Biochimica et Biophysica Acta, 1016 (1990) 107–114
Elsevier

BBABIO 43176

Chemical modification of spinach plastocyanin using 4-chloro-3,5-dinitrobenzoic acid: characterization of four singly-modified forms

Elizabeth L. Gross, April Curtiss, Stewart R. Durell and Dorothy White

The Department of Biochemistry, The Ohio State University, Columbus, OH (U.S.A.)

(Received 11 July 1989)
(Revised manuscript received 21 November 1989)

Key words: Plastocyanin; Chemical modification; Cytochrome *f*; (Spinach)

Chemical modification of plastocyanin was carried out using 4-chloro-3,5-dinitrobenzoic acid, which has the effect of replacing positive charges on amino groups with negatively charged carboxyl groups. Four singly-modified forms were obtained which were separated using anion exchange FPLC. The four forms were modified at the N-terminal valine and at lysines 54, 71 and 77. The rates of reaction with mammalian cytochrome *c* were increased for all four modified plastocyanins. In contrast, the rates of reaction with cytochrome *f* were inhibited for the forms modified at residues 1, 54 and 77, whereas no effect was observed for the form modified at residue 71. Modification had no effect on either the midpoint redox potential or the reaction with $K_3Fe(CN)_6$. These results are consistent with a model in which charged residues on plastocyanin located at or near the binding site for cytochrome *f* recognize the positively-charged binding site on cytochrome *f*. In contrast, charged residues located at points on plastocyanin distant from the cytochrome *f* binding site recognize the net negative charge on the cytochrome *f* molecule. Based on these considerations, Glu-68 may be within the interaction sphere of cytochrome *f*, suggesting that cytochrome *f* may donate electrons to plastocyanin at either Tyr-83 or His-87.

Introduction

Plastocyanin is a 10.5 kDa blue copper protein which functions in the chloroplast electron transport chain [1–3]. The crystal structures have been determined for both oxidized [4,5] and reduced [6] poplar plastocyanin. The copper atom is coordinated to two histidine residues (37 and 87), a methionine and a cysteine residue.

NMR studies have shown that there are two potential binding sites on plastocyanin for redox partners [7,8]. One site is located at His-87 at the top of the plastocyanin molecule as shown in Fig. 1. We have designated this location as Site 1. Negatively charged redox agents such as ferricyanide are thought to interact with plastocyanin at this site. The second site is in the vicinity of Tyr-83 (shown on the right-hand side of Fig. 1a). We have designated this location as Site 2. Tyr-83 is surrounded by a patch of negatively charged reisdues (42–45 and 59–61), which are highly conserved in higher plant plastocyanins [1]. Positively charged redox agents such as $Co(phen)_3^{2+,3+}$ are thought to interact with plastocyanin at this site [3]. The existence of two potential binding sites raises the question as to which of the site(s) are used by plastocyanin's physiological reaction partners, cytochrome *f* and P700.

We have used chemical modification techniques to answer this question. In earlier studies [9–11], carboxyl groups on plastocyanin were modified using ethylenediamine plus a water-soluble carbodiimide. This reaction replaces a negatively charged carboxyl group with a positively charged amino group. Three singly modified forms were obtained which were modified at residues 42–45 and 59–61 and 68, respectively. Residues 42–45 and 59–61 are part of the conserved negative patch and, hence, are closer to binding Site 2 than is residue 68. In contrast, residue 68 and residues 59–61 are closer to His-87 and Site 1 than are residues 42–45.

Greater inhibition of cytochrome *f* oxidation was observed for plastocyanins modified at residues 42–45 and 59–61 than for plastocyanin modified at residue 68,

Abbreviations: CDNB, 4-chloro-3,5-dinitrobenzoic acid; DEAE, diethylaminoethyl; DPPC, diphenylcarbonyl chloride; EDA, ethylenediamine; FPLC, fast protein liquid chromatography; HPLC, high-pressure liquid chromatography; P700, reaction center chlorophyll of Photosystem I; TFA: trifluoroacetic acid.

Correspondence: E.L. Gross, Department of Biochemistry, The Ohio State University, Columbus, OH 43210, U.S.A.

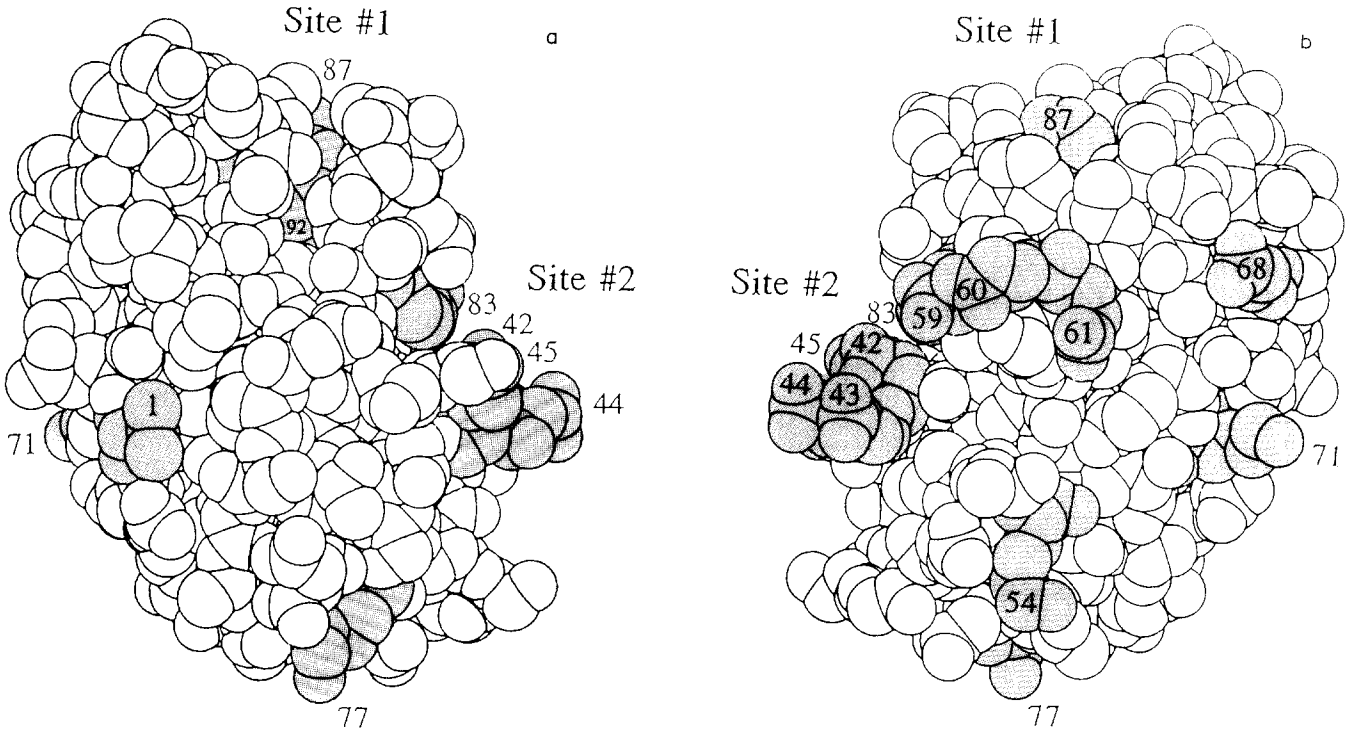
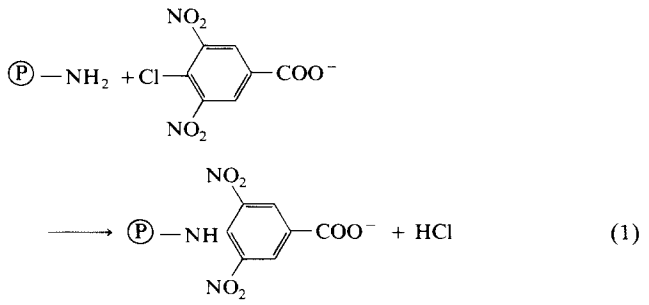


Fig. 1. Molecular graphics representation of spinach plastocyanin. X-ray coordinates of poplar plastocyanin [4] were obtained from the Brookhaven Protein Data Bank [19]. 22 amino-acid substitutions were made to convert poplar to spinach plastocyanin. The plastocyanin molecule is displayed using the Quanta program from Polygen Inc. which is available to us courtesy of the Ohio Supercomputer Center. The program was run on a Control Data-Silicon Graphics 4D.70 GT graphics workstation. The ligands to the copper center, Tyr-83, the anion residues at residues 42–45, 59–61, 68, the amino terminus and lysines 54, 71 and 77 are shown. The two proposed binding sites on plastocyanin for reaction partners are also shown.

indicating that cytochrome *f* interacts with plastocyanin at Site 2. These results confirm those of Beoku-Betts and Sykes [12]. In contrast, sitmulation of the reaction of plastocyanin with P700 was observed for modifications at residues 68 and 59–61 with no effect for the modification at residues 42–45. These results indicate that Photosystem I interacts with plastocyanin at Site 1.

These studies were incomplete in that only three singly modified forms were obtained, all of which were located close to one of the two potential binding sites. To continue these studies, it was necessary to obtain a greater number of chemically modified forms of plastocyanin. Therefore, we chose to modify the lysine residues of plastocyanin using, CDNB which replaces a positively charged amino group with a negatively charged carboxyl group according to Eqn. 1.



(1)

CDNB modification of lysine residues has been reported for both mammalian cytochrome *c* [13,14] and chloroplast cytochrome *f* [15]. Reaction with CDNB increases the net negative charge on the plastocyanin molecule. Thus, the change in net charge is in a direction opposite to that observed for the carboxyl group modifications. This allows an increased range of net charges for a study of the effect of molecular charge on the properties of plastocyanin.

Plastocyanin contains six lysine residues. We have modified three of the lysine residues (54, 71 and 77) in addition to the α-amino group of the N-terminal value residue. Our results are presented below.

Methods

Isolation and chemical modificaiton of plastocyanin. Plastocyanin was isolated from spinach chloroplasts using the method of Davis and San Pietro [16] without further purification. CDNB was recrystallized. 0.4 ml of CDNB (5 mg/ml) were added to 3.0 ml of reduced plastocyanin (0.4–0.6 mM) in 0.3 M carbonate buffer (pH 9.0). This reaction mixture was allowed to stir slowly at room temperature for 24 h with a second aliquot of CDNB added after 7 h of incubation. The reaction was stopped by chromatography on a Bio-Gel

P-10 column equilibrated with 25 mM Tris-HCl (pH 8.2) after which the plastocyanin was concentrated using an Amicon cell with a YM-2 membrane.

The extent of CDNB incorporation was determined by measuring the absorption of the CDNB peak at 422 nm using an extinction coefficient of 6.9 $mM^{-1} \cdot cm^{-1}$ [13]. This value was compared to the concentration of plastocyanin which was determined by measuring the extinction at 597 nm after addition of excess potassium ferricyanide using an extinction coefficient of 4.9 $mM^{-1} \cdot cm^{-1}$ [16]. The ratio of these two values represents the extent of incorporation. An average of 1 mol of CDNB was incorporated per mol of plastocyanin with a recovery of plastocyanin of approx. 90%.

Separation of modified forms. The mixture of modified plastocyanins was separated using a Pharmacia FPLC equipped with a Mono Q HR 5/5 anion-exchange column. The initial (A) buffer was 25 mM Tris-HCl (pH 8.2). The gradient (B) buffer was 25 mM Tris-HCl (pH 8.2)/1.0 M NaCl. The buffers were filtered through a 0.2 μm GVWP Millipore filter and degassed prior to use. The mixture of modified forms eluted between 15 and 60% of the B buffer. Fractions 2, 3, 4 and 6 (Fig. 2) were further purified by a second FPLC step as follows. Each fraction was divided into two parts: a leading half from the beginning of the peak to the point of maximum absorbance and a trailing half which was taken from the absorbance maximum to the end of the peak. Each of these fractions was rechromatographed using the same conditions as for the first FPLC separation. It was found that, in each case, the trailing halves of each peak were pure as determined by HPLC of the tryptic peptides (see below). In contrast, the leading halves were contaminated with the previous fraction. Therefore, the repurified trailing portions of the peaks 2, 3, 4 and 6 were used for the studies described below. The extent of modification for each fraction was determined as described above.

Determination of the location of the modifications. The location of the CDNB moiety was determined for peaks F2, F3, F4 and F6. Each sample was denatured by immersion in a boiling water bath for 5 min. It was then digested with 1% (w/v) DPCC-treated trypsin in 0.001 M HCl. The digest was filtered through a 0.22 μm Millipore GVWP filter. The tryptic peptides were separated using reverse-phase chromatography on a Varian HPLC with a Brownlee RP-8 column equilibrated with 0.1% TFA in water. The peptides were eluted using a 1–99% gradient of 9 : 1 acetonitrile/water over 28 min. The solvents were filtered through a 0.2 μm filter (Millipore GVWP for the TFA/H_2O and Millipore GVHP for the acetonitrile) and degassed prior to use.

Modification of lysine residues results in the loss of a tryptic cleavage site. This is manifest on the HPLC trace by the loss of two tryptic peptides and the appearance of a new tryptic peptide at another location. This new peptide is a ditryptic peptide. Only one new peptide was observed for F2, F3, F4 and F6. In each case, the new peptide was subject to amino-acid analysis and the determination of the first five amino acids in the sequence.

The amino-acid analysis was performed at the Ohio State University Biochemical Instrumentation Center using a Waters Pico-Tag Amino Acid Analysis System. The peptide sequence analysis was also performed by the Biochemical Instrument Center using an Applied Biosystems Model 470A/900A. The results show that each peak has a single modification in a different localtion on the plastocyanin molecule.

Molecular mass determination of P2x. Gel filtration using Bio-Gel P-4 was used to determine the molecular mass of tryptic peptide P2x, the new tryptic peptide obtained from fraction F2. Bio-Gel P-4 excludes molecules with a size greater than 4000 Da. DNP-glycine (241 Da) and cytochrome *c* (12 kDa) were used to determine the internal and excluded volumes of the column, respectively. Authentic tryptic peptide 1 (3500 Da) was used for comparison purposes.

Isolation of cytochrome f and cytochrome c. Cytochrome *f* was isolated from turnip leaves using the procedure of Molnar et al. [17], through the DEAE purification step. This resulted in a purity index of 0.7. The cytochrome *c* from horse heart (Type VI) was used without further purification and was dissolved in 25 mM Tris-succinate buffer (pH 7.0).

Kinetics of the interaction of the CDNB-modified plastocyanins with cytochrome f, cytochrome c and potassium ferricyanide. The kinetics of the interaction of the CDNB-modified plastocyanins with cytochrome *f*, mammalian cytochrome *c* and potassium ferricyanide were measured using the stopped-flow attachment of the Aminco-DW2a spectrophotometer. In each case, second-order rate constants were determined using pseudo-first-order conditions. The reaction of oxidized plastocyanin with reduced cytochrome *f* was monitored at 422 nm using a slit width of 6 nm. The cytochrome *f* and plastocyanin concentrations were 0.5 and 2.5 μM, respectively. These conditions were sufficient to drive the reaction nearly to completion. The reactions were carried out in 25 mM Tris-succinate buffer (pH 7.0), with sufficient NaCl added to attain an ionic strength of 0.300 M. The reaction temperature was 20 °C. The kinetics were recorded on a Bascomb-Turner Model 2110 electronic recorder with a data acquisition rate of one data point per millisecond. The calculations were performed using a Kaypro computer using Hyperplot. The conditions for the oxidation of cytochrome *c* were identical to those used for cytochrome *f*. Reaction of the CDNB-modified plastocyanins with potassium ferricyanide was determined by monitoring the oxidation of reduced plastocyanin at 597 nm. The conditions were the same as described above except that the plasto-

cyanin and ferricyanide concentrations were 5.0 and 50 μM, respectively.

Cytochrome *f* was reduced by reaction with an excess of sodium ascorbate which was removed by gel filtration using a Bio-Gel P-10 column. The reduced state was maintained provided the cytochrome *f* sample was stored at 4° C.

Cytochrome *c* was reduced by addition of excess sodium ascorbate which was removed by dialysis against 25 mM Tris-succinate buffer (pH 7.0) for 1 to 2 h under nitrogen. The reduced cytochrome *c* was kept under nitrogen to maintain the reduced state.

Plastocyanin was oxidized or reduced by addition of an excess of potassium ferricyanide or sodium ascorbate, respectively. In both cases, the excess reagents were removed by gel-filtration as described above.

Determination of the midpoint redox potentials of the CDNB-modified plastocyanins and comparison with calculated values. Midpoint redox potentials were determined as described by Burkey and Gross [10]. The CDNB – modified plastocyanins (at 5 μM) were incubated in 25 mM Tris-succinate buffer (pH 7.0) plus sufficient NaCl to attain an ionic strength of 0.3 M and various ratios of ferricyanide to ferrocyanide.

The experimental values for the midpoint redox potentials of the modified plastocyanins were compared with those calculated using Honig's Del-Phi program (Klapper et al. [18]). The atomic coordinates of plastocyanin were provided by the Brookhaven Protein Data Bank [19]. Program variables include, the dielectric constant inside and outside the protein, the ionic strength and the size of the Stern layer. We have also used this program to calculate the effects of the EDA modifications of plastocyanin on the midpoint redox potentials [20].

Materials. Bio-Gel P-10, P-4 were obtained from Bio-Rad Laboratories. CDNB, trifluoroacetic acid, Tris, DPCC-treated trypsin, and cytochrome *c* were obtained from Sigma Chemical Co. Potassium ferricyanide, HPLC grade water and acetonitrile came from J.T. Baker Chemicals. All other chemicals were of reagent grade.

Results

Separation of CDNB-modified forms of plastocyanin

The FPLC separation of the CDNB-modified forms of plastocyanin is shown in Fig. 2. Nine peaks were obtained which were designated F1 to F9. Peak F1 was found to consist of unmodified plastocyanin. Peaks F2, F3, F4 and F6 were all singly modified forms. The extent of incorporation is shown in Table I. Peaks F5, F7, F8 and F9 were present in very small quantities and represent doubly or multiply modified forms.

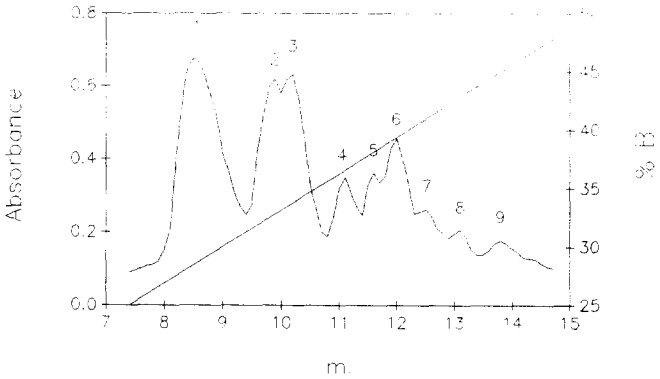


Fig. 2. FPLC separation of CDNB-modified forms of PC. Chemical modification and separation of the CDNB-modified forms of plastocyanin were carried out as described in the Methods section.

Determination of the location of the CDNB modification sites

Spinach plastocyanin contains seven tryptic cleavage sites as shown in Fig. 3. The HPLC trace of the unmodified fraction F1 is shown in Fig. 4a. The identification of the various peaks is also shown. The elution pattern for F1 is identical to that observed for control plastocyanin (see also Anderson et al. [11]). The HPLC elution profile for fraction F3 is shown in Fig. 4b. It can be seen that the tryptic peptides 3 and 4 disappeared and a new peak appeared at 22 ml. The remaining extinction in the vicinity of Peak 3 is due to a shift in baseline. This peak was designated P3x. This was the only new peptide observed in F3 confirming the purity of the fraction. The amino-acid composition of P3x is consistent with the presence of tryptic peptides 3 and 4. The first five amino acids were Ile, Ser, Met, Ser, Glu, which are the same as the first five amino-acid residues of tryptic peptide 3 (Fig. 3). These results taken together

TABLE I

Incorporation of CDNB into modified plastocyanins

The CDNB modification of plastocyanin and the amounts of CDNB incorporated were determined as described in the Methods sections. The error in the determinations of the incorporation of CDNB is ± 0.2.

Fraction	Incorporation of CDNB (mol CDNB/mol plastocyanin)	
	after first FPLC	after second FPLC
F1	0.06	0.04
F2	0.8	0.8
F3	1.1	1.0
F4	1.5	1.2
F5	1.9	
F6	1.6	1.3
F7	2.4	
F8	3.0	
F9	3.6	

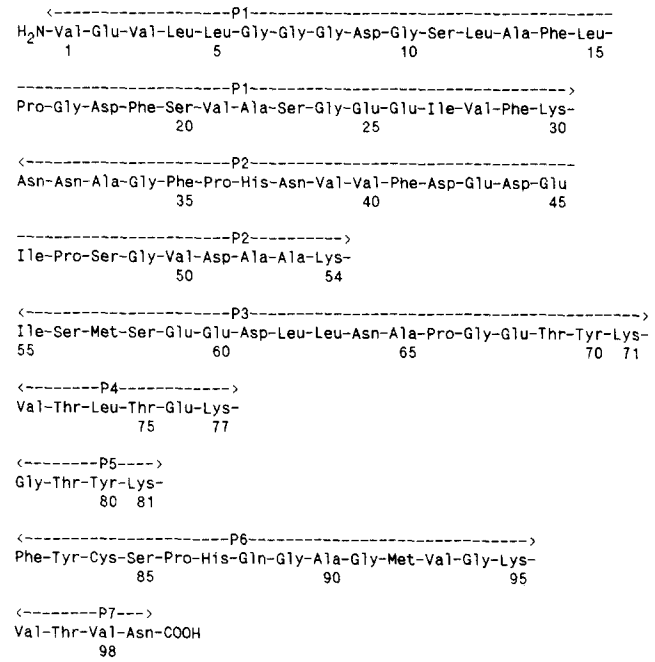


Fig. 3. Amino-acid sequence of spinach PC. The amino-acid sequence was taken from Boulter et al. [1]. The trypsin cleavage sites are shown.

indicate that F3 contains a single CDNB moiety located at Lys-71.

Peak F2 contained a new peptide designated P2x eluting just after tryptic peptide 1. The amino-acid analysis was consistent with either peptide 1 alone or peptide 1 + 2 (not shown). However, we were unable to obtain an amino-acid sequence, suggesting a blocked N-terminus. Gel filtration on Bio-Gel P4 showed that P2x eluted in the same position as authentic tryptic peptide 1. Moreover, chromatography of a mixture of P2x and authentic tryptic peptide 1 yielded only a single peak. In contrast, tryptic peptide 1 + 2 would have been excluded from the gel filtration column, since it has a molecular mass of 5400 Da. These results taken together indicate that the single CDNB moiety in F2 is located on the α-amino group of the N-terminal valine residue.

Peak F4 showed a decrease in peptides 4 and 5 and a new tryptic peptide which appeared as a shoulder of tryptic peptide 2. This new peptide was designated P4x. The sequence of the first five amino acids confirmed its identidy as peptide 4 + 5. Therefore, peak F4 is modified at lysine-77.

Peak F6 showed a decrease in peptides 2 and 3 and a new tryptic peptide which appears as a shoulder of tryptic peptide 1. This peptide was designated P6x. The amino-acid analysis indicated a composition of peptides 1, 2 and 3. The peptide sequence yielded two amino acids per cycle indication two peptides present. These were identified as the first five amino aicds of peptide 1 and peptide 2. Thus, peak F6 is modified between peptides 2 and 3 with peptide 1 as a contaminant. Since peptide 1 elutes from the HPLC as a leading shoulder to peptide F6x, this contaminant is difficult to separate. Thus, the site of the modification in F6 is located at residue 54. Please note that fraction F6 is pure. The contamination is only at the level of the HPLC separation of the tryptic peptides not at the level of the FPLC separation of the modified forms. The location of all four modification sites is shown in Fig. 1.

The kinetics of the reaction of the CDNB-plastocyanins with $K_3Fe(CN)_6$, cytochrome c and cytochrome f

The choice of reaction partners for this study was as follows. $K_3Fe(CN)_6$ was chosen because it is known to react at Site 1. In contrast, the preponderance of evidence indicates that both cytochrome *c* [21] and cytochrome *f* [11,12] interact with Site 2 on plastocyanin (however, see Rush et al. [22]). Although they interact at the same reaction site, cytochromes *c* and *f* differ from one another with respect to net charge. Cytochrome *c* has a net positive charge [24] whereas cytochrome *f* is a negatively charged molecule with a positively charged binding site [25]. Hence, different results would be expected if the interaction with plastocyanin were determined by the net charge on the cytochrome *f* molecule, rather than the charge at the site of interaction.

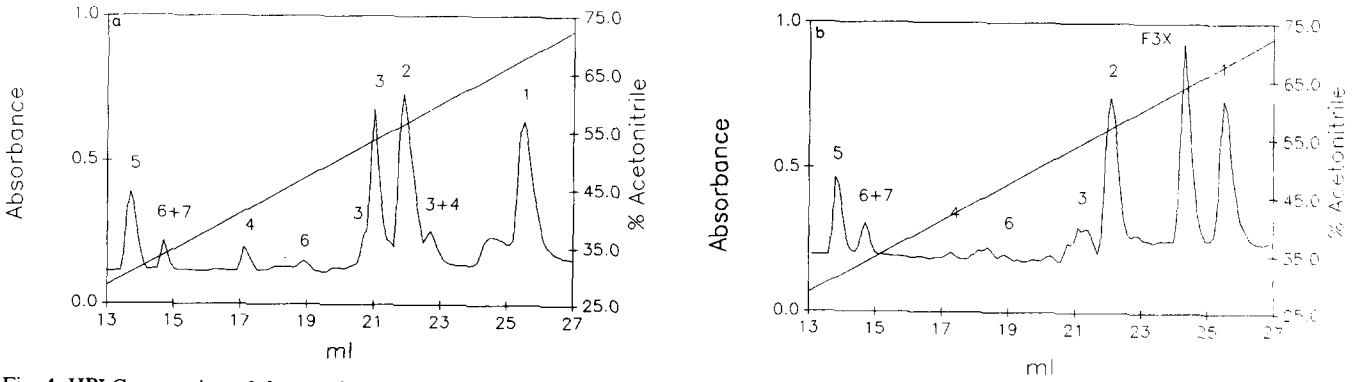


Fig. 4. HPLC separation of the tryptic peptides for Fractions 1 and 3. Tryptic digestion of the CDNB-modified plastocyanins and their separation using reversed phase HPLC were carried out as described in the methods section. (a) Fraction 1 (unmodified PC); (b) CDNB-modified fraction 3 from the FPLC.

TABLE II

Rate of reaction of CDNB-plastocyanins with $K_3Fe(CN)_6$, cytochrome c and cytochrome f

Rates of plastocyanin, cytochrome *c* and cytochrome *f* oxidation were determined as described in the Methods section. Distances to the copper center and Tyr-83 were calculated using the coordinates provided by the Brookhaven Protein Data Bank.

Fraction	Residue modified	Distance to		k ($M^{-1} \cdot s^{-1} \times 10^{-6}$)		
		Cu (Å)	Tyr-83 (Å)	$K_3Fe(CN)_6$	cyt *c*	cyt *f*
Control PC	–	–	–	0.160 ± 0.006	1.10 ± 0.04	26.0 ± 1.2
F1	–	–	–	0.176 ± 0.008	1.2 ± 0.1	31.9 ± 3.6
F2	1	16.2	23.6	0.188 ± 0.006	5.7 ± 1.1	20.0 ± 2.0
F3	71	15.0	22.1	0.176 ± 0.016	5.8 ± 0.8	28.7 ± 3.6
F4	77	30.1	25.7	0.180 ± 0.022	17.4 ± 1.9	9.5 ± 1.4
F6	54	25.0	19.1	0.174 ± 0.012	5.7 ± 0.5	20.8 ± 3.5

TABLE III

The effect of CDNB modification on the midpoint redox potential of plastocyanin

Experimental and calculated values of the midpoint redox potentials were determined as described in the Methods section. The errors for the experimental determinations is ± 3 mV.

Fraction	Residue modified	Distance to Cu (Å)	Midpoint redox potentials		
			experimental		calculated
			$E^{\circ\prime}$ (mV)	$\Delta E^{\circ\prime}_{mod\text{-}cont.}$ (mV)	$\Delta E^{\circ\prime}_{mod\text{-}cont.}$ (mV)
F1	–	–	367	–	–
F2	1	19.5	368	+1	−12
F3	71	15.0	364	−3	−4
F6	54	25.0	367	0	−1

None of the CDNB-modified plastocyanins showed any effect on the oxidation of reduced plastocyanin by $K_3Fe(CN)_6$ (Table II). In contrast, all of the modified forms showed significant stimulation of activity using cytochrome *c* as an electron donor. It is interesting that Fraction 4, modified at residue 77, showed the greatest stimulation in spite of the large distance from this location to either the copper center (30 Å) or Tyr-83 (26 Å).

The second-order rate constant observed for cytochrome *f* oxidation was $(26.0 \pm 1.2) \cdot 10^6$ $M^{-1} \cdot s^{-1}$ compared to a value of $36 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ observed by Wood [26]. However, Wood used a lower ionic strength (0.1 M compared to 0.3 M used in our experiments). We used a higher ionic strength in our experiments in order to slow the reaction to the point where we could accurately measure it under our conditions.

The results obtained for the CDNB-modified PC's were mixed. No effect was observed for Form 3 modified at Lys-71. However, partial inhibition was observed for Form 1 modified at the N-terminus and Form 6 modified at Lys-54. Severe inhibition was observed for Form 4 modified at Lys-77.

The effect of CDNB modification on the midpoint redox potentials

The effects of modification on the midpoint redox potentials are shown in Table III. In each case, the shift in midpoint redox potential was less than the error in making the measurements.

We also calculated the effect of modification using the Del-Phi program of Klapper et al. [18]. This program allows the user to vary ionic strength, dielectric constant inside and outside the protein and the thickness of the Stern layer. An ionic strength of 0.15 M * and dielectric constants of 2 and 80 inside and outside the protein, respectively, were used. The calculated results agree with the experimental results except in the case of the N-terminal valine. However, the deviation may be due to some uncertianty in the exact location of that residue [27]. In the case of the carboxyl group modifications [20], it was found that a slight movement of a charged residue away from the protein surface significantly decreased the predicted effect on the midpoint redox potential.

Discussion and Conclusions

Four singly-modified forms of plastocyanin were obtained upon reaction of plastocyanin with CDNB. The

* A change in ionic strength from 0.15 M to 0.3 M has been shown to have almost no effect on the midpoint redox potential, since the electric field is communicated to the copper center primarily through the low dielectric medium of the protein [20].

locations of each of these modifications are shown in Fig. 1. It can be seen that all four modification sites are located on the lower half of the plastocyanin molecule and are distant from either Site 1 or Site 2.

The negative results obtained for the interaction of the CDNB-modified forms of plastocyanin with ferricyanide can be explained on the basis of the large distance between the sites of modification and the copper center (15–25 Å) and the small size of the ferricyanide anion. The cytochrome *c* results reflect the increased net negative charge on the plastocyanin molecule as a result of the modifications. The increased net negative charge would be expected to increase the attraction to a positively charged electron acceptor. It is a net charge effect opposed to a site-specific effect, since the degree of stimulation of the rate of cytochrome *c* oxidation is independent of the location of the modification. In this respect, the present results differ from those previously observed for the carboxyl group modifications [11].

There are several possible explanations for the decrease in rate of cytochrome *f* oxidation observed for three of the CDNB-modified forms of plastocyanin. The first is that modification caused a negative shift in redox potential which decreased the driving force for the reaction [25]. The driving force is small due to the small difference between the midpoint redox potentials of cytochrome *f* (+360 mV) [24] and plastocyanin (+372 mV [11]). A negative-shift in potential would decrease this still further. However, no shift in midpoint potential was observed which is reasonable due to the large distance between the sites of modification and the copper center.

Steric hindrance is a second possibility due to the large size of the CDNB moiety. For this reason plastocyanin was modified using dansyl chloride (Gross and Curtiss, unpublished results). A singly modified form was obtained with the dansyl moiety located at the N-terminus. No inhibition of cytochrome *f* oxidation was observed for the dansyl modification. If steric hindrance were a problem, inhibition should have been observed, since the dansyl group is even larger than the CDNB moiety. In addition, steric hindrance would not be expected for modifications located far from either binding site.

A third possible mechanism for the modification-induced inhibition of the reaction rates is that the dipole axis has been changed interfering with the correct positioning of the plastocyanin molecule for electron transfer [22]. Calculations of the dipole moments of the CDNB-modified plastocyanins show little or no effect. Moreover, changes in the dipole axis would be expected to produce similar effects for the interaction with both cytochrome *c* and cytochrome *f*. However, Form 4, modified at Lys-77, showed a large stimulation of cytochrome *c* oxidation but severe inhibition of cytochrome *f* oxidation. These results are inconsistent with the dipole moment hypothesis.

A fourth explanation is that of charge repulsion. The rates of reaction with the modified plastocyanins would be determined by the net charge of the cytochrome *f* molecule, not the charge at the binding site. If the rates of interaction were determined by the charge at the positively charged binding site on cytochrome *f*, increasing the net negative charge on plastocyanin would be expected to increase the rate of reaction. The results obtained previously for the carboxyl group modifications [11] indicated that it was the charge at the binding site on cytochrome *f* which determined the interaction with plastocyanin, not the net charge on the cytochrome *f* molecule.

One possible reason for the difference in the results obtained for the two types of modification concerns the location of the sites of modification in the two cases. All of the amino group modifications are located far from the proposed cytochrome *f* binding site (Site 2). In this case, the net charge on both of the interaction partners may be more important than the charge at the binding site. In the case of the carboxyl group modifications, two of the sites (residues (42–45 and 59–61) are located at the binding site for cytochrome *f* where it is predicted that specific charges would have the most effect. However, inhibition of the rate of cytochrome *f* oxidation was also observed for the form of plastocyanin modified at residue 68 which is located close to the other binding site. These results could be explained as follows. The primary site of recognition for cytochrome *f* on plastocyanin may be at Site 2 but the final site of electron donation may be at Site 1 [22]. Thus, plastocyanin may initially dock at cytochrome *f* using binding Site 2 but may diffuse or rotate in such a manner as to align Site 1 with the cytochrome *f* heme prior to electron transport [28]. Alternatively, cytochrome *f* is a large molecule which may be able to react with both binding sites simultaneously. This explanation is consistent with the results of Takabe et al. [29] who found that formation of a covalent adduct between plastocyanin and cytochrome *f* altered the kinetics of the interaction of plastocyanin with ferricyanide. Further experiments are necessary in order to distinguish between these two alternatives.

An added complication is that the electron transfer process requires binding of the two proteins followed by transfer of the electron and dissociation of the complex. In principle, chemical modification could affect any of these processes. Because of the very high second-order rate constant for cytochrome *f* oxidation and the low binding constant for the cytochrome-*f*–plastocyanin complex, it is impossible to measure a K_m. However, the results reported here are consistent with binding

phenomena and experiments are being conducted to determine effects of modification on the binding constants.

Acknowledgments

This research was supported in part by Grant GM30560 from the National Institutes of Health. We wish to thank the NSF Young Scholars Program for the support of D.W. We wish to thank Jane Tolley of the Biochemical Instrumentation Center for determining the amino-acid contents and sequences for the tryptic peptides.

References

1 Boulter, D., Haslett, B.G., Peacock, D., Ramshaw, J.A.M. and Scawen, M.D. (1977) Int. Rev. Biochem. 13, 1–40.
2 Freeman, H.C. (1981) Coord. Chem. 21, 29–51.
3 Sykes, A.G. (1985) Chem. Soc. Rev. 14, 283–315.
4 Coleman, P.M., Freeman, H.C., Guss, J.M., Murata, M., Norris, V.A., Ramshaw, J.A.M. and Venkatappa, M.P. (1978) Nature 272, 319–324.
5 Guss, J.M. and Freeman, H.C. (1983) J. Mol. Biol. 169, 521–563.
6 Guss, J.M. Harrowell, P.R., Murata, M., Norris, V.A. and Freeman, H.C. (1986) J. Mol. Biol. 192, 361–387.
7 Cookson, D.J., Hayes, M.T. and Wright, P.E. (1980) Biochim. Biophys. Acta 591, 162–176.
8 Hanford, P.M., Hill, H.A.O., Lee, R.W.-K., Henderson, R.A. and Sykes, A.G. (1980) J. Inorg. Biochem. 13, 83–88.
9 Burkey, K.O. and Gross, E.L. (1981) Biochem. 20, 5495–5499.
10 Burkey, K.O. and Gross, E.L. (1982) Biochem. 21, 5886–5890.
11 Anderson, G.P., Sanderson, D.G., Lee, C.H., Durell, S., Anderson, L.B. and Gross, E.L. (1987) Biochim. Biophys. Acta 894, 386–398.
12 Beoku-Betts, D. and Sykes, A.G. (1985) Inorg. Chem. 24, 1142–1147.
13 Brautigan, D.L., Ferguson-Miller, S. and Margoliash, E. (1978) J. Biol. Chem. 253, 130–139.
14 Osheroff, N., Brautigan, D.L. and Margoliash, E. (1980) J. Biol. Chem. 255, 8245–8251.
15 Takenaka, K. and Takabe, T. (1984) J. Biochem. 96, 1813–1821.
16 Davis, D.J. and San Pietro, A. (1979) Anal. Biochem. 95, 254–259.
17 Molnar, S.A., Anderson, G.P. and Gross, E.L. (1987) Biochim. Biophys. Acta 894, 327–331.
18 Klapper, I., Hastrom, R., Fine, R., Sharp, K. and Honig, B. (1986) Proteins 1, 47–59.
19 Bernstein, F.C., Koetzle, T.F., Williams, G.J.B., Meyer, Jr., E.F., Brice, M.D., Rogers, J.R., Kennard, O., Shimanouchi, T. and Tasumi, M. (1977) J. Mol. Biol. 112, 535–542.
20 Durell, S.R., Labanowski, J.K. and Gross, E.L. (1990) Arch. Biochem. Biophys., in press.
21 King, G.C., Binstead, R.A. and Wright, P.E. (1985) Biochim. Biophys. Acta 806, 262–271.
22 Rush, J.D., Levine, F. and Koppenol, W.H. (1988) Biochem. 27, 5876–5884.
23 Margoliash, E. and Bosshard, H.R. (1983) Trends Biochem. Sci. 8, 316–320.
24 Gray, J.C. (1978) Eur. J. Biochem. 92, 133–141.
25 Tollin, G., Meyer, T.E. and Cusanovitch, M.A. (1986) Biochim. Biophys. Acta 853, 29–41.
26 Wood, P.M. (1974) Biochim. Biophys. Acta 357, 370–379.
27 Guss, J.M. and Freeman, H.C. (1983) J. Mol. Biol. 169, 521–563.
28 Northrup, S.H., Boles, J.O. and Reynolds, J.C.L. (1988) Science 241, 67–70.
29 Takabe, T. and Ishikawa, H. (1989) J. Biochem. 105, 98–102.