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

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Abstract. Spinach plastocyanin was modified by 7-chloro-4-nitrobenz-2-oxa-1, 3-diazole (NBD-Cl) whose alkylamine derivative was fluorescent. The V_{max} for electron transport from plastocyanin to P_{700}^+ was decreased by 30% after modification. The K_m 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 (A_{480}) and fluorescence (F_{550}) 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[4]) 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 faciliated 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

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[4] **Abbreviations:** Chl, Chlorophyll; NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1, 3-diazole; PSI, Photosystem I; PC, Plastocyanin.

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 Borchert and Wessels (1970). The purified plastocyanin had a final A_{278}/A_{597} 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_{597} or according to a modified Lowry's method (Larson *et al.*, 1986). Oxidized plastocyanin was obtained by adding excess $K_3Fe(CN)_6$ which was subsquently 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 $\varepsilon_{480}=26{,}000\ M^{-1}\,cm^{-1}$ for amino derivative of NBD and $\varepsilon_{597}=4.9\ mM^{-2}\,cm^{-1}$ 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 μg 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 substracting 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 μM) 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 spectophotometer (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 NH_4HCO_3 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.

Ressults

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 growed. On the other hand, the absorbance of oxidized

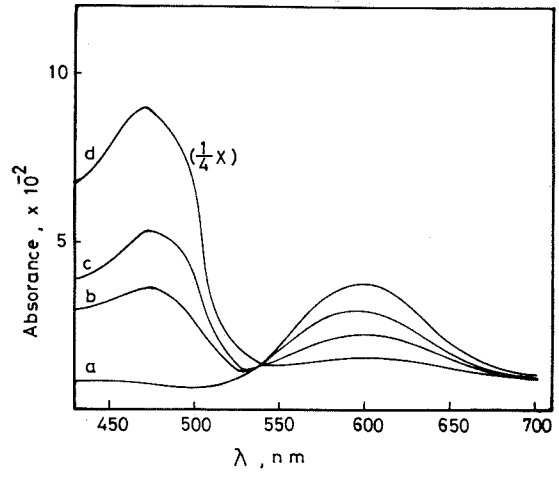

Fig. 1. Absorption spectra of plastocyanin during incubation wilh NBD-Cl. The modification of plastocyanin with NBD-Cl was carried out as described under "Materials and Methods". Incubation time: (a) control, (b) 1 h, (c) 2.5 h, (d) 24 h.

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 550nm (Aboderin *et al.*, 1973). Figure 4 displays the fluorescence emission spectrum of control plastocyanin and NBD-plastocyanin.

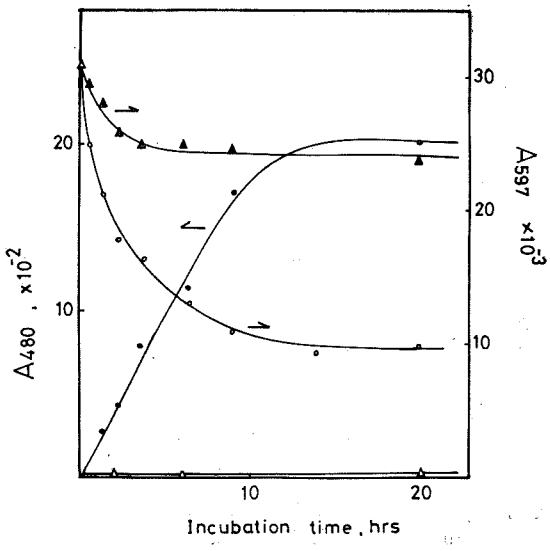

Fig. 2. Time course of change of absorption peak for NBD-plastocyanin. Conditions were the same as in Fig. 1. (▲) control at A_{597}, (○) A_{597}, (●) A_{480}.

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

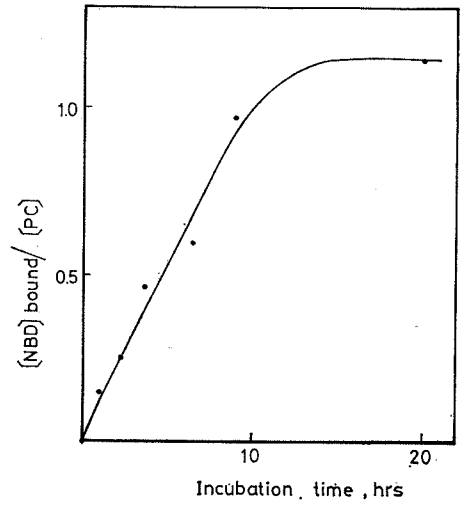

Fig. 3. Time course of incorporation of NBD-moiety on plastocyanin. The reaction conditions and calculation of molecular fraction incorporated were described under "Materials and Methods".

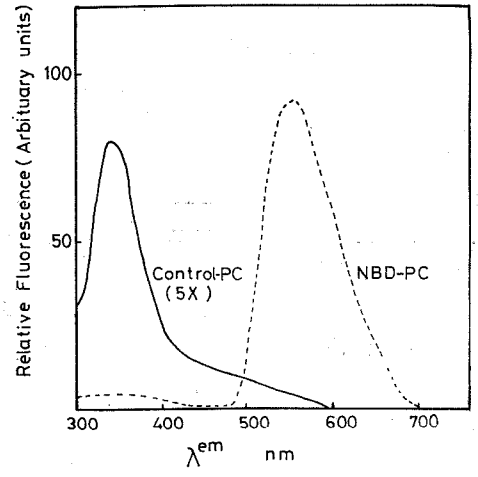

Fig. 4. The relative fluorescence emission spectra of plastocyanin and NBD-plastocyanin. Conditons were described as under "Materials and Method". (——) Control plastocyanin, (·····) NBD-plastocyanin.

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_{480} (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 μM to 4–5.5 μM. 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 modificaition 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}^+.

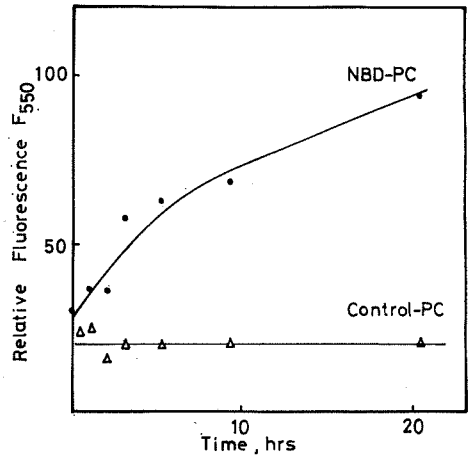

Fig. 5. Time course of growth of F_{550} for NBD-plastocyanin. Conditions were as in Fig. 4. (●) NBD-plastocyanin, (△) Control plastocyanin.

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_{480} and F_{550} of NBD-plastocyanin

Urea showed no significant effect on A_{480} of NBD-plastocyanin even at concentration of 8.0 M (Fig. 6a). On the other hand, heating of NBD-plastocyanin increased A_{480} by 67% within 100 min. The hydrolysis of NBD-moiety from NBD-plastocyanin was found to be negligible

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

Experiment[a]	Treatment	V_{max} ($\frac{\mu mol\ P_{700}^+\ reduced}{mg\ Chl.\ h}$)	K_m (μM)	Inhibition of V_{max} (%)
I	Control	76.0	15.4	100
	Modified	43.5	4.2	43
II	Control	56.0	12.5	100
	Modified	38.5	5.6	31

[a] Reaction mixture contained: 15μg Chl/ml PSI, 10mM Tris-Cl, pH 8.0, 5mM $MgCl_2$, 50 μM ascorbate. The volume of reaction mixture was 3 ml.

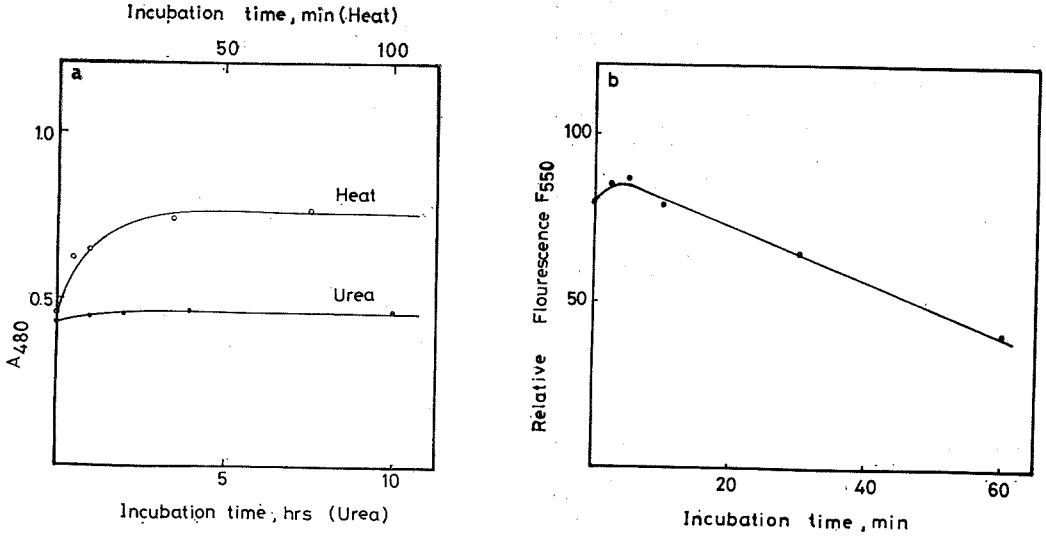

Fig. 6. (a) The change for A_{480} of NBD-plastocyanin upon treatment of denaturation agents, heat (○) and urea (●). (b) The time course of A_{480} during heat treatment.

(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 2M NH_2OH (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 likly 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 cytohrome *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

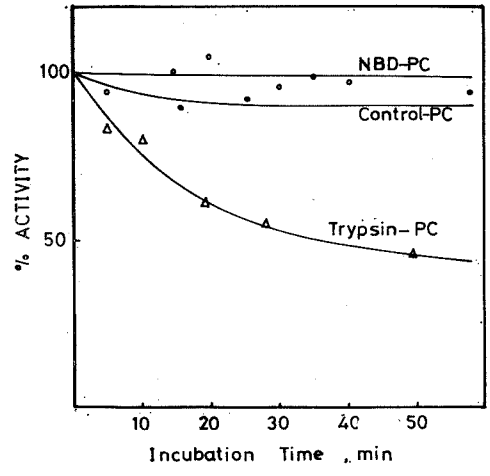

Fig. 7. The relative resistance of modified plastocyanin to tryptic digestion. The reaction conditions of control plastocyanin were the same as that of others except the addition of trypsin. (●) control, (○) NBD-plastocyanin, (△) trypsin digested plastocyanin.

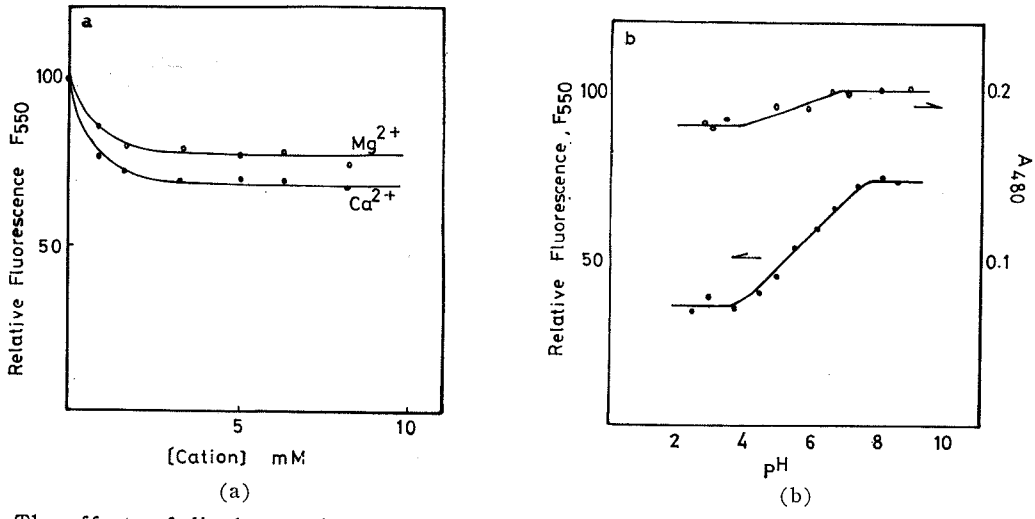

Fig. 8. The effects of divalent cations and pH on NBD-plastocyanin. The conditions were the same as described in Fig. 4, except the various concentrations of cations and pH indicated. (a): (○) Mg^{+2}, (●) Ca^{+2}, (b): (○) A_{480}, (●) F_{550}.

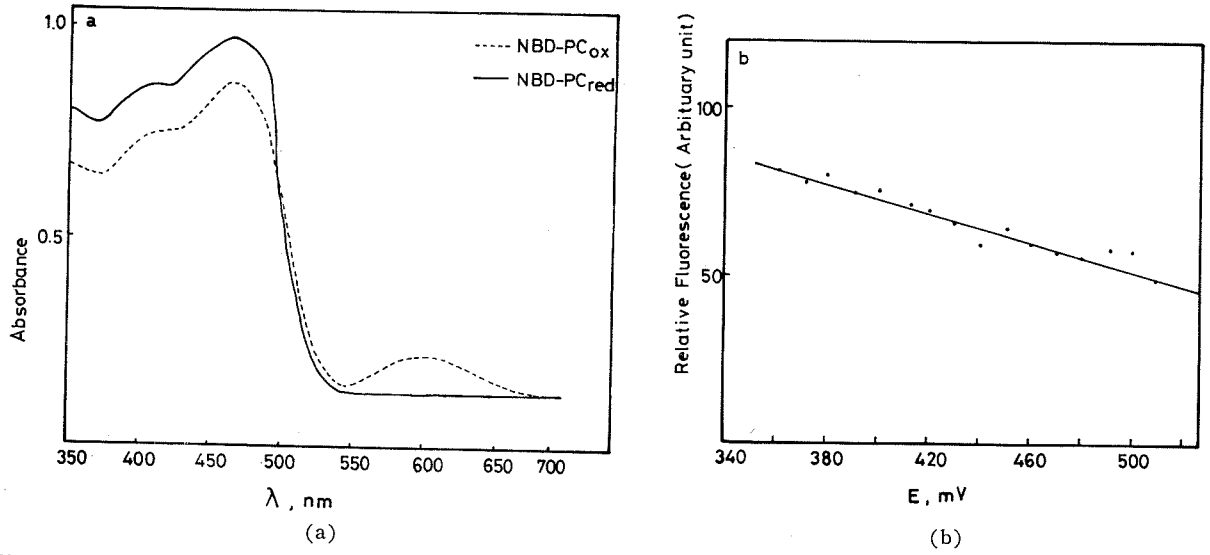

Fig. 9. (a) The absorption spectra of NBD-plastocyanin in oxidized or reduced conditions. The preparation of oxidized or reduced form of NBD-plastocyanin was described under "Materials and Methods". (——) reduced form, (·····) oxidized form. (b) The dependence of fluorescence (F_{550}) of plastocyanin on redox state. The redox potential was maintained by ferricyanide/ferrocyanide couples as described under "Materials and Methods"

decreased F_{550} by 35% for Ca^{2+} and 25% for Mg^{2+}, respectively (Fig. 8). Obviously, the fluorescence as probe to the environmental change is much more sensitive than absorption (Lakowicz, 1983).

The A_{480} and F_{500} 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 A_{480} and F_{550} 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_{480} while F_{550}

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

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

Redox state	A_{480}	F_{550} (arbitrary units)
Reduced	0.91 (100%)	100
Oxidized	0.80 (88%)	64

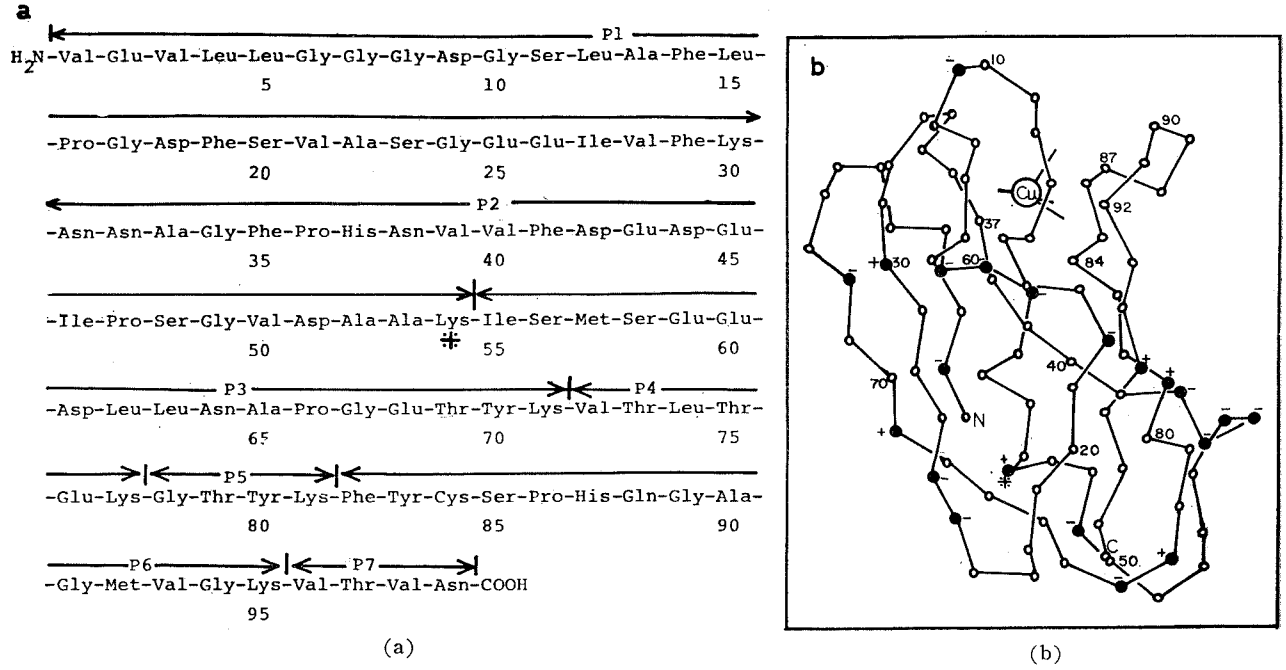

Fig. 10. (a) Primary structure of spinach plastocyanin [reprinted from Scawen *et al.* (1975)]. The peptides produced by trypsin cleavage of plastocyanin are shown. The lysine residue (Lys-54) modified by NBD-Cl was indicated with asterisk. (b) Amino acid sequence of spinach plastocyanin projected on 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 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 (Boulter *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-55, 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 plastocaynin 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 (Boulter *et al.*, 1977). However, Gross *et al.* (1985) found that concentration of $CaCl_2$ at 10 mM was suffficient to saturate the interaction of plastocyanin with P_{700} and had no efffect on plastocyanin fluorescence. In this report, lower concentraton of $CaCl_2$ (2.5 mM)

decrease 20% NBD-plastocyanin fluorescence, although increase in concentration of $CaCl_2$ (or $MgCl_2$) has no further effect. The change of fluorescence of NBD-plastocyanin 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 fluoroscence of NBD-plastocyanin are pH dependent. There are maximal at pH above 8 and minimal at pH below 4. This result reveals that the conformation of plastocyanin is pH dependent. Thus, the conformation of plastocyanin 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 plastocyanin 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-plastocyanin 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 plastocyanin. Modification of Lys-54 induced conformational change of plastocyanin and diminishes electron transport between plastocyanin and PSI.

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菠菜藍質體的修飾——備製與特性

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菠菜藍質體經由 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) 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 P_{700} 電子傳遞 V_{max} 降低 30%，K_m 則由 15μM 降低至 5 μM，並且還原電位差提高約+10mV。經修飾的藍質體在 A_{480} 的吸光度與 F_{550} 的螢光度可作爲探測藍質體在環境因子影響下造成的結構變化，主要的修飾位置被鑑定是在藍質體 Lysine-54。