Chemical modification of plastocyanin with 7-chloro-4-nitrobenz-2-oxa-1,3-diazole: Preparation and characterization

May Yun Wang, May Yu Tsai, Rong Long Pan and Elizabeth L. Gross

Institute of Radiation Biology, National Tsing Hua University
Hsin Chu, Taiwan 30043, Republic of China
(Received August 1, 1988; Accepted November 29, 1988)

Abstract. Spinach plastocyanin was modified by 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) whose alkyamine derivative was fluorescent. The $V_{m}$ for electron transport from plastocyanin to P$_{700}^{+}$ was decreased by 30% after modification. The $K_{d}$ for P$_{700}^{+}$ reduction was also decreased from 15 μM to 5 μM. Meanwhile, the modification increased 10 mV more positive of redox potential. The absorption (ΔA$_{680}$) and fluorescence (F$_{680}$) of NBD-plastocyanin were investigated as a probe to study its conformational change against environment. The major labelled group on plastocyanin was identified as lysine-54.

Key words: Chemical modification; NBD chloride; P$_{700}$ reduction; Photosystem I; Plastocyanin; Spinacia oleracea L.

Introduction

Plastocyanin (PC) is a blue or type one copper protein which mediates electron transport between cytochrome f and P$_{700}^{+}$ in photosynthesis (Boulter et al., 1977; Plesnica and Bendall, 1973; Wood, 1974; Haehnel et al., 1980). Plastocyanin is a small protein with a single polypeptide chain, a molecular weight of 10,500 and a single type one Cu atom (Boulter et al., 1977). The copper is coordinated to 4 ligands (two histidines, a cysteine and a methionine) in a distorted tetrahedral geometry (Gross et al., 1985). Though amino acid sequence of plastocyanin from various species was reported (Colman et al., 1978; Boulter, 1977), little is known about its structure and conformational change during mediating electron flow between its partners.

Recent studies have shown that electron transport between plastocyanin and P$_{700}^{+}$ is facilitated in the presence of Mg$^{2+}$ or polycations such as polylysine in both spinach chloroplasts (Lockau, 1979; Tamura et al., 1980; Haehnel et al., 1980) and isolated photosystem I (PSI) particles (Lien and San Pietro 1979; Davis et al., 1980). It was suggested that Mg$^{2+}$ and polycations stimulate electron transport by screening the negative charges on both spinach
plastocyanin and the PSI complex (Boutler et al., 1979). This notion was supported by that the cation requirement was abolished when the charge on PSI complex (Burkey and Gross, 1981a) or plastocyanin molecule (Davis et al., 1980; Burkey and Gross, 1981b) was converted from negative to positive by chemical modification of carboxylate groups.

Obviously, the carboxylate groups on plastocyanin molecules are very important in the binding of plastocyanin to PSI particles. However, interesting question arises whether the amino groups on plastocyanin molecule play any roles in the electron flow between plastocyanin and PSI particles. In this work, we modified amino group of plastocyanin with a fluorescent chemical modifier NBD-Cl. We report here on the preparation and characterization of a plastocyanin modified at a lysine residue with NBD-Cl. We also investigated the effect of chemical modification on electron flow between plastocyanin and PSI.

Materials and Methods

Isolation of PSI Particles and Plastocyanin

Isolation of PSI particles from spinach was carried out according to Shiozawa et al. (1974) as modified by Burkey and Gross (1981). Chlorophyll concentrations were determined according to the method of Arnon (1949). Plastocyanin was isolated according to Borchart and Wessels (1970). The purified plastocyanin had a final $A_{280}/A_{600}$ ratio of 1.2–1.5 and were eluted as single peak by both Sephadex G-25 gel filtration and DEAE-cellulose ion-exchange chromatography. Plastocyanin concentration were measured according to Davis and San Pietro (1979) using an extinction coefficient of 4.9 mM$^{-1}$cm$^{-1}$ at $A_{600}$ or according to a modified Lowry's method (Larson et al., 1986). Oxidized plastocyanin was obtained by adding excess $K_2Fe(CN)_6$ which was subsequently removed by centrifuge filtration (Sephadex G-10). Plastocyanin was reduced using substantial amount of sodium ascorbate which was also removed by the centrifuge column.

Chemical Modification of Plastocyanin

The chemical modification of plastocyanin (0.3 mM) was carried out by reacting the plastocyanin with NBD-Cl (NBD-Cl/plastocyanin molar ratio=50/1) in the 50 mM borate buffer (pH 9.0) at room temperature in the dark. The degree of modification was monitored by the appearance of absorption peak at 480 nm which indicates the reaction of NBD-Cl with amino group (Aboderin et al., 1973). Separation of unreacted NBD-Cl from NBD-plastocyanin was through centrifuge filtration (Sephadex G-10). The NBD/plastocyanin ratio was determined using the molar extinction coefficient of $\epsilon_{480}=26,000$ M$^{-1}$cm$^{-1}$ for amino derivative of NBD and $\epsilon_{405}=4.9$ mM$^{-1}$cm$^{-1}$ for exhaustedly oxidized plastocyanin, respectively (Aboderin et al., 1973; Burkey and Gross, 1982).

Kinetics of $P_{700}^{+}$ Reduction

The kinetics of $P_{700}^{+}$ reduction were determined on an Amico DW-2a spectrophotometer according to the method of Gross (1979). The reaction mixture contained 10 $\mu$g Chl/ml of PSI, 10 mM Tris-Cl (pH 8.0), 50 mM ascorbate and 5 mM MgCl$_2$. The initial rate of $P_{700}^{+}$ reduction was measured by subtracting the background reduction rate using ascorbate as electron donor.

Absorption and Fluorescence Measurements

Absorption were measured using a Hitachi Model 200–20 Spectrophotometer (Hitachi, Japan). Plastocyanin (15–25 $\mu$M) was suspended in 10 mM concentration of Tris-Cl buffer (pH 8.0). The absorption spectrum was scanned from 400 nm to 700 nm. The absorbance of plastocyanin was
found to vary linearly with concentration from 20 to 100 μM, indicating the absence of aggregation.

Fluorescence spectra were obtained on a Hitachi Model 650-60 Fluorescence spectrophotometer (Hitachi, Japan). The concentration of plastocyanin was 2.5 μM in 50 mM borate buffer (pH 8.5). The excitation wavelength was 278 nm, while the emission spectra were scanned from 300 nm to 700 nm. The slit width for the emission monochromator was 10 nm.

**Measurement of the Oxidation-reduction Midpoint Potential**

The midpoint redox potential was obtained according to the method described by Davis and San Pietro (Davis and San Pietro, 1979; Burkey and Gross, 1981).

**Tryptic Digestion and Peptide Mapping of the Modified Plastocyanin**

The location of NBD binding residues was determined by monitoring the absorption of NBD-derivative following trypsin digestion and HPLC separation of digested peptides. NBD-plastocyanin in 10 mM NH₄HCO₃ was denatured at 100°C for 10 min. Trypsin in 1 mM HCl was added to the denatured plastocyanin solution at room temperature for 4 hours. A second aliquot of trypsin was then added which gave a final trypsin concentration of 0.001% (w/v) and the digestion continued for another 20 hours. The reaction mixture was lyophilized and resuspended in an aliquot of distilled water. The peptides were separated with HPLC using a μ-Bondapak C-18 column (Millipore, Waters Chromatography Division). The peptides were eluted out using 45% gradient of 9:1 acetonitrile/0.1% trifluoroacetic acid in water. The main peak was eluted at retention time of 3.52 min. This eluant peak contained 47.4% of total concentration of NBD-peptides. The amino acid composition of peptides were determined by Regional Instrument Center at National Taiwan University, NSC.

**Materials**

Spinach (Spinacia oleracea L.) was obtained from local market. NBD-Cl, Sephadex G-10 and G-25, and trypsin were purchased from Sigma. DEAE-cellulose was from Bio-Rad. HPLC grade acetonitrile, trifluoroacetic acid were obtained from Merck. Deionized water was processed using Millipore RO-60 apparatus+ Milli-Q Water Purification (Millipore Inc.) at this institute. All other chemicals were of reagent grade.

**Results**

**The Preparation of NBD-plastocyanin**

Figure 1 depicts the appearance of absorbance of NBD-plastocyanin. The absorption at 480 nm indicated its N-NBD-alkylamine derivative (Aboderin et al., 1973). As the incubation time increased the absorbance at 480 nm grewed. On the other hand, the absorbance of oxidized

![Fig. 1. Absorption spectra of plastocyanin during incubation with NBD-Cl. The modification of plastocyanin with NBD-Cl was carried out as described under "Materials and Methods". Incubation time: (a) control, (b) 1 h, (c) 2.5 h, (d) 24 h.](image-url)

10
5

Absorbance, x 10^2

450 500 550 600 650 700

λ, nm

Fig. 1. Absorption spectra of plastocyanin during incubation with NBD-Cl. The modification of plastocyanin with NBD-Cl was carried out as described under "Materials and Methods". Incubation time: (a) control, (b) 1 h, (c) 2.5 h, (d) 24 h.
plastocyanin at 597 nm decreased as modification proceeded.

Figure 2 shows the time course of modification of plastocyanin with NBD-Cl. The reaction took 20 to 24 hours to reach a plateau. The time course for the decrease of absorbance at 597 nm is also shown in Fig. 2. The control plastocyanin was also monitored by adding ethanol/ethylene glycol (1:1, v/v) mixture to the final concentration of 6 mM. The ethanol/ethylene glycol mixture carried over in NBD-Cl treatment has obviously no significant (<15%) effect on plastocyanin.

The binding of NBD moiety to plastocyanin was thereby determined by the difference in the absorbance at 480 nm. After incubation of 20 to 24 hours, the NBD/plastocyanin ratio reached 1.0-1.15 (Fig. 3). Isoelectrofocusing pattern showed only one species of modified plastocyanin presented (data not shown).

N-NBD-amino derivative gives strong fluorescence at 550 nm (Aboderin et al., 1973). Figure 4 displays the fluorescence emission spectrum of control plastocyanin and NBD-plastocyanin.

Modification of plastocyanin with NBD-Cl quenched the fluorescence peak of plastocyanin at 340 nm. However, it appeared a new peak at 550 nm when the excitation wavelength was at 278 nm. NBD-Cl has a strong absorption peak at 350 nm where no change was found.

Fig. 2. Time course of change of absorption peak for NBD-plastocyanin. Conditions were the same as in Fig. 1. (▲) control at A400, (●) A597, (○) A480.

Fig. 3. Time course of incorporation of NBD-moieity on plastocyanin. The reaction conditions and calculation of molecular fraction incorporated were described under "Materials and Methods".

Fig. 4. The relative fluorescence emission spectra of plastocyanin and NBD-plastocyanin. Conditions were described as under "Materials and Methods". (——) Control plastocyanin, (-----) NBD-plastocyanin.
during its reaction with amino groups (data not shown). It is believed that the shift of fluorescence from 340 nm to 550 nm is due to the energy transfer from the tyrosine of plastocyanin to NBD-chromophore. Figure 5 depicts the time course for the growth of fluorescence at 550 nm. This agrees with the situation when determined by the increase in $A_{460}$ (cf., Figs. 3 and 5).

**Effect of the Chemical Modification on $P_{700^+}$ Reduction**

The modification of plastocyanine affected the $V_{max}$ and $K_m$ of $P_{700^+}$ reduction (Table 1). The modified plastocyanin caused 30-35% inhibition in $V_{max}$. The $K_m$ was also decreased from 12-15 $\mu$M to 4-5.5 $\mu$M. The decrease in both $K_m$ and $V_{max}$ suggests that the effect might be due to partial retardation in electron transfer from plastocyanin to $P_{700^+}$ in plastocyanin-$P_{700^+}$ complex rather than in the affinity of these two entities. The modification of amino group on plastocyanin might cause a conformational change of the protein resulting in the decrease of the electron flow from plastocyanin to $P_{700^+}$.

**The Effect of Modification on Redox Potential of Plastocyanin**

The midpoint potential for the reduction of plastocyanin was determined from the ratio of oxidized to reduced form at various redox potential poised by ferricyanide/ferrocyanide couples. The redox potential was measured to be 0.395 V which is +10 mV more positive than control plastocyanin (data not shown). The slight increase in redox potential might retard the electron transport to its endogenous electron acceptor, $P_{700^+}$, and thus decreased $V_{max}$.

**Factors Which Affect $A_{460}$ and $F_{550}$ of NBD-plastocyanin**

Urea showed no significant effect on $A_{460}$ of NBD-plastocyanin even at concentration of 8.0 M (Fig. 6a). On the other hand, heating of NBD-plastocyanin increased $A_{460}$ by 67% within 100 min. The hydrolysis of NBD-moiety from NBD-plastocyanin was found to be negligible

![Graph](image)

**Fig. 5.** Time course of growth of $P_{700}$ for NBD-plastocyanin. Conditions were as in Fig. 4. (●) NBD-plastocyanin, (△) Control plastocyanin.

**Table 1. The effect of NBD-Cl modification of plastocyanin on $P_{700^+}$ reduction**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>$V_{max}$ ($\mu$mol $P_{700^+}$ reduced/mg Chl. h)</th>
<th>$K_m$ ($\mu$M)</th>
<th>Inhibition of $V_{max}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>76.0</td>
<td>15.4</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Modified</td>
<td>43.5</td>
<td>4.2</td>
<td>43</td>
</tr>
<tr>
<td>II</td>
<td>Control</td>
<td>56.0</td>
<td>12.5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Modified</td>
<td>38.5</td>
<td>5.6</td>
<td>31</td>
</tr>
</tbody>
</table>

*Reaction mixture contained: 15μg Chl/ml PSI, 10mM Tris-Cl, pH 8.0, 5mM MgCl₂, 50 μM ascorbate. The volume of reaction mixture was 3 ml.*
(data not shown). If the change of $A_{480}$ reflects a conformational change in plastocyanin, then the resistance to 8 M urea for almost 15 hours or to boiling temperature for 100 min demonstrates that the structure of plastocyanin is very stable against these denaturation agents (cf., Boulter et al., 1977). Nevertheless, $A_{480}$ disappeared after addition of 2 M NH₄OH (data not shown) indicating the reversible modification by NBD-Cl. However, heat treatment increased $F_{550}$ slightly within 5 min while prolonged heating decreased the fluorescence at this wavelength (Fig. 6b). Furthermore, the treatment also shifted the fluorescence peak from 550 to 565 nm after 30-60 min of incubation (data not shown). It is likely that the environment of NBD-moiety on plastocyanin was changed upon the heat treatment. Modified plastocyanin was also more resistant to tryptic digestion than control plastocyanin (Fig. 7). Incubation of plastocyanin with 1% of trypsin for 50 min inhibited 50% of $P_{700}^-$ reduction. However, trypsin showed no effect on modified plastocyanin within 120 min of incubation.

Divalent cations have significant influence on the behavior of NBD-plastocyanin as electron carrier between cytochrome $f$ and $P_{700}^-$. The effect of divalent cations such as Mg$^{2+}$ and Ca$^{2+}$ was examined. There was no change in $A_{480}$ (data not shown) while these cations
Fig. 8. The effects of divalent cations and pH on NBD-plastocyanin. The conditions were the same as described in Fig. 4, except the various concentrations of cations and pH indicated. (a): (○) Mg²⁺, (●) Ca²⁺, (b): (○) A₄₈₀, (●) F₅₅₀.

Fig. 9. (a) The absorption spectra of NBD-plastocyanin in oxidized or reduced conditions. The preparation of oxidized or reduced form of NBD-plastocyanin was described under "Materials and Methods". (—) reduced form, (⋯⋯⋯) oxidized form. (b) The dependence of fluorescence (F₅₅₀) of plastocyanin on redox state. The redox potential was maintained by ferricyanide/ferrocyanide couples as described under "Materials and Methods".

decreased F₅₅₀ by 35% for Ca²⁺ and 25% for Mg²⁺, respectively (Fig. 8). Obviously, the fluorescence as probe to the environmental change is much more sensitive than absorption (Lakowicz, 1983).

The A₄₈₀ and F₅₅₀ at pH 2 to 10 were compared (Fig. 8b). At pH higher than 7.5 both absorption and fluorescence reached maximum, while at pH below 4.0 were minimum. The transition pH of either case was at pH 6. The pH profile of A₄₈₀ and F₅₅₀ might be the indicators for conformational change of NBD-plastocyanin.
under different pH. Isoelectrofocusing pattern showed that pI of modified plastocyanin was not different from that of control plastocyanin (pI = 3.0; Davis et al., 1980). The modification of lysine by NBD-Cl, which converts only one positive charge into a neutral residue, is negligible in the change of net charge in plastocyanin.

It has been shown that the conformation of plastocyanin is redox dependent (Draheim et al., 1985). Figure 9 shows that absorbance and fluorescence of NBD-plastocyanin are also redox dependent. For control plastocyanin, the absorbance at 278 nm increased upon reduction (see Fig. 5 of Draheim et al., 1985). The absorbance of reduced NBD-plastocyanin at 480 nm was slightly higher than its oxidized form (Fig. 9a). Figure 9b depicts that the fluorescence at 550 nm decreased as redox potential increased. For the reduced form, the fluorescence was higher than its oxidized form, which is coincident with that of absorbance at 480 nm. However, fluorescence is more sensitive than absorbance to the redox state of plastocyanin (Table 2). The reduction of NBD-plastocyanin caused 12% decrease in A, while F, decreased.

### Table 2. The effect of redox state on the absorbance and fluorescence of NBD-modified plastocyanin

<table>
<thead>
<tr>
<th>Redox state</th>
<th>A,</th>
<th>F,</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced</td>
<td>0.91 (100%)</td>
<td>100</td>
</tr>
<tr>
<td>Oxidized</td>
<td>0.80 (88%)</td>
<td>64</td>
</tr>
</tbody>
</table>

The concentration of NBD-modified plastocyanin was 40 µg/ml. The medium contained Tris-Cl 5 mM, pH 7.8.

---

**Fig. 10.** (a) Primary structure of spinach plastocyanin (reprinted from Scawen et al. (1975)). The peptides produced by trypsin cleavage of plastocyanin are shown. The lysine residue (Lys-54) modified by NBD-Cl was indicated with an asterisk. (b) Amino acid sequence of spinach plastocyanin projected on to the three-dimensional structure of poplar plastocyanin. The three-dimensional structure of plastocyanin was produced from Colman et al. (1978). The circles represent the carbons of amino acids. The dark circles represent the charged amino acids in spinach plastocyanin. The letters N and C denote the amino and carboxyl terminal residues, respectively. The Lys-54 modified by NBD-Cl is labelled with an asterisk.
declined down to about 64%.

Identification of Labelled Plastocyanin Peptides

The fraction containing NBD-peptide was analyzed to determine amino acid composition. It was found that the peptide P2-P3 was most likely the peptide obtained from HPLC (data not shown; cf., Fig. 10a). It is thus suggested that amino group of lysine-54 is the one modified by NBD-Cl. Figure 10b has been constructed by superimposing the amino acid sequence of spinach plastocyanin (Boultier et al., 1977) on a projection of the three dimensional structure of poplar plastocyanin. The Lys-54 is labelled with asterisk as shown in Fig. 10.

Discussion and Conclusions

Plastocyanin contains six lysines, one cysteine and two to three tyrosines, depending on species. NBD-Cl is well known for its reaction with lysine, cysteine or tyrosine. However, only the N-NBD-alkylamine derivative is fluorescent (Aboderin et al., 1973). This property offers an advantage to use NBD-Cl as probe to study the protein conformational change around its lysine residues. In this report, lysine-54 of plastocyanin was identified as the amino residue modified. The reason why lysine-54 is the major residue modified still remains unknown. It is possible that lysine-81 and lysine-95 are close to negative carboxylate cluster and the rest of lysine are peripheral on plastocyanin molecule while only lysine-54 is surrounded by more hydrophobic amino acid residues (Ile-53, Val-21, Val-72, etc.; see Fig. 10). The hydrophobic surrounding may offer an environment for the anchor of NBD-Cl to access lysine-54.

On three dimensional projection (Fig. 10b), Lys-54 lays on the other side of negative carboxylate cluster which is very important for the binding of plastocyanin to PSI particles (Burkey and Gross, 1981). From the results shown above, lysine-54 on plastocyanin may be involved in electron flow from plastocyanin to PSI. The decreases of both $V_{max}$ and $K_m$ in NBD-plastocyanin demonstrate that modification of Lys-54 results in retarding electron flow even though the binding affinity of plastocyanin-PSI complex is enhanced. Despite that the modification of plastocyanin by NBD-Cl neutralizes the positive charge of lysine-54, the pI of modified plastocyanin shows no difference from that of parent protein which is a very acidic protein (pI=3.0; Davis et al., 1980). The inhibition of NBD-modification on electron flow seems not due to the charge screening of plastocyanin. The conversion of positive lysine residue into bulky hydrophobic NBD-moiety may cause a conformational distortion which is unfavorable for electron flow.

Draheim et al. (1985) has shown that the copper center of oxidized plastocyanin is rigid and invariant. However, the protein portion of molecule seems flexible with respect to the environment change. The redox-dependent conformational change involved both tyrosine and phenylalanine. Our results indicate that the vicinity of lysine-54 may also subjected to redox-dependent conformational change. Therefore, the redox state may influence the structure of whole molecule rather than the aromatic amino acids only.

Salts have been shown to promote the interaction of plastocyanin with $P_{700}^+$ (Lockau, 1979; Lien and San Pietro, 1979; Tamura et al., 1980; Haehnel et al., 1980), while inhibiting that between plastocyanin and cytochrome f. These results have been interpreted as charge screening (Boultier et al., 1977). However, Gross et al. (1985) found that concentration of CaCl$_2$ at 10 mM was sufficient to saturate the interaction of plastocyanin with $P_{700}$ and had no effect on plastocyanin fluorescence. In this report, lower concentration of CaCl$_2$ (2.5 mM)
decrease 20% NBD-plastoycyanin fluorescence, although increase in concentration of CaCl₂ (or MgCl₂) has no further effect. The change of fluorescence of NBD-plastoycyanin reflects possible conformational change upon salt addition. The result is consistent with the notion that higher salt concentrations are probably due to change in hydrophobic interaction rather than simple charge screening (Gross et al., 1985). The absorbance and fluorescence of NBD-plastoycyanin are pH dependent. There are maximal at pH above 8 and minimal at pH below 4. This result reveals that the conformation of plastoycyanin is pH dependent. Thus, the conformation of plastoycyanin will be different when chloroplasts are illuminated and pH in lumen of thylakoid drops. In this case, the proton motive force generated under illumination controls the rate of electron flow between plastoycyanin and its physiological reaction partners. It is likely that pH affects electron transport through protonation of carboxyl groups, tyrosine and histidine (Gross et al., 1985). Affinity of PSI-plastoycyanin complex through charge screening of negative charge (carboxylate) cluster (Burkey and Gross, 1981) and through conformational change exerted by lysine residue (this work).

In conclusion, NBD-Cl is a good modifier for plastoycyanin. Modification of Lys-54 induced conformational change of plastoycyanin and diminishes electron transport between plastoycyanin and PSI.

**Acknowledgements:** We gratefully acknowledge Mrs. J.H. Chow for her editorial and secretarial assistance.

**Literature Cited**


菠菜藍質體的修飾—備製與特性

王美雲 蔡梅玉 潘榮隆 E. L. Gross

國立清華大學輻射生物研究所

菠菜藍質體經由 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-CI) 化學修飾，其修飾衍生藍質體具有螢光作用。化學修飾使由藍質體至 P570 電子傳遞 Vmax 降低 30%，Km 則由 15μM 降低至 5μM，並且還原電位差提高約 +10mV。經修飾的藍質體在 A480 的吸光度與 P570 的螢光度可作為探測藍質體在環境因子影響下造成的結構變化，主要的修飾位置被範定是在藍質體 Lysine-54。