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COVALENT MODIFICATION OF CHLOROPLAST PHOTOSYSTEM II POLYPEPTIDES BY *p*-NITROTHIOPHENOL

J.E. MULLET [a], C.J. ARNTZEN [a], Y. KOBAYASHI [b] and Y. INOUE [b,*]

[a] *USDA/SEA/AR, Department of Botany, University of Illinois, Urbana, IL 61801 (U.S.A.) and* [b] *RIKEN, The Institute of Physical and Chemical Research, Wako-shi, Saitama 351 (Japan)*

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Summary

Illumination of the chlorophyll *a/b* light-harvesting complex in the presence of *p*-nitrothio[^{14}C]phenol caused quenching of fluorescence emission at 685 nm (77 K) relative to 695 nm and covalent modification of light-harvesting complex polypeptides. Fluorescence quenching saturated with one *p*-nitrothiophenol bound per light-harvesting complex polypeptide (10—13 chlorophylls); 1/2 maximal quenching occurred with one *p*-nitrothiophenol bound per light-harvesting complex polypeptides (190—247 chlorophylls). This result provides direct evidence for excitation energy transfer between light-harvesting complex subunits which contain 4—6 polypeptides plus 40—78 chlorophylls per complex.

Illumination of chloroplasts or Photosystem II (PS II) particles in the presence of *p*-nitrothio[^{14}C]phenol caused inhibition of PS II activity and labeling of several polypeptides including those of 42—48 kilodaltons previously identified as PS II reaction center polypeptides. In chloroplasts, inhibition of oxygen evolution accelerated *p*-nitrothiophenol modification reactions; DCMU or donors to PS II decreased *p*-nitrothiophenol modification. These results are consistent with the hypothesis that accumulation of oxidizing equivalents on the donor side of PS II creates a 'reactive state' in which polypeptides of PS II are susceptible to *p*-nitrothiophenol modification.

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* To whom reprint requests should be addressed.

Abbreviations: CCCP, carbonyl cyanide m-chlorophenylhydrazone; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; LHC, chlorophyll *a/b* light-harvesting complex; PS II, Photosystem II; Chl, chlorophyll; Tricine, *N*-tris(hydroxymethyl)methylglycine.

Introduction

Illumination of chloroplast membranes causes the formation of a membrane potential, a pH gradient, and creates localized regions of charge density in reaction center complexes [1]. These light-induced phenomena, in turn, may alter the interactions between electron-transport components [2] and may elicit structural perturbations within membrane bound protein complexes. This latter idea is supported by the selective light-dependent chemical modification of chloroplast proteins by *p*-nitrothiophenol [3—5] and diazoniumbenzenesulfonic acid [6].

Lipophilic thiol compounds have been reported to inhibit chloroplast activity at three sites. Sireci et al. [7] reported that lipophilic thiol compounds inhibit reduction of ferricyanide when forward electron flow toward Photosystem I is blocked by addition of DBMIB(2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone) or KCN. These authors suggested that lipophilic thiols inhibit cyclic electron flow around PS II. Additional sites of thiol inhibition have been investigated extensively by Kobayashi et al. [3—5]. The light-dependent action of *p*-nitrothiophenyl has been shown to occur at two sites; the first involved selective quenching of 685 nm fluorescence emission (77 K) and the second inhibited the donor side of PS II [3,5]. The action of *p*-nitrothiophenol in these studies was reported to be regulated by the oxidation state of the donor side of PS II [4].

In the present study, *p*-nitrothiophenol modification of proteins in chloroplast membranes, LHC and PS II particles was examined. Simultaneous measurement of PS II activity, fluorescence and radioactive labeling revealed conditions leading to light-induced exposure of *p*-nitrothiophenol binding sites.

Materials and Methods

Chloroplasts were isolated from spinach leaves by grinding for 10 s, 4°C, in 0.4 M sorbitol, 0.05 M Tricine-NaOH, pH 7.8, 5 mM $MgCl_2$, followed by filtering through eight layers of cheesecloth and centrifugation at $3000 \times g$ for 5 min. The chloroplast pellet obtained was resuspended in 0.02 M Tricine-NaOH, pH 7.8, 5 mM $MgCl_2$ and centrifuged at $1000 \times g$ for 15 s. The resulting supernatant was collected and centrifuged at $5000 \times g$ for 5 min. The chloroplast pellet obtained was resuspended in 0.2 M sorbitol, 0.02 M Tricine-NaOH, pH 7.8, and 5 mM $MgCl_2$. Chlorophyll concentrations were determined by the method of Arnon [8]. Tris-treatment of chloroplast fractions was done as previously described [4]. Tris-treated chloroplasts were pelleted by centrifugation at $5000 \times g$ for 5 min and resuspended in 0.2 M sorbitol, 0.02 M Tricine-NaOH, pH 7.8, 5 mM $MgCl_2$.

LHC was prepared from peas by the method of Burke et al. [9] modified as previously reported [10]. PS II particles were prepared from peas by the procedure of Mullet and Arntzen [11]. ^{14}C-Labeled *p*-nitrothiophenol (8.72 mC/mmol) was obtained from New England Nuclear (Boston). Stock solutions of *p*-nitrothiophenol (1 mM) were prepared in the presence of 1 mM dithiothreitol (to reduce disulfide bridges) and stored in darkness under N_2. The stock solution of *p*-nitrothiophenol was centrifuged at $5000 \times g$ for 10 min immediately prior to use.

^{14}C-Radioactivity was determined by liquid scintillation counting. Chlorophyll-containing samples were bleached (prior to liquid scintillation counting) by heating at 70°C in 5% H_2O_2, 1% SDS for 30 min. Unlabeled *p*-nitrothiophenol was obtained from Tokyo Kasei Co. (Tokyo) and purified before use [12]. Gel electrophoresis of radioactive samples was done as previously described [13]. ^{14}C-Labeled polypeptides were detected by autoadiography of 2,5-diphenyloxazole-incorporated gels [14]. PS II activity and 77 K fluorescence emission was measured as previously described [3,4]. Chloroplasts were illuminated for 10 min in the presence of *p*-nitrothiophenol (75 μM). LHC and PS II were resuspended in 20 mM Tricine-NaOH, pH 7.8 for the *p*-nitrothiophenol treatment.

Results

A. Sites of p-nitrothiophenol modification

Illumination of chloroplasts in the presence of *p*-nitrothiophenol results in inhibition of PS II activity and quenching of 685 nm fluorescence emission [3—5]. It has been suggested that these changes result from light dependent covalent modification of PS II polypeptides by *p*-nitrothiophenol [4]. In order to identify the polypeptides modified by *p*-nitrothiophenol, chloroplast membranes were illuminated for 10 min in the presence of *p*-nitrothio[^{14}C]phenol and the membrane polypeptides were subsequently separated on SDS-polyacrylamide gels (Fig. 1, MEMB). Chloroplast membranes exposed to *p*-nitrothio[^{14}C]phenol in the dark were not labeled. ^{14}C-Labeled polypeptides were identified by fluorography (Fig. 1, ARG MEMB = autoradiogram of membrane polypeptides). This analysis revealed that treatment of chloroplast membranes with *p*-nitrothio[^{14}C]phenol labeled several polypeptides including polypeptides of 25—29 kilodaltons previously reported to be components of the LHC [9]. To verify this point, isolated LHC particles (slot-LHC) were incubated with *p*-nitrothio[^{14}C]phenol in the light and then subjected to gel separation and fluorography (Fig. 1, LHC = stained polypeptides; ARG LHC = autoradiogram of LHC sample). These results indicate that polypeptides of the LHC are accessible to *p*-nitrothiophenol modification in chloroplasts and in isolated LHC particles.

Chloroplast membrane polypeptides of 29—34 kilodaltons were labeled by *p*-nitrothio[^{14}C]phenol treatment (Fig. 1, ARG MEMB). Polypeptides of 32 kilodaltons have recently been identified as constituents of PS II [10,15]. *p*-Nitrothio[^{14}C]phenol modification of chloroplast membranes also labeled polypeptides of 42—48 kilodaltons. Polypeptides of this molecular weight are found in PS II particles (see slot, PS II) and have been tentatively identified as reaction center Chl-proteins [16]. *p*-Nitrothiophenol modification of PS II polypeptides of 42—48 kilodaltons was confirmed using isolated PS II particles (Fig. 1. PS II = stained gel of separated PS II polypeptides; ARG PS II = autoradiogram of PS II polypeptide sample).

p-Nitrothio[^{14}C]phenol treatment of chloroplast membranes labeled polypeptides of 55—65 kilodaltons (Fig. 1, ARG-MEMB). Polypeptides of this molecular weight have been reported in ATPase preparations (α and β subunits of the ATPase; 58 and 55 kilodaltons, respectively).

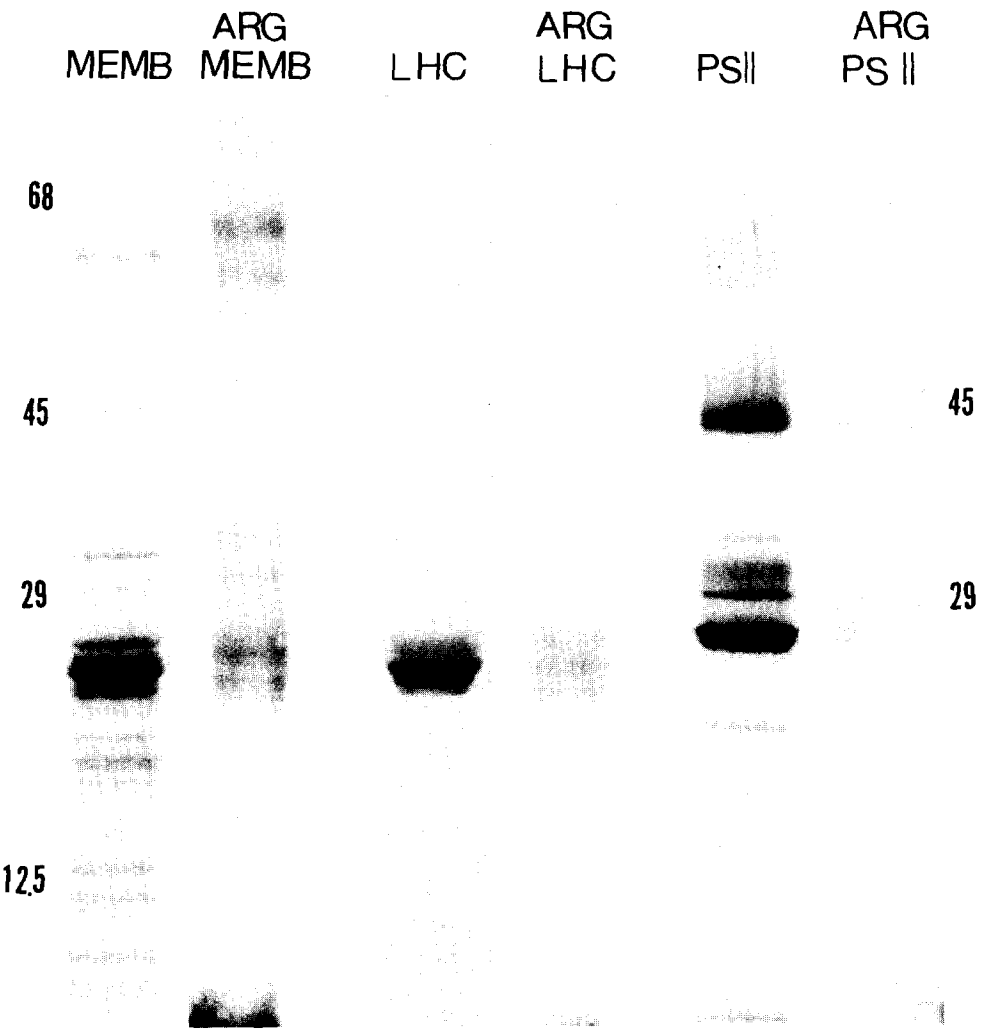

Fig. 1. SDS-polyacrylamide gradient gel (7.5—15%) and corresponding autoradiograms (labeled ARG) showing *p*-nitrothio[^{14}C]phenol labeled polypeptides which were modified after illumination in the presence of *p*-nitrothio[^{14}C]phenol. The following samples are shown; chloroplast membranes (MEMB and ARG MEMB), LHC (LHC and ARG LHC) and PS II (PS II and ARG PS II). Molecular weight markers are shown at the right, bovine serum albumin (68 000), ovalbumin (45 000), carbonic anhydrase (29 000), cytochrome *c* (12 500).

B. Modification of PS II activity by p-nitrothiophenol

Treatment of chloroplast membranes with *p*-nitrothiophenol has been shown to inhibit PSII mediated photoreduction of DCIP (±diphenyl carbazide as donor to PS II) [3,4]. This observation was verified in the present study (see Table I). Chloroplasts treated with *p*-nitrothiophenol in the dark were not inhibited and were not labeled by *p*-nitrothio[^{14}C]phenol (Table I). Illumination of chloroplast membranes for 10 min in the presence of 75 μM *p*-nitrothiophenol inhibited PS II mediated DCIP photoreduction by approx. 45%. Chloroplast membrane polypeptides were labeled by *p*-nitrothio[^{14}C]phenol

TABLE I

THE EFFECT OF VARIOUS COMPOUNDS ON *p*-NITROTHIOPHENOL MODIFICATION OF PS II ACTIVITY AND PROTEIN LABELING IN CHLOROPLASTS

Control and Tris-treated chloroplasts were incubated for 10 min in either actinic light or kept in the dark in reaction mixtures containing 75 μM *p*-nitrothiophenol plus the following additions where indicated: 50 μM DCMU, 100 μM diphenyl carbazide, 50 μM phenylenediamine plus 1 mM ascorbate, 100 μM tetraphenyl boron. Control rates of DCIP photoreduction were 300—350 μmol DCIP reduced per mg Chl per h. Maximum labeling by *p*-nitrothio[^{14}C]phenol (value of 100 in the table) = 0.15 μmol *p*-nitrothiophenol per mg Chl. N.D., rates or amounts not determined.

Condition	Relative PS II activity (diphenyl carbazide → DCIP)		*p*-Nitrothio[^{14}C]phenol labeling (relative)	
	Cloroplasts	Tris-treated chloroplasts	Cloroplasts	Tris-treated chloroplasts
Control (minus *p*-nitrothiophenol)	100	105	0	0
Dark	100	110	2	2
Light	55	17	100	218
+ DCMU	75	60	60	93
+ diphenyl carbazide	N.D.	35	N.D.	115
+ phenylenediamine plus ascorbate	N.D.	50	N.D.	95
+ tetraphenyl boron	17	3	48	62

concomitantly with inhibition of PS II activity (see Fig. 1 and Table I). Light-dependent *p*-nitrothiophenol inhibition of PS II activity and ^{14}C-labeling was accelerated in Tris-treated chloroplast membranes. Tris treatment of the chloroplast membranes inactivated oxygen evolution as has been previously reported [17].

p-Nitrothiophenol-induced inactivation of PS II could be partially blocked by DCMU. DCMU also blocked *p*-nitrothio[^{14}C]phenol labeling of chloroplast membrane polypeptides (Table I). Diphenyl carbazide, a donor to PS II, has previously been reported to protect chloroplast membranes from *p*-nitrothiophenol-induced inactivation of PS II [4]. The data in Table I show that diphenyl carbazide also partially blocks *p*-nitrothiophenol-induced inactivation of PS II in Tris-treated chloroplasts. The presence of diphenyl carbazide or phenylenediamine plus ascorbate during sample illumination also partially blocked *p*-nitrothio[^{14}C]phenol labeling of chloroplast polypeptides.

Tetraphenyl boron has been reported to inactivate oxygen evolution [18]. The data in Table I show that tetraphenyl boron increased *p*-nitrothiophenol-induced inactivation of PS II activity in chloroplasts and Tris-treated chloroplasts. In contrast, tetraphenyl boron decreased *p*-nitrothio[^{14}C]phenol incorporation into chloroplast polypeptides (Table I). This result suggested that tetraphenyl boron selectively blocked *p*-nitrothiophenol incorporation; incorporation into sites which affect PS II activity was accelerated whereas overall incorporation of *p*-nitrothiophenol was reduced.

C. p-Nitrothiophenol modification of fluorescence emission

Chloroplast membranes exhibit major fluorescence emission peaks at 77 K at 685, 695 and 736 nm. Fluorescence emission at 685 nm and 695 nm have been reported to originate from the PS II-LHC Chl-protein complex in chloroplasts

[19]. Isolated LHC particles have also been reported to exhibit fluorescence emission at 681—683 nm [20,21] and also at 695—699 nm when the LHC complexes are aggregated into membrane-like sheets [20]. Incubation of chloroplasts, LHC, or PS II with *p*-nitrothiophenol in the dark did not modify 685 nm or 695 nm fluorescence emission (Table II). Illumination of chloroplasts, LHC or PS II particles in the presence of *p*-nitrothiophenol caused quenching of fluorescence emission peaking at 685 nm relative to the fluorescence emission peaking at 695 nm. In chloroplasts, the relative emissions at 695 and 736 nm were changed only slightly. The *p*-nitrothiophenol-induced quenching of 685 nm fluorescence is expressed in Table II as a change in the ratio of the intensity of fluorescence, 695 nm/685 nm.

In chloroplasts, donors to PS II such as diphenyl carbazide or phenylenediamine plus ascorbate, were found to paritially block *p*-nitrothiophenol-induced quenching of fluorescence emission whereas treatments which inhibited oxygen evolution (CCCP or Tris-treatment) were found to increase *p*-nitrothiophenol-induced quenching. *p*-Nitrothiophenol-induced quenching observed in chloroplasts, Tris-treated chloroplasts and CCCP-treated chloroplasts could be partially blocked (75%) by DCMU. In contrast, DCMU did not block *p*-nitrothiophenol-induced quenching of fluorescence in PS II particles even though DCMU (10^{-4} M) inhibited greater than 85% of PS II mediated DCIP photoreduction in these particles [11]. Chloroplast membranes also became insensitive to the DCMU blocking affect on *p*-nitrothiophenol modification after treatment with 6 M urea. Urea-treated chloroplasts exhibited increased *p*-nitrothiophenol-induced quenching of fluorescence which was not blocked in the presence of DCMU (Table II). Urea treatment, in the dark, did not affect the fluorescence emission ratio.

TABLE II

THE EFFECT OF VARIOUS COMPOUNDS ON *p*-NITROTHIOPHENOL MODIFICATION OF THE RATIO OF FLUORESCENCE EMITTED AT 695 nm DIVIDED BY FLUORESCENCE OF 685 nm AT 77 K

Chloroplasts, Tris-treated chloroplasts, PS II and LHC particles (25 μg Chl/ml) were illuminated (10 min, white actinic light) or kept in the dark in reaction mixtures containing 75 μg *p*-nitrothiophenol plus the following additions where indicated: 50 μM DCMU, 100 μM tetraphenyl boron, 50 μM CCCP, 100 μM diphenyl carbazide, 50 μM phenylenediamine plus 1 mM ascorbate, 6 M urea.

Condition	F_{695}/F_{685} at 77 K			
	Chloroplasts	Tris-treated chloroplasts	PS-II	LHC
Control (minus *p*-nitrothiophenol)	1.3	1.3	0.75	0.70
Dark	1.3	1.3	0.72	0.70
Light	2.5	3.9	1.51	1.90
+ DCMU	1.6	1.9	1.40	—
+ tetraphenyl boron	1.3	1.3	0.96	1.00
+ CCCP	3.9	—		
+ CCCP plus DCMU	1.7	—		
+ diphenyl carbazide	—	1.9		
+ phenylenediamine plus ascorbate	—	1.7		
+ urea	3.1	—		
+ urea plus DCMU	2.9	—		

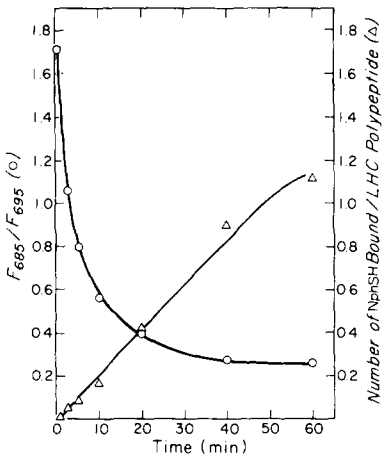

Fig. 2. Time course of incorporation of *p*-nitrothio[^{14}C]phenol into the polypeptides of the LHC and ratio of fluorescence emission at 685 nm divided by fluorescence emission at 695 nm at 77 K, which occurs during illumination of LHC in the presence of 75 μM *p*-nitrothiophenol.

Tetraphenyl boron was particularly effective in blocking *p*-nitrothiophenol modification of fluorescence. This compound blocked *p*-nitrothiophenol-induced quenching of fluorescence at 685 nm in chloroplasts, Tris-treated chloroplasts and partially blocked fluorescence quenching in PS II particles and LHC (Table II).

D. p-Nitrothiophenol, a probe of excitation energy transfer between LHC subunit

LHC subunits exhibit 77 K fluorescence emission at 685 nm and 695 nm [20]; fluorescence at 685 nm is quenched relative to fluorescence at 695 nm when LHC is modified by *p*-nitrothiophenol (see Table II). Fig. 2 shows a time course of *p*-nitrothiophenol modification of F_{685}/F_{695} and incorporation of ^{14}C-label into the LHC particles. At maximal quenching approx. 1 mol *p*-nitrothiophenol was bound per LHC polypeptide. At 1/2 maximal quenching 1 mol *p*-nitrothiophenol was bound per 19 LHC polypeptides.

Discussion

A. Site of p-nitrothiophenol modification

Lipophilic thiol compounds have been reported to modify cyclic electron flow around PS II [7], PS II fluorescence emission [3—5], and PS II activity [3—5]. Light pretreatment is not required to observe inhibition of cyclic electron flow around PS II by aliphatic or aromatic thiol compounds [7]. In contrast, in studies with aromatic thiol reagents (i.e. *p*-nitrothiophenol), inhibition of PS II activity and modification of PS II fluorescence emission were reported only when samples were illuminated in the presence of the lipophilic compound [4]. The absorptive properties of the aromatic ring may therefore participate in light-mediated activation of the thiol group involved in modification of PS II activity and fluorescence.

It has previously been reported that illumination of chloroplast membranes

in the presence of *p*-nitrothiophenol inhibits PS II activity [4]. Fig. 1 showed that polypeptides of 42—48 kilodaltons, previously reported to be Chl-proteins of the PS II reaction center [16], are covalently labeled with *p*-nitrothio[^{14}C]-phenol. This observation suggests that *p*-nitrothiophenol inhibits PS II activity by covalently modifying polypeptides involved in the primary PS II photoreactions.

p-Nitrothiophenol binding to isolated LHC subunits yielded labeled LHC polypeptides (Fig. 1, ARG LHC). Previous studies have revealed that *p*-nitrothiophenol may bind to proteins by acyl-thiol or disulfide bonds [22]. The LHC polypeptides have been shown to lack free sulfhydryl groups [10]. Therefore it is probable that *p*-nitrothiophenol is bonded to LHC via an acyl-thiol bond.

B. Formation of the 'reactive state'

Light-dependent incorporation of *p*-nitrothio[^{14}C]phenol into chloroplast membrane polypeptides was shown. Treatments which blocked oxygen evolution (CCCP or Tris-treatment) increased *p*-nitrothio[^{14}C]phenol labelling and accelerated inhibition of PS II activity (Table I) and quenching of 77 K fluorescence emission at 685 nm (Table II). DCMU, an inhibitor of electron transport in PS II, was found to partially block (75%) the *p*-nitrothiophenol-induced changes in chloroplasts and Tris-treated chloroplasts (Table I). In contrast, DCMU did not block *p*-nitrothiophenol-induced fluorescence changes in PS II particles. This result can be explained in the following way. *p*-Nitrothiophenol modification of chloroplast membranes is hypothesized to involve two light-requiring steps. The first establishes a 'reactive state' in which sites for *p*-nitrothiophenol modification are exposed. The second step involves a light requiring conversion reaction which results in covalent binding of *p*-nitrothiophenol to polypeptides. According to this hypothesis, the rate of *p*-nitrothiophenol modification of chloroplast membranes is dependent on the lifetime of the 'reactive state' (i.e. exposure of sites for *p*-nitrothiophenol modification). In contrast, *p*-nitrothiophenol sites in PS II or LHC particles are hypothesized to be exposed at all times due to the solubilization procedure utilized to isolate these complexes. *p*-Nitrothiophenol modification of PS II particles or LHC thus requires only the conversion reaction and should be insensitive to inhibitors of the 'reactive state' such as DCMU. The results in Table II on PS II particles are consistent with this idea. Treatments which expose sites in chloroplast membanes in an irreversible manner should render the preparations insensitive to DCMU. To test this possibility, urea, a chaotropic agent, was added to chloroplast membranes prior to *p*-nitrothiophenol modification. This treatment accelerated *p*-nitrothiophenol modification of fluorescence and caused *p*-nitrothiophenol-modification to become insensitive to DCMU (Table II). It is suggested that urea caused irreversible exposure of *p*-nitrothiophenol binding sites in the chloroplast membranes.

The 'reactive state' in chloroplasts is correlated with the oxidized state of the donor side of PS II [5]. Tris-treatment or CCCP, which increased *p*-nitrothiophenol modification (Table I), inhibits oxygen evolution and caused accumulation of oxidizing equivalents on the donor side of PS II. DCMU, which partially blocks *p*-nitrothiophenol modification, inhibits turnover of PS II reaction

centers and thereby blocks accumulation of oxidizing equivalents. Donors to PS II, diphenyl carbazide and phenylenediamine plus ascorbate, were found to decrease *p*-nitrothiophenol modification of fluorescence in chloroplasts (Table II). Donors to PS II would decrease the number and lifetime of oxidizing equivalents on the donor side of PS II thereby shortening the lifetime of the 'reactive state' and decreasing *p*-nitrothiophenol modification reactions.

The 'reactive state' may have been previously measured by prompt fluorescence. The analysis of Duysens [23] of F_0 and F_{max} in prompt fluorescence, led to the hypothesis that energy coupling between antennae chlorophyll in the PS II-LHC complex exists in two conformations. It was suggested that a decrease in coupling between Chl in the PS II antennae and Chl in the PS II reaction center occurs in the presence of oxidized P-680. In the present work a P-680$^+$ induced decrease in antennae coupling would correspond to formation of the 'reactive state' and exposure of *p*-nitrothiophenol reactive sites.

C. Excitation energy transfer between LHC subunits and chlorophyll species in LHC

Isolated LHC particles consist of polypeptides of 25—29 kilodaltons plus associated chlorophylls *a* and *b* structurally organized into subunits 80 Å in diameter [20]. It can be estimated, based on average protein density, that each LHC subunit consists of 4—6 LHC polypeptides plus 40—78 chlorophylls (each polypeptide has been reported to bind 10—13 chlorophylls) [9,10].

p-Nitrothiophenol modification of LHC quenches 685 nm fluorescence emission relative to fluorescence emission at 695 nm (Fig. 2). This effect has been previously reported in chloroplast membranes [3]. The data in Fig. 2 reveal that 1/2 maximal fluorescence quenching occurs when one *p*-nitrothiophenol was bound per approximately 19 LHC polypeptides (190—280 chlorophylls). Saturation of fluorescence quenching required one *p*-nitrothiophenol bound per LCH polypeptide (10—13 chlorophylls). This observation may be explained by assuming that excitation energy transfer occurs between the purified LHC subunits. At 1/2 maximal quenching one *p*-nitrothiophenol is calculated to be capable of quenching excitatione energy from a minimum of two 80 Å LHC subunits (i.e. 8—12 LHC polypeptides plus chlorophyll). The reason why fluorescence emission at 695 nm exhibits relatively less quenching compared to fluorescence at 685 nm may be related to the physical distance of the chlorophyll species which emitts fluorescence at 695 nm from the *p*-nitrothiophenol binding site and/or limited up-hill transfer at 77 K, which energetically isolates this chlorophyll from quenching sites.

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