSI particles from spinach. They concluded that the donor was able to interact with $P700^+$ more effectively as the charge on the donor became more positive. They also observed that $MgCl_2$ increased the binding of donors with isoelectric points below 5.0 while inhibiting the binding of donors with isoelectric points above 5.0. Inhibition by $MgCl_2$ was observed in some preparations of chemically modified plastocyanin. All of the above experiments suggest an electrostatic regulation of the plastocyanin–PSI interaction. Neutral or basic donors bind effectively to the negatively charged PSI complex. Acidic donors such as spinach plastocyanin require cations to screen negative charges on the two molecules in order to facilitate their interaction.

Oxidation–Reduction Potential of Plastocyanin. The oxidation–reduction midpoint potential of plastocyanin was found to be altered by the modification process (see Table II). A midpoint potential of ~380 mV was found for control plastocyanin which was close to the value of 370 mV previously reported for spinach plastocyanin (Boulter et al., 1977). Modification of plastocyanin resulted in a 40-mV increase in the midpoint potential. One possible explanation for this change in midpoint potential involves differences in the affinity of modified plastocyanin for ferricyanide compared to that for ferrocyanide [see Dutton & Wilson (1974) for a discussion of the effects of specific interactions on redox potentials]. This phenomenon should be dependent on the total concentration of the redox mediators. However, the redox potential of modified plastocyanin was identical when the measurement was performed under conditions where the ferrocyanide plus ferricyanide concentration was held constant at either 0.33 or 1.0 mM (data not shown). Therefore, the positive increase in the redox potential indicates a stabilization of the reduced Cu(I) state of plastocyanin compared to the oxidized Cu(II)

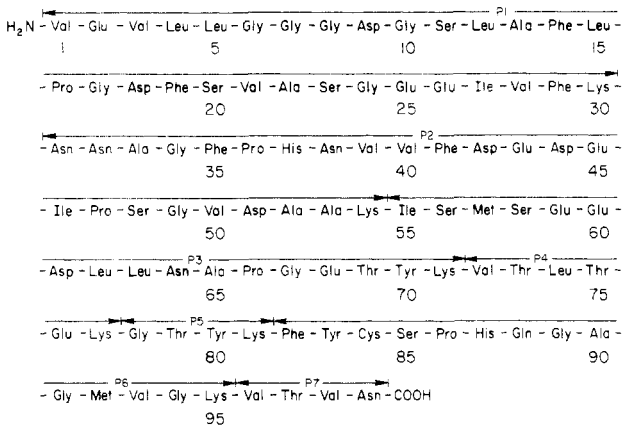

FIGURE 2: Primary structure of spinach plastocyanin [reprinted from Scawen et al. (1975)]. The peptides produced by trypsin cleavage of plastocyanin are shown.

state. Brill et al. (1964) suggested that the steric constraints of a protein were not likely to provide a very flexible geometry of ligands for binding copper. This steric constraint would tend to stabilize the Cu(I) ion because it has less rigid stereochemical demands (Brill et al., 1964). The coordination geometry of poplar plastocyanin was found to be distorted from the normal tetragonal or planar geometry found for Cu(II) complexes of low molecular weight (Colman et al., 1978). The stabilization of Cu(I) in modified spinach plastocyanin may be due to conformational alterations brought about by incorporation of ethylenediamine molecules near the copper site, which altered the ligand geometry. The incorporated ethylenediamine molecules not only change the local ionic environment but would also create a modified amino acid residue with a more bulky side chain. These changes could produce steric strain in the copper environment which would stabilize the Cu(I). A portion of this strain must be the result of some type of ionic phenomenon because reduction of ionic interactions by adding NaCl lowered the redox potential of modified plastocyanin in the direction of control plastocyanin by ~22 mV (see Table II).

Effect of Chemical Modification on the Plastocyanin Molecule. Our chemical modification procedure did not alter the oxidized absorption spectrum (400–825 nm) of plastocyanin (data not shown). $NaDodSO_4$–polyacrylamide gel electrophoresis was used to show that both the modified and control plastocyanins have molecular weights of 11 000 daltons. Therefore, no cross-linking of polypeptide chains occurred due to covalent attachment of carboxyl groups from two plastocyanin molecules through one molecule of ethylenediamine.

[^{14}C]Ethylenediamine was used to determine the number of modified plastocyanin carboxyl groups. The results of four labeling experiments showed that 4.3 ± 0.7 molecules of ethylenediamine were incorporated per plastocyanin molecule. Including the carboxyl terminus of the protein, spinach plastocyanin contains 16 carboxyl groups (see Figure 2). Therefore, the modification of an average of four of these groups resulted in large alterations both in the ability of plastocyanin to donate electrons to $P700^+$ and in the midpoint potential of the molecule.

Identification of Labeled Plastocyanin Peptides. Spinach plastocyanin contains six lysine residues and no arginine residues. Because trypsin hydrolyzes peptide bonds on the carboxyl side of lysine and arginine, this proteolytic enzyme would be expected to cleave plastocyanin into the seven peptides shown in Figure 2. Peptide P2 was identified in two spots on the peptide map, which contained 1.3 and 2.1 mol of ethylenediamine/mol of peptide, respectively. Peptide P3 was also found in duplicate spots which contained 1.9 and 1.2 mol of ethylenediamine/mol of peptide. Peptide P4, which contains only one glutamic acid residue, was found as both an unlabeled peptide and as a labeled peptide containing 0.8 mol of ethylenediamine/mol of peptide. This separation of identical peptides was the result of differences in the incorporation of ethylenediamine into various plastocyanin molecules. One large spot in the peptide map was not resolved because the amino acid content did not correlate with one of the plastocyanin peptides. This spot contained [^{14}C]ethylenediamine and must consist of a mixture of components. One component must be peptide P1 because this was the only plastocyanin peptide not found in any of the spots on the peptide map. Other unknown components might be segments of plastocyanin that were not completely digested by trypsin and therefore contained a combination of two or more of the peptides shown in Figure 2.

The fact that several of the individual peptides contained varying amounts of ethylenediamine would suggest that the chemically modified plastocyanin was really a heterogeneous mixture of plastocyanin molecules modified to different extents. This was confirmed by using polyacrylamide gel electrophoresis which separated the plastocyanin molecules on the basis of charge. Native plastocyanin migrated with a relative mobility[2] of 0.85. The modified plastocyanin contained four major bands with relative mobilities of 0.21, 0.34, 0.47, and 0.62. Two conclusions can be made from these results. First, all of the plastocyanin was chemically modified to some extent because the modified plastocyanin contained no protein which migrated with native plastocyanin. Second, the modified plastocyanin was a mixture of four major populations of plastocyanin which have different charges due to different extents of modification. All of the modified plastocyanin proteins migrated with smaller relative mobilities than the control. This indicated that modified plastocyanin was less negatively charged than the control plastocyanin, which is an extremely acidic protein (p*I* = 3.0; Davis et al., 1980). This would be expected since the chemical modification reaction replaces negatively charged carboxyl groups with positively charged amino groups. However, at neutral pH the net charge of the modified plastocyanin proteins is probably still slightly negative or neutral for two reasons. First, the replacement of an average of four carboxyl groups on plastocyanin with amino groups by chemical modification results in a protein with eleven amino groups and twelve carboxyl groups. This modified protein would have an approximately neutral charge at pH 7. Second, the modified plastocyanin did not bind to CM-Sephadex which is a cation-exchange resin that binds the positively charged plastocyanin from *A. variabilis* (Lightbody & Krogmann, 1967).

Physiological Role of Modified Plastocyanin Amino Acids. A high degree of similarity exists between the sequences of plastocyanin isolated from a variety of sources (Boulter et al., 1977). High-resolution NMR studies indicate that the tertiary structures of many plastocyanins, including those from spinach and *A. variabilis*, are very similar (Freeman et al., 1977; Markley et al., 1975). The X-ray diffraction structure has been determined for poplar plastocyanin (Colman et al., 1978). Davis et al. (1980) pointed out that if the amino acid sequence of spinach plastocyanin is projected onto the structure of poplar plastocyanin, there is a ring of negatively charged residues around the middle of the molecule. They also pointed out that if the sequence of *A. variabilis* is projected onto the structure

[2] The relative mobility is defined as the distance a protein has migrated into a gel divided by the distance that the bromophenol blue dye has migrated into the gel.

of poplar plastocyanin, the ring of negative charges is missing. Because they observed a 100-fold decrease in K_m for *A. variabilis* plastocyanin compared to that of spinach plastocyanin, they suggested that the differences in the interaction of these donors with PSI were due to changes in the net charge of this ring. Amino acids 42–45, 59–61, and 68 (see Figure 2) of spinach plastocyanin are acidic residues located in this ring which are highly conserved in higher plants (Boulter et al., 1977). These sequences are located within tryptic peptides P2 and P3 which were labeled with [^{14}C]ethylenediamine in our preparation of modified plastocyanin. Therefore, using chemical modification to replace a portion of the negative carboxyl groups in this ring with positively charged amino groups allowed modified plastocyanin to donate electrons to $P700^+$ in the absence of cations as well as altering the redox potential of the molecule.

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