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The effect of ethylenediamine chemical modification of plastocyanin on the rate of cytochrome *f* oxidation and P-700$^+$ reduction

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Chemical modification of plastocyanin was carried out using ethylenediamine plus a water-soluble carbodiimide, which has the effect of replacing a negatively charged carboxylate group with a positively charged amino group at pH 6–8. The conditions were adjusted to produce a series of singly and doubly modified forms of plastocyanin. Differences in charge configuration allowed separation of these forms on a Pharmacia fast protein liquid chromatograph using a Mono Q anion exchange column. These forms were used to study the interaction of plastocyanin with its reaction partner cytochrome *f*. The rate of cytochrome *f* oxidation was progressively inhibited upon incorporation of increasing numbers of ethylenediamine moieties indicating a positively charged binding site on cytochrome *f*. However, differential inhibition was obtained for the various singly modified forms allowing mapping of the binding site on plastocyanin. The greatest inhibition was found for forms modified at negatively charged residues Nos. 42–45 and Nos. 59–61 which comprise a negative patch surrounding Tyr-83. In contrast, the form modified at residue No. 68, on the opposite side of the globular plastocyanin molecule, showed the least inhibition. It can be concluded that the binding site for cytochrome *f* is located in the vicinity of residues Nos. 42–45 and Nos. 59–61. Modification of plastocyanin at residues Nos. 42–45 showed no effect on the rate of P-700$^+$ reduction, suggesting that these residues are not involved in the binding of Photosystem I. However, an increase in the rate of P-700$^+$ reduction was observed for plastocyanins modified at residue No. 68 or Nos. 59–61, which is consistent with the idea that the reaction domain of Photosystem I is negatively charged and Photosystem I binds at the top of the molecule and accepts electrons via His-87 in plastocyanin. These results raise the possibility that plastocyanin can bind both cytochrome *f* and Photosystem I simultaneously. The effect of ethylenediamine modification on the formal potential of plastocyanin was also examined. The formal potential of control plastocyanin was found to be $+372 \pm 5$ mV vs. normal hydrogen electrode at pH 7. All modified forms showed a positive shift in formal potential. Singly modified forms showed increases in formal potentials between +8 and +18 mV with the largest increases being observed for plastocyanins modified at residues Nos. 42–45 or Nos. 59–61.

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Abbreviations: DEAE, diethylaminoethyl; DPPC, diphenylcarbonylchloride; FPLC, fast protein liquid chromatography; HPLC, high-pressure liquid chromatography; P-700, reaction center chlorophyll of Photosystem I; BHMF, 1,1-bis(hydroxymethyl)ferrocene; DPM, disintegrations per min.

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Introduction

Plastocyanin is a 10.5 kDa blue copper protein which functions in chloroplast electron transport [1–4]. The crystal structures have been determined for oxidized [5,6], reduced [3] and apo-plastocyanin [7]. The copper is coordinated to two histidines (37 and 87), a methionine and a cysteine

in a distorted tetrahedral geometry (Fig. 1). NMR experiments [8,9] revealed two distinct interaction sites for redox agents. It was concluded that negatively charged donors such as ferricyanide were found to bind at the top of the molecule near His-87 and to react with the copper via an outer-sphere mechanism. In contrast, positively charged molecules such as cobalt phenanthroline were postulated to bind near Tyr-83. Tyr-83 is surrounded by a patch of negatively charged residues (Nos. 42–45 and Nos. 59–61) which are highly conserved in higher plant plastocyanins [1]. The existence of two binding sites for the artificial redox agents raises the possibility that plastocyanin's two reaction partners, cytochrome *f* and P-700, may bind to two different sites on the plastocyanin molecule. Farver and Pecht [10] and Farver et al. [11] observed that a derivative obtained by reduction with Cr(II), which gave a product modified by Cr(III) attachment at residues Nos. 42–45, inhibited the reaction with P-700, but not cytochrome *f*. They concluded that the binding site for P-700 is located in this region of the plastocyanin molecule. In contrast, Beoku-Betts et al. [12] provided evidence that these residues comprise at least part of the binding site for cytochrome *f*. The negatively charged residues surrounding Tyr-83 have also been implicated as a site for the binding of mammalian cytochrome *c* [13].

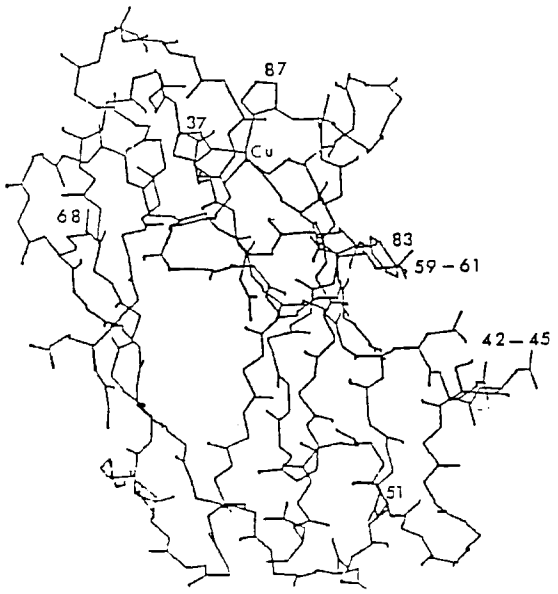

Fig. 1. Computer graphics representation of poplar plastocyanin. The X-ray coordinates for oxidized poplar (*Populus nigra*) plastocyanin as determined by Coleman et al. [5] were obtained from the Brookhaven Protein Data Bank [57] and displayed using a commercial graphics program called IDEAS. The peptide backbone, the histidine ligands to the copper, Tyr-83 and the relevant negative charges are shown.

To answer definitively the question of the location of the binding sites for cytochrome *f* and P-700, we have used a different type of chemical modification: namely, the reaction of carboxyl groups with ethylenediamine in the presence of a water-soluble carbodiimide [14] (Eqn. 1). This reaction replaces a negatively charged carboxyl group with a positively charged amino group.

$$\text{Ⓟ-}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{O}^- + \text{NH}_2(\text{CH}_2)_2\text{NH}_3^+ \rightarrow \text{Ⓟ-}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{NH}(\text{CH}_2)_2\text{NH}_3^+ \quad (1)$$

Using this reaction, Burkey and Gross [15,16] obtained four forms of chemically modified plastocyanins containing between two and six moles of ethylenediamine per mol plastocyanin. The rate of $P\text{-}700^+$ reduction increased with increasing extents of modification. However, all forms showed an approx. +40 mV shift in formal potential. Incorporation of 4–6 mol ethylenediamine per mol plastocyanin also caused conformational changes in plastocyanin as measured by near-ultraviolet circular dichroism [17].

Using a modification of the procedure of Burkey and Gross [16], several singly and doubly modified plastocyanins have been obtained which were used to map the binding sites on plastocyanin for cytochrome *f* and P-700. This approach was used by Margoliash's group [18,19] to map the binding site on mammalian cytochrome *c* for its reaction partners. The effect of location and extent of ethylenediamine modification on the formal potential of plastocyanin has also been determined.

Methods

Isolation of plastocyanin. Plastocyanin was isolated from spinach according to the method of Davis and San Pietro [20]. It was further purified using a Pharmacia FPLC equipped with a Mono

Q HR 5/5 anion-exchange column. Reduced plastocyanin was applied to the Mono Q column equilibrated with 25 mM Tris-HCl (pH 8.2) and was eluted with a linear (0–0.5 M) NaCl gradient. The final A_{278}/A_{597} ratio for oxidized spinach plastocyanin was 1.12. Plastocyanin isolated by this method showed no tendency to autoreduce at pH 7. Absorption and circular dichroic spectra were measured as described by Draheim et al. [21].

Chemical modification of plastocyanin. Plastocyanin was concentrated to 0.3–0.5 mM using ultrafiltration. Although the extent of modification is independent of the oxidation state, modification was carried out on oxidized plastocyanin. The plastocyanin (in 50 mM sodium borate, pH 8.0) was diluted with a known volume of a 2.0 M stock solution of ethylenediamine (pH 6.0) to give a final concentration of 20 mM. Solid 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was added to a final concentration of 5 mM. The pH was adjusted to 6.0 ± 0.1 using 0.1 M HCl. The reaction was allowed to proceed for 1 h at 4°C after which it was stopped by desalting on a Bio-Gel P-10 column equilibrated with 50 mM sodium borate (pH 8.0).

Separation of the ethylenediamine-modified forms of plastocyanin. The plastocyanin was reduced with sodium ascorbate and desalted on a Bio-gel P-10 column equilibrated with 25 mM Tris-HCl (pH 8.2). The mixture of modified plastocyanins was then separated on a Mono Q column as described above (See also Fig. 2). Eleven peaks were obtained. Peak A consists of control, unmodified plastocyanin. The other forms all vary in either extent or location of modification. Peak H could be separated into two forms, H and H′, by a second FPLC step. Each form was chromatographed a second time on the FPLC in order to eliminate any overlapping of peaks for the determination of the location of the label and rates of cytochrome *f* oxidation and P-700$^+$ reduction.

Determination of the extent of modification. The extent and location of ethylenediamine modification was determined using [^{14}C]ethylenediamine. 25 μCi [1,2-^{14}C]ethylenediaminedihydrochloride (25 μCi/mmol) were added to the reaction mixture containing unlabelled ethylenediamine that is described above. The ethylenediamine/plastocyanin ratios are listed in Table I. Peak A contained no label and corresponds to unmodified, control plastocyanin. B consists of a mixture of singly modified and unmodified plastocyanin. C, E, and F are singly modified forms, whereas G,

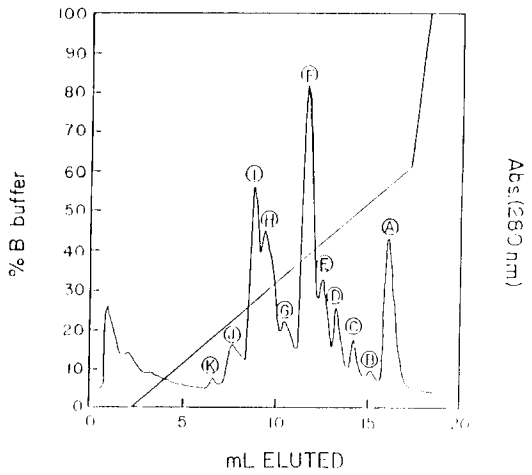

Fig. 2. FPLC separation of EDA-modified plastocyanin. The FPLC separation was carried out using a linear NaCl gradient in 25 mM Tris-HCl (pH 8.2). The B buffer contained 25 mM Tris-HCl (pH 8.2)+0.5 M NaCl. Other conditions were as described in Materials and Methods. Peak A consists of control plastocyanin. The identity of the other peaks is given in Table I. Peak H could be further resolved into two peaks by repeated FPLC chromatography.

TABLE I

INCORPORATION OF ETHYLENEDIAMINE INTO MODIFIED PLASTOCYANIN

The ethylenediamine (EDA) -modified forms correspond to the peaks shown in Fig. 2. The mol ethylenediamine/mol plastocyanin ratios are the average of two determinations and are accurate to ±0.1. Refer to the Materials and Methods section for the details of this determination.

EDA-modified form	mol EDA/mol plastocyanin
A (control)	0
B	0.4
C	1.0
D	1.5
E	1.0
F	1.0
G	2.0
H′	2.0
H	2.0
I	2.0
J	3.0
K	3.0

H, H′ and I are doubly modified forms, and J and K are both triply modified forms. D appears to be a mixture of singly and doubly modified forms. Since forms B an D are mixtures, they will not be considered further.

Determination of the location of the label. Heat-denatured samples of the different forms of plastocyanin were digested for 1 h using 1% DPCC-treated trypsin. The tryptic peptides were separated using reverse-phase chromatography on an LDC HPLC with an Anspec RP-8 column equilibrated with a 0.1% solution of trifluoroacetic acid in water. The peptides were eluted using a 0–45% gradient of a 9 : 1 acetonitrile/water solution containing 0.1% trifluoroacetic acid. The HPLC profile is shown in Fig. 3. The peptides were lyophilized and hydrolyzed under vacuum at 110 °C for 24 h in the presence of 6 M HCl + 0.2% phenol. The amino acid composition was determined by amino acid analysis using a Beckman 119 CL Amino Acid Analyzer. The primary amino acid sequence of spinach plastocyanin with the seven expected tryptic peptides is shown in Fig. 4. The HPLC peaks from left to the right, in Fig. 3 correspond to pep 5, pep 4, pep 6–7, pep 3, pep 2, pep 6 and pep 1. The radioactivity in each peptide ws determined using a Beckman LS-230 liquid scintillation counter. The presence of the modification did not appear to alter the elution pattern of the peptides. No new peptides were observed, indicating that the carbodiimide caused no cross-linking. The label appears almost exclusively in peptides 2 and 3 (Table II).

Peptides 2 and 3 each contain residues which are part of the negative patch consisting of residues Nos. 42–45 and Nos. 59–61. However, each also has another negatively charged residue near the carboxyl terminus of the peptide, at residues Nos. 51 and 68, respectively. These carboxyl groups are located at the bottom of the molecule and on the left side of the plastocyanin molecule pictured in Fig. 1a. Thus, it is very important to determine whether the peptide is modified in one of the negative patches or at the other locations. To do this, the tryptic peptides of plastocyanin were treated with a mixture of carboxypeptidases A and B which will sequentially remove the carboxyterminal residues up to one residue prior to the proline residue, releasing residues Nos. 51

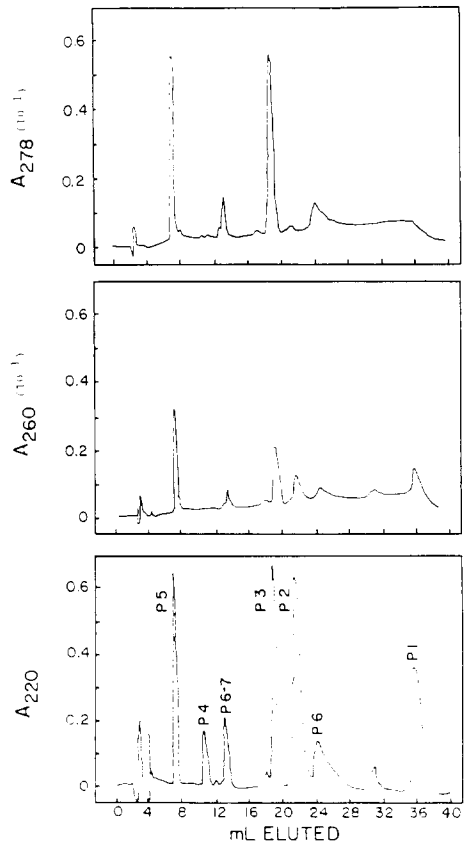

Fig. 3. HPLC separation of the tryptic peptides of plastocyanin. The tryptic peptides were eluted using a 0.5 exponential gradient of 0–45% acetonitrile containing 10% trifluoroacetic acid over 30 min, after which the gradient automatically reset to 0.1% trifluoroacetic acid. The elution profile was monitored at three wavelengths (220, 260 and 278 nm) in order to aid in the identification of the tryptic peptides. Their identity was further confirmed by amino acid analysis. Other conditions were as described in Materials and Methods.

and 68 as free amino acids. The radiolabelled peptides isolated from the HPLC were lyophilized and resuspended in 0.5 ml of 25 mM Tris-HCl (pH 7.6) + 100 mM NaCl. The peptides were digested for 15 min with 10 units of carboxypeptidase B. 20 units of carboxypeptidase A were added to the solution and allowed to react for 1 h at room temperature. The positively charged free amino acids were then separated from the negatively charged peptide by FPLC on a Mono Q column using a linear salt gradient as described above. The samples were then counted to determine whether the free amino acids or the peptides contained the radioactive label.

TABLE II

LOCATION OF THE ETHYLENEDIAMINE (EDA) INCORPORATED INTO PLASTOCYANIN

The ethylenediamine-modified forms correspond to the peaks shown in Fig. 2. See Fig. 4 for peptide nomenclature. The locations were determined as described in the Materials and Methods section. The percentages represent an average of two separate determinations.

EDA-modified form	Primary location			Secondary location		
	Peptide-labelled	Residue-labelled	% of total label	peptide-labelled	residue-labelled	% of total label
Singly modified forms:						
C	3	68	100	–	–	0
E	3	59–61	62	2	42–45	38
F	2	42–45	80	3	59–61	20
Doubly modified forms						
G	2	42–45	72	3	68	28
H	2	42–45	58	3	59–61	42
H′	2	51	66	3	68	34
I	2	42–45	53	3	59–61	47

Fig. 5 shows the amount of radioactivity present in each fraction from the FPLC column for carboxypeptidase-treated plastocyanin forms C and F. In the case of form C, the radioactivity eluted immediately indicating that the label was released with the free amino acids. This shows that form C is labelled at residue No. 68. The small number of counts in fractions 4–7 represents background radioactivity. In contrast, for form F, the radioactivity was retained on the column until fraction 7, indicating that this form is labelled at residues Nos. 42–45.

The purity of the various forms is given in Table II. Form C contains 100% of the label at residue No. 68. In contrast, forms E and F both contain some plastocyanin molecules labelled at the other location within the negative patch. This is not a problem, since both fractions showed similar rates of cytochrome *f* oxidation indicating that the pure fractions would also have similar rates of cytochrome *f* oxidation. Also, knowledge of the degree of contamination allows one to correct the rate constants for the presence of the other form in cases in which it makes a difference in the results.

Isolation of cytochrome f and Photosystem I. In the initial studies, cytochrome *f* was isolated from turnip leaves by the procedure of Matsuzaki et al. [22]. The cytochrome *f* had a purity index ratio of 0.3 ($A_{545.5}/A_{277}$) after Sephadex column chromatography. In later studies, the procedure of Anderson et al. [23] was used up through the CM-Sephadex chromatography step. This resulted in cytochrome *f* with a purity index ratio of 0.5.

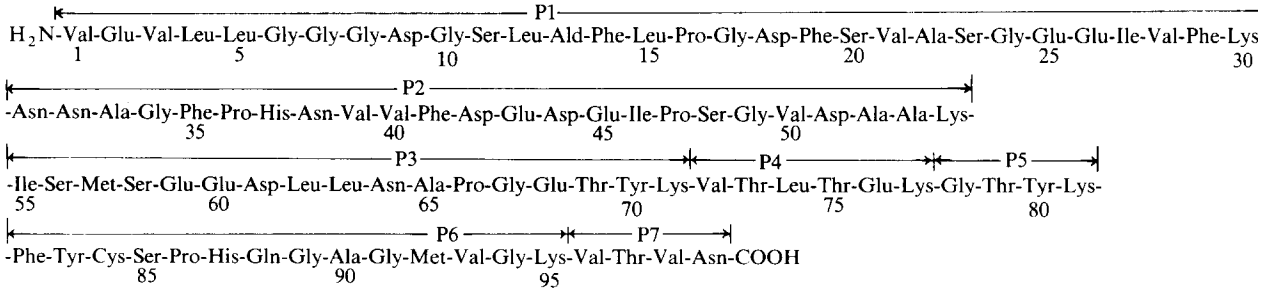

Fig. 4. Tryptic peptides of spinach plastocyanin. The primary structure of spinach plastocyanin was taken from Boulter et al. [1]. The points of tryptic cleavage are indicated.

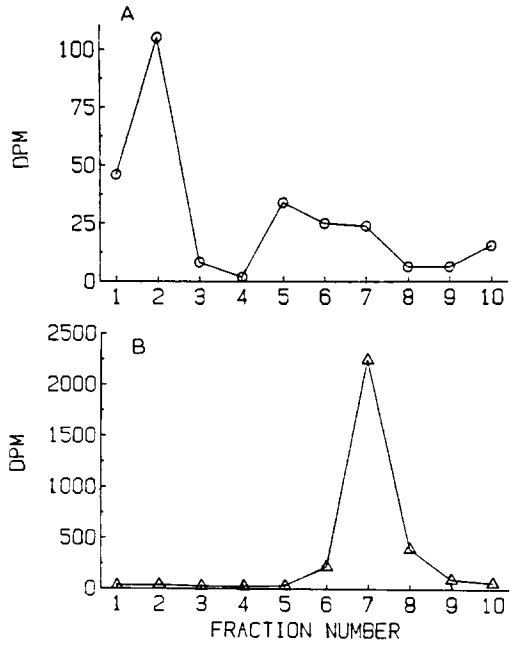

Fig. 5. FPLC separation of carboxypeptidase-treated tryptic peptides. (A) Carboxypeptidase-treated ethylenediamine-modified form C. The number of DPM in fractions 4–8 represent the background. (B) Carboxypeptidase-treated ethylenediamine-modified form F. Conditions were as described in Materials and Methods.

Photosystem I particles were isolated according to the method of Shiozawa et al. [24]. The P-700 content was calculated using an extinction coefficient of 64 $mM^{-1} \cdot cm^{-1}$ [25]. The preparations used for this study had chlorophyll *a*/P-700 ratios of 30–60 and contained the 70 kDa pigment containing subunit plus all of the small subunits.

Measurements of the cytochrome f/plastocyanin reaction kinetics. Second-order rate constants for the oxidation of cytochrome *f* by plastocyanin were determined for both control and modified plastocyanins using an Aminco DW-2a spectrophotometer with a stopped-flow attachment. Second-order rate constants were obtained using equal concentrations of plastocyanin and cytochrome *f* (1 μM) in 10 mM phosphate buffer (pH 7.0) + 100 mM NaCl, except where indicated. The oxidation of cytochrome *f* was monitored by observing the decrease in absorbance at 421 nm with the reference wavelength at 650 nm. A difference (reduced-minus-oxidized) extinction coefficient of 123.4 $M^{-1} \cdot cm^{-1}$ at 421 nm was determined for the cytochrome *f* used in this study. The kinetics were recorded on a Bascomb-Turner Model 2110 electronic recorder at a rate of one data point per ms. The oxidation of cytochrome *f* does not go to completion due to the fact that the redox potentials of the two reaction partners differ by less than 30 mV [26]. Therefore, only the initial points of the time course were used to calculate the second-order rate constants in order to eliminate problems caused by the back reaction.

Rates of $P\text{-}700^+$ reduction were determined according to the method of Gross [27] using an Aminco DW-2A spectrophotometer with side illumination. The reaction mixture contained 0.2 μM P-700, 0.2 μM plastocyanin, 10 mM Tris-HCl (pH 8.2), 1 mM ascorbate and either 2.5 or 5.0 mM $MgCl_2$ as indicated.

Determination of the formal potentials of the modified plastocyanins. The midpoint redox potentials of control plastocyanin and the ethylenediamine-modified forms of plastocyanin were measured using the equilibrium method described by Sanderson and Anderson [28] and Sanderson et al. [29]. The electrode was of their filar design employed in a thin-layer, optically transparent electrochemical cell. Plastocyanin (at 0.6 mM) was suspended in 10 mM phosphate buffer (pH 7) + 50 mM NaCl along with 0.12 mM 1,1-bis(hydroxymethyl)ferrocene as a redox mediator.

Materials. DEAE-cellulose and Biogel P-10 were obtained from BioRad Laboratories. Ethylenediamine dihydrochloride, Tris and DPCC-treated trypsin were obtained from the Sigma Chemical Co. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide was obtained from the Pierce Chemical Co. [1,2-^{14}C]ethylenediamine was obtained from New England Nuclear. HPLC grade acetonitrile and water were obtained from Burdick and Jackson Laboratories, Inc. All other chemicals were of reagent grade.

Results and Discussion

The effect of ethylenediamine-modification on the conformation of plastocyanin

It is important to determine the effect of chemical modification on the conformation of plastocyanin in order ascertain whether any observed effects on activity are caused by protein confor-

mational changes. None of the ethylenediamine-modified forms showed a change in the A_{278}/A_{597} ratio indicating no loss of copper. Moreover, it was shown previously that incorporation of 4–6 mol ethylenediamine per mol plastocyanin had no effect on the secondary structure as determined by far-ultraviolet circular dichroic spectroscopy [17]. These results show that modification did not cause denaturation of the protein. Moreover, in the case of the singly modified forms, there were no changes in either the near-ultraviolet absorption or circular dichroic spectra indicating the absence of conformational changes (not shown).

The effect of ethylenediamine-modification on the rate of cytochrome f oxidation

Second-order rate constants were determined for the oxidation of cytochrome *f* (II) by the various forms of EDA-modified plastocyanins:

$$\text{plastocyanin(II)} + \text{cytochrome } f\text{(II)} \leftrightarrow \text{plastocyanin(I)} + \text{cytochrome } f\text{(III)} \quad (2)$$

The rate of cytochrome *f* oxidation was inhibited for all of the ethylenediamine derivatives tested (Table III). The effects can be divided into two classes: those dependent on the total number of charges introduced, and those where the number of charges is constant but the location of the modification is varied.

Fig. 6 shows a plot of the natural logarithm of the second-order rate constant (ln k) for cytochrome *f* oxidation as a function of the number of ethylenediamine moieties incorporated into plastocyanin. It can be seen that the inhibition of cytochrome *f* oxidation increases as the number of ethylenediamine moieties incorporated increases. This is consistent with previous evidence that the binding site on cytochrome *f* is positively charged [30–32].

The progressive decrease in ln k observed upon addition of ethylenediamine moieties can be explained as follows. The substitution of a positive for a negative charge at sites on the surface of a protein will have the effect of increasing the free energy of activation ($\Delta G^{\ddagger}$) for the electron-transfer reaction (Eqn. 2). In fact, the free energy of activation should be proportional to the number of charges added. According to Eyring rate theory [33], ln k is proportional to $\Delta G^{\ddagger}$ (Eqn. 3):

$$\ln k = -\Delta G^{\ddagger}/RT + \text{constant} \quad (3)$$

where k is the second-order rate constant, $\Delta G^{\ddagger}$ is the free energy of activation, R is the gas constant and T is the absolute temperature. Thus ln k will also be proportional to the number of positive charges added.

TABLE III

THE EFFECT OF ETHYLENEDIAMINE (EDA) MODIFICATION ON THE RATE OF CYTOCHROME *f* OXIDATION

The ethylenediamine-modified forms are labelled as indicated in Fig. 2. Label locations are as indicated in Table II. The rates of cytochrome *f* oxidation were determined as described in the Materials and Methods section. Each value represents an average of four runs. Cytochrome *f* was isolated as described by Matsuzaki et al. [22].

EDA-modified form	Location of label	Rate constant (10^{-7} M^{-1} $\cdot s^{-1}$)	% inhibition of cytochrome *f* oxidation
A (control)	–	7.8 ±0.9	–
C	68	5.0 ±0.7	36
E	59–61	2.4 ±0.2	69
F	42–45	1.8 ±0.4	77
G	42–45, 68	1.1 ±0.2	86
H	42–45, 59–61	0.65 ±0.04	89
H′	51, 68	0.82 ±0.04	82
I	42–45, 59–61	0.37 ±0.06	95
J	triple	0.43 ±0.03	95

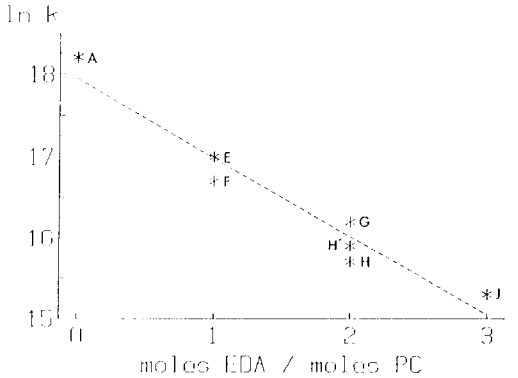

Fig. 6. Plot of ln k vs. number of ethylenediamine molecules incorporated into plastocyanin. Rates of cytochrome *f* oxidation were determined as described in Materials and Methods after which the second-order rate constants (k) were calculated. Ln k is proportional to $\Delta G^{\ddagger}$ according to Eqn. 2.

If only singly modified forms are considered, the inhibition appears to be dependent on the location of the ethylenediamine moiety (Table II). Form C, labelled at residue No. 68 showed significantly less inhibition than form E, labelled at residues Nos. 59–61 or form F labelled at residues Nos. 42–45. Correcting the data obtained for form E for 40% contamination with a form labelled at residues Nos. 42–45 decreases the per cent inhibition from 69% to 64%. The corrected value is within the limits of error in determining the rate constants for cytochrome *f* oxidation.

Fig. 7 shows the effect of increasing the ionic strength on the rate of cytochrome *f* oxidation for control and ethylenediamine-modified plastocyanins. In all cases, the rate decreased with increasing ionic strength. This is consistent with the hypothesis that negative charges on plastocyanin interact with positive charges on cytochrome *f*. The double modified forms H and I also show the decrease in rate as the ionic strength is increased, indicating that the binding site on plastocyanin still has a net negative charge even with two positive charges incorporated into the region of residues Nos 42–45 and Nos. 59–61. These results suggest that at least five negative charges on plastocyanin are involved in the binding of cytochrome *f*.

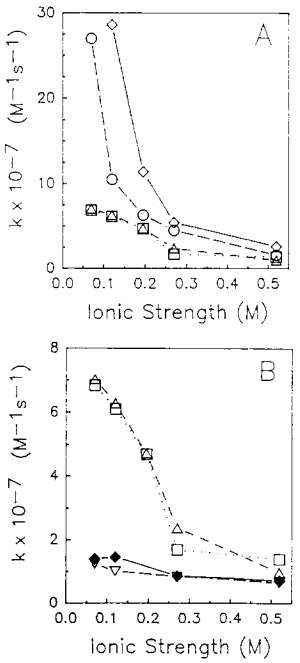

Fig. 7. The effect of ionic strength on the rate of cytochrome *f* oxidation by control and ethylenediamine-modified plastocyanins. Cytochrome *f* was isolated according to the method of Anderson et al. [22]. Rates of cytochrome *f* oxidation were determined as described in Materials and Methods after which the second-order rate constants (k) were calculated. ◇——◇, ○— — —○, □·····□, △— — —△, (A) control; form C; form E; form F; (B) □·····□, form E; △— — —△, form F; ▽— — —y, form H; ◆——, form I.

Fig. 7 also shows that the rate of cytochrome *f* oxidation by form C modified at residue No. 68 is higher than that for form E (modified at residues Nos. 59–61) or form F (modified at residues Nos. 42–45) at all ionic strengths tested. The per cent inhibition decreases significantly as the ionic strength is increased for form C (Table IV). In contrast, the ionic strength dependence of the rate constants is less for forms E and F. This agrees with previous observations that under high salt conditions, the difference in the rate of reaction of two molecules is determined more by the difference in formal charge at their binding sites [34,35] than by the net charge on the molecule.

The ionic strength dependence of the rate constants can be analyzed using the Debye–Marcus equation (Eqn. 4) [36,37], which relates the logarithm of the rate constant k to the charge on the molecules (Z_A, Z_B) and their radii (R_A and R_B)

$$\ln k = \ln k_\infty - 3600\left(\frac{e^{-\kappa R_B}}{1+\kappa R_A} + \frac{e^{-\kappa R_A}}{1+\kappa R_B}\right)\left(\frac{Z_A Z_B}{R^{\ddagger}_{AB}}\right) \qquad (4)$$

TABLE IV

THE EFFECT OF NaCl ON THE INTERACTION OF THE ETHYLENEDIAMINE (EDA) -MODIFIED PLASTOCYANINS WITH CYTOCHROME *f*

The ethylenediamine-modified forms are as designated in Fig. 2. The label locations were taken from table II. The rates of cytochrome *f* oxidation were taken from Fig. 7.

	Inhibition of cytochrome *f* oxidation		
	(%)	(%)	(%)
EDA-modified form	C	E	F
Location of label	68	59–61	42–45
100 mM NaCl concn.	57	78	78
175 mM NaCl concn.	45	59	59
250 mM NaCl concn.	33	71	61

$R^{\ddagger}_{AB}$ is the radius of the transition state complex and $\kappa = 0.329\,\mu^{-1/2}$. Takabe et al. [38] have modified this treatment so that Z_A and Z_B are the charges at the respective binding sites and R_A and R_B are their respective radii. μ is the ionic strength. Using the values of Takabe et al. [38] for the charge ($Z_A = +10.5$) and radius ($R_A = 1.07$ nm) for the interaction domain on cytochrome f, we obtained a value of 0.96 nm for the radius of the reaction domain in plastocyanin. This is slightly larger than the value of 0.856 nm obtained by Takabe et al. [38]. We obtained a value of -5.5 for Z_B, the charge at the binding site. This is significantly greater than the value of -3.24 obtained by Takabe et al. [38]. However, it is consistent with the results presented in FIg. 7 which indicates that more than five negative charges on plastocyanin are involved with its interaction with cytochrome *f*. The values of Z_B for the singly and doubly modified forms are shown in Table V).

The results presented above show that the cytochrome *f* binding site on plastocyanin spans Tyr-83. In view of this, it is interesting that the incorporation of a nitro moiety into Tyr-83 [39] had no effect on the rate of cytochrome *f* oxidation. However, unlike the ethylenediamine modification, the nitrotyrosine modification caused no change in charge on plastocyanin at pH 7. Since the addition of the nitro group had no effect on activity, it is unlikely that the inhibition observed for ethylenediamine derivatives E and F is due to steric hindrance. The results presented here confirm the work of Beoku-Betts et al. [12] that residues Nos. 42–45 are part of the binding site for cytochrome *f*.

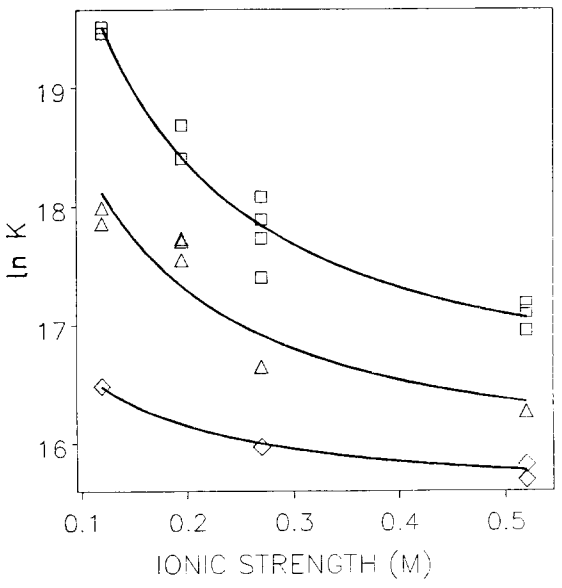

Fig. 8. ln *k* vs. ionic strength for the interaction of modified plastocyanins with cytochrome *f*. The data shown in Fig. 7 were fitted with Eqn. 3 using the SAS statistical software package. □, Control; △, Form F; ◇, Form H.

TABLE V

CALCULATION OF THE NUMBER OF CHARGES AT THE BINDING SITE ON PLASTOCYANIN FOR CYTOCHROME *f*

The designation of the forms is as given in Tables I and II. Rate constants for cytochrome *f* oxidation were taken from Fig. 7. The data were fit to Eqn. 3, using the following parameters: charge of cytochrome $f = +10.5$; radius of interaction for cytochrome *f* 1.07 nm; radius of interaction for plastocyanin = 0.96 nm. The SAS statistical software package was used to fit the data. ln *k* is the second-order rate constant. EDA, ethylenediamine.

Form	Mol EDA per mol plastocyanin	Charge at binding site	ln k_∞	Residual S.E. per point
Control	0	−5.5	18.82	0.0335
E	1	−4.3	18.22	0.0760
F	1	−4.0	18.22	0.0730
I	2	−1.6	17.92	0.00244
H	2	−0.9	17.92	0.0164

The small effect on activity observed upon modification of residue No. 68 (form C) can be explained on the following basis. Koppenol and Margoliash [39] argued, in the case of cytochrome *c*, that the effects observed upon modification of residues at other locations than the binding site were due to changes in the dipole moment which caused incorrect orientation upon binding. Plastocyanin (Fig. 1) like cytochrome *c*, has a large charge asymmetry, since seven of the thirteen negative charges are located in the negative patch surrounding Tyr-83 and some of the positive charges are located on the opposite side of the molecule. This charge asymmetry may orient plastocyanin so that the negative charges surrounding Tyr-83 can bind to positive charges surrounding the heme cleft on cytochrome *f*. As for cytochrome *f*, modification at sites other than the binding site may cause incorrect orientation which

would decrease the reactivity of plastocyanin with cytochrome *f*.

The effect of ethylenediamine modification on the rate of P-700$^+$ reduction

Both plastocyanin [1] and Photosystem I [41] have a net negative charge at pH 7. For this reason, divalent cations are required for the reaction of plastocyanin with P-700$^+$ [42–44]. Divalent cations provide sufficient shielding to allow the two reactants to approach each other without attenuating the local electrostatic effects at the binding site produced by chemical modification [34,35].

Table VI shows the effect of ethylenediamine modification on the rate of P-700$^+$ reduction for two different $MgCl_2$ concentrations: 2.5 and 5.0 mM. Form F, modified at residues Nos. 42–45, showed no effect on P-700$^+$ reduction for either $MgCl_2$ concentration tested. In addition, there was no effect at other pH values between 6.0 and 8.0. In contrast, form C, modified at residue No. 68, and form E, modified at residues Nos. 59–61, showed an increase in activity. The increase in activity is consistent with the replacement of a negative charge by a positive one. The increase in activity for form E may be underestimated due to contamination with a form modified at residues Nos. 42–45, which showed no effect on the rate of P-700$^+$ reduction. The participation of residues Nos. 59–61 is confirmed by results obtained with the nitrotyrosine modification. In the case of the nitrotyrosine derivative, the rate of P-700$^+$ reduction was inhibited only under conditions in which the hydroxyl group was deprotonated. No effect was observed when the hydroxyl group was protonated ruling out steric effects. Tollin et al. [35] also implicated residues Nos. 59–61 and No. 68 in the interaction of FMN semiquinone with plastocyanin. These results suggest that Photosystem I binds at the top of the molecule and interacts with the copper center via His-87. Thus, these results disagree with those of Farver and Pecht [10,11] who implicated residues Nos. 42–45 as part of the binding site for P-700.

The effect of ethylenediamine-modification on the formal potential of plastocyanin

The formal potential for control plastocyanin was found to be $+372 \pm 5$ mV vs. normal hydrogen electrode at pH 7, which agrees well with previous determinations [16,45–47]. BHMF was used as the mediator because ferricyanide did not show Nernstian behavior when reacting with the gold electrode. The formal potential was independent of BHMF concentration for ratios of plastocyanin/mediator between 0.11 and 10 [48]. It was also independent of salt concentration between 3.3 and 50 mM NaCl added to 10 mM phosphate buffer (pH 7.0).

All modified forms showed a positive shift in formal potential (Table VII). However, the magnitude of the shift was dependent on both the number and location of the ethylenediamine molecules incorporated. The various singly modified forms showed shifts in formal potential from +8 mV for plastocyanin modified at residue No. 68 to +20 mV for plastocyanin modified at residues Nos. 59–61.

TABLE VI

THE EFFECT OF ETHYLENEDIAMINE MODIFICATION ON THE RATE OF P-700$^+$ REDUCTION

The ethylenediamine (EDA) -modified forms are as designated in Fig. 2. The location of the label is given in Table II. Rates of P-700$^+$ reduction were determined as described in the Materials and Methods section. Units for the rate of P-700$^+$ reduction are mol P-700 reduced-mole per P-700 per s. The standard deviation in the per cent stimulation was ±15%.

EDA-modified form	Location	+2.5 mM $MgCl_2$		+5 mM $MgCl_2$	
		rate	% stimulation	rate	% stimulation
A	–	6.5	–	10.5	–
C	68	7.8	20	14.4	37
E	59–61	8.1	25	13.4	28
F	42–45	6.3	−3	10.8	3
H	42–45; 59–61	10.3	58	15.2	45
H′	51–68	8.8	35	15.2	45
I	42–45; 59–61	8.3	28	14.7	40

TABLE VII

FORMAL POTENTIALS OF ETHYLENEDIAMINE-MODIFIED FORMS OF PLASTOCYANIN

The ethylenediamine (EDA) -modified forms of plastocyanin are labelled as in Fig. 2. The location of the label was taken from Table II. The formal potentials were determined as described in the Materials and Methods section. The distance was calculated using the coordinates of oxidized poplar plastocyanin taken from the Brookhaven Protein Data Bank. The distance is measured to the average of the 'east face' region Nos. 42–45, and Nos. 59–61.

EDA-modified form from Cu	Location	Distance of label (nm)	$E^{O'}$	$E^{O'}$
A (control)	–	–	+372	–
C	68	1.24	+380	+8
E	59–61	1.5	+392	+20
F	42–45	1.97	+389	+17
G	42–45; 68	–	+397	+27
H′, H [a]	–	–	+377	+5
I	42–45; 59–61	–	−387	+15

[a] This value was determined for the mixture of H and H′.

The shift in $E^{O'}$ is not a simple $1/r$-function of the distance from the derivatization site to the copper center. Derivatization at residue Nr. 68, which is located 1.2 nm from the copper, has a comparable effect to derivatization at residues Nos. 42–45 which are approx. 2 nm distant from the copper site. This may be due to differences in local dielectric constant for different parts of the plastocyanin molecule. Moreover, there seems to be a saturation effect, since some of the doubly modified forms show a smaller shift in formal potential than the corresponding singly modified forms. These results contrast with those of Schejter et al. [49], who found a progressive change in the formal potential of mammalian cytochrome *c* upon increasing the number of negative maleic acid moieties incorporated.

According to Marcus theory [36,50], the rate of electron transfer between two molecules is dependent on both electrostatic effects involved in binding and the difference in formal potential between the two reaction partners. The correlation between the reaction rate and the difference in formal potential was documented by Meyer et al. [50] for a series of cytochromes and high potential non-heme iron proteins under high salt conditions where electrostatic effects could be neglected.

Under our conditions, electrostatic effects dominate the reaction rates as can be seen from the following argument. The formal potentials of cytochrome *f* and P-700 are +360 [26] and +430 [51] mV, respectively. Thus, in the case of the reaction between plastocyanin and cytochrome *f*, ethylenediamine modification of plastocyanin should increase the reaction rate for its interaction with cytochrome *f* and decrease that with P-700 if the difference in formal potential were the principal determinant of reaction rates. Since the opposite is true, it can be concluded that electrostatic factors are more important in controlling reaction rates than is the difference in formal potential between the reaction partners.

The results presented above implicate the negatively charged amino acid residues Nos. 42–45 and Nos. 59–61 in the interaction of plastocyanin with cytochrome *f*. The simplest assumption is that cytochrome *f* binds to the negative charges and donates electrons to plastocyanin via the tunneling pathway starting at Tyr-83. However, due to the size of cytochrome *f* (33 kD) the possibility cannot be ruled out that cytochrome *f* binds at residues Nos. 42–45 but donates electrons at the top of the plastocyanin molecule via His-87. Indirect evidence for electron donation at tyrosine 83 comes from an NMR study [13] of the interaction of plastocyanin with mammalian cytochrome *c*. In this case, it was shown that the heme edge interacts with plastocyanin in the vicinity of Tyr-83. On the other hand, plastocyanin containing a nitrotyrosine derivative of Tyr-83 showed no change in its reactivity with cytochrome *f* [52]. Some change might have been expected if Tyr-83 were an integral part of the electron-transfer pathway.

If cytochrome *f* binds to plastocyanin in the vicinity of Tyr-83, and Photosystem I binds at the top of the molecule in the vicinity of His-87, it may be possible for both cytochrome *f* and Photosystem I to bind to plastocyanin simultaneously forming a ternary complex. This is impossible in the case of mitochondrial cytochrome *c*, since both reaction partners bind to the same domain on the cytochrome *c* molecule [53]. The possibility of a ternary complex of cytochrome *f*, plas-

tocyanin and Photosystem I has been suggested by Haehnel [54]. However, it would be unlikely if Photosystem I and the b_6/f complex are separated by a considerable distance in the thylakoid membrane [55], in which case plastocyanin would act as a mobile electron carrier [44,56].

Conclusions

A comparison of the rates of cytochrome *f* oxidation using a series of single modified ethylenediamine derivatives of plastocyanin showed that the binding site for cytochrome *f* spans the region containing Tyr-83 to include the negatively charged residues Nos. 42–45 and Nos. 59–61. In contrast, residues Nos. 59–61 and No. 68 were implicated in the binding of Photosystem I, suggesting that it interacts at His-87. All modified forms showed a positive shift in formal potential. However, the effects were greatest for the forms modified at residues Nos. 42–45 and Nos. 59–61, indicating the importance of this region of the plastocyanin molecule in regulating electron transfer.

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