Biochemica et Biophysica Acta, 423 (1976) 80–90

BBA 47017

INHIBITORY EFFECT OF *p*-NITROTHIOPHENOL IN THE LIGHT ON THE PHOTOSYSTEM II ACTIVITY OF SPINACH CHLOROPLASTS

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(Received June 24th 1975)

SUMMARY

The treatment of spinach chloroplasts with *p*-nitrothiophenol in the light at acidic and neutral pH's caused specific inhibition of the Photosystem II activity, whereas the same treatment in the dark did not affect the activity at all. The photosystem I activity was not inhibited by *p*-nitrothiophenol both in the light and in the dark. The inhibition was accompanied by changes of fluorescence from chloroplasts. As observed at room temperature, the 685-nm band was lowered by the *p*-nitrothiophenol treatment in the light and, at liquid nitrogen temperature, the relative height of the 695-nm band to the 685-nm band increased and the 695-nm band shifted to longer wavelengths. The action spectra for these effects of *p*-nitrothiophenol on the activity and fluorescence showed a peak at 670 nm with a red drop at longer wavelengths. It was concluded that the light absorbed by Photosystem II is responsible for the chemical modification of chloroplasts with *p*-nitrothiophenol to causing the specific inhibition of Photosystem II.

INTRODUCTION

Whole chloroplasts and subchloroplast particles or membranes undergo conformational changes on illumination. The changes in these dimensional orders have been observed directly be means of a Coulter counter [1] or electron microscope [2] and indirectly as a change of light-scattering [3]. For smaller particles such as the coupling factor on thylakoid membranes, Ryrie and Jagendorf [4, 5] succeeded in observing a difference in the degree of tritium labelling between samples in the light and in the dark. The success suggested that chemical modification is a useful tool for detecting a conformational change of the order of biopolymers by light. In fact, McCarty et al. [6, 7] found that *N*-ethylmaleimide is incorporated into the coupling factor in the light but not in the dark, and McCarty [8] showed that 1-ethyl-3(dimethylaminophenol)-carbodiimide inhibits the electron transport

Abbreviations: NphSH, *p*-nitrothiophenol; Cl_2Ind, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

between the two photosystems and photophosphorylation only in the light. More recent studies by Dilley and co-workers [9–11] revealed that *p*-diazonium benzene sulfonate in the light inhibits the oxidation of water by Photosystem II and the electron flow on the reducing side of Photosystem I. They suggested that a conformational change of membranes is responsible for these inhibitions by *p*-diazonium benzene sulfonate.

The requirement of light for the specific inhibition by a chemical reagent will provide a useful approach in the studies of functional aspects of membrane topography and changes of the tertiary structure of proteins. A reagent, *p*-nitrothiophenol (NphSH) was examined in the present study for its effects on spinach chloroplasts in the light and in the dark. It was found that this reagent specifically inhibits the Photosystem II activity of chloroplasts only in the light. The fluorescence from the chloroplasts was also changed by the action of this reagent in the light. The present paper describes these activity and fluorescence changes together with the action spectra for these changes.

MATERIALS AND METHODS

Treatment of chloroplasts with NphSH

Chloroplasts isolated from spinach leaves by the procedure described previously [12] were suspended in a medium containing 50 mM Tris/HCl (pH 7.4), 0.4 M sucrose and 10 mM NaCl. A solution (4.5 ml) of NphSH in the same medium was added to the chloroplast suspension containing 150 μg of chlorophyll in 0.5 ml, and the mixture was incubated at 20 °C under illumination. The reagent, NphSH (Tokyo Kasei Co.) was purified before use according to Fromm and Wittmann [13]. In most of the experiments except for the measurements of activity-intensity curves or action spectra, the red light from a 300-watt Canon projector through a red glass filter (V-R65, Toshiba Kasei Co. transmitting the light longer than 630 nm), a neutral density filter (transmittance = 10 %, Toshiba Kasei Co.) and a 10-cm layer of water was used for the illumination. The light intensity at the surface of the reaction vessel (path length = 1.5 cm) was 140 μW/cm^2. The same mixture was incubated in complete darkness to obtain a control. After incubation for a desired period in the dark or in the light, the chloroplasts in the mixture were sedimented by centrifugation at 10000 $\times g$ for 5 min, and the supernatant containing unreacted NphSH was discarded. The sediment was then re-suspended in the isolation medium and subjected to the measurement of the Hill activity. These procedures were carried out after the incubation under dim green safety light.

In the measurements of activity-intensity curves or action spectra, monochromatic light from a Bausch and Lomb grating monochromator equipped with a 45-watt halogen lamp was used for the illumination. The light intensity in the measurement of activity-intensity curves was controlled with a combination of neutral density filters (Toshiba Kasei Co.). The light intensity in the measurement of action spectra was 6.5 μW/cm^2 which was controlled with the slit in the monochromator. The half-band width of monochromatic light was 6$\pm$1 nm between 550 and 750 nm. Light intensities were measured with a Kipp and Zonen thermopile model E2 standardized with a standard lamp (National Bureau of Standards, U.S.A.).

Activity measurements

The rate of photoreduction of 2,6-dichlorophenolindophenol by the chloroplasts treated with NphSH was measured by following the absorbance decrease at 593 nm in the same manner as in the previous study [14]. The reaction mixture contained 150 μM Cl_2Ind and chloroplasts containing 18 μg of chlorophyll in 3 ml of the isolation medium. The rate of oxygen evolution or uptake was measured with a Clark-type oxygen electrode. The reaction was run at 18 °C under illumination with the light from a 300-watt Canon projector through a 10-cm layer of water. The reaction mixture for oxygen evolution contained 2 mM ferricyanide and chloroplasts containing 200 μg of chlorophyll in 5 ml of the above medium. The reaction mixture for oxygen uptake contained 150 μM Cl_2Ind, 1 mM ascorbate, 10 μM 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea (DCMU), 0.5 mM methylviologen, 0.5 mM sodium azide, 5 mM $MgCl_2$ and chloroplasts containing 200 μg of chlorophyll in 5 ml of the medium. The concentration of chlorophyll was determined according to the method of MacKinney [15].

Fluorescence measurements

The fluorescence spectrum in the red region of a reaction mixture at liquid nitrogen temperature (77 °K) was measured with a Shimadzu recording fluorometer model RF-502. The fluorescence excited with the 440-nm light from a monochromator with a 500-watt xenon lamp was lead to another monochromator through a Toshiba filter (V-R60, Toshiba Kasei Co.) transmitting the light longer than 580 nm. The fluorescence intensity through the monochromator was measured with a photomultiplier, R-666 (Hamamatsu TV), having a red sensitive photocathode of Ga-As. Fluorescence spectra were not corrected for the variation of photomultiplier sensitivity and monochromator efficiency with wavelength. It was confirmed in a separate experiment that NphSH in the reaction mixture does not interfer with the fluorescence measurement. Intensity of the reaction mixture at room temperature was measured at a maximum wavelength, 685 nm. The fluorescence reached a maximum intensity within 2 s after onset of actinic light (440 nm). The maximum intensity of a mixture with chloroplasts treated with NphSH in the light was compared with that of its control treated in the dark.

RESULTS

The effect on the Photosystem II activity

Table I summarizes the effects of NTP on the activities of Photosystems I and II in the light and in the dark. The Photosystem II activity as measured either as oxygen evolution supported by the electron transport from water to ferricyanide or as Cl_2Ind photoreduction was inhibited by illumination of chloroplasts with red light for 10 min in the presence of NphSH, whereas the same activity was not affected at all by the same treatment in the dark. A similar experiment on the Photosystem I activity measured as the oxygen uptake mediated by the electron transport from $Cl_2Ind \cdot H_2$ to methylviologen showed no inhibition by NphSH both in the light and in the dark. Addition of an artificial electron donor, diphenylcarbazide, to the reaction mixture after the NphSH treatment in the light did not restore the lost Photosystem II activity of Cl_2Ind photoreduction. Further

TABLE I

EFFECTS OF THE NphSH TREATMENT IN THE LIGHT AND IN THE DARK ON THE PHOTOSYSTEM I ACTIVITY OF METHYLVIOLOGEN-MEDIATED O_2 UPTAKE AND ON THE PHOTOSYSTEM II ACTIVITIES OF FERRICYANIDE-DEPENDENT O_2 EVOLUTION AND Cl_2Ind PHOTOREDUCTION

Chloroplasts (Chl) (30 μg/ml) were treated with 100 μM NphSH at pH 7.4 for 10 min under illumination with red light at an intensity of 140 $\mu W/cm^2$ or in the dark.

Assay	Control	NphSH treatment	
		Light (10 min)	Dark (10 min)
O_2 uptake (μmol $O_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$)	520	528	517
O_2 evolution (μmol $O_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$)	89	17	86
Cl_2Ind photoreduction (μmol $Cl_2Ind \cdot mg^{-1}$ Chl $\cdot h^{-1}$)	152	21	158

inhibition experiments were conducted by measuring Cl_2Ind phtoreduction as a measure of the Photosystem II activity.

Curve A in Fig. 1 shows the process of inhibition measured with 100 μM NphSH under illumination with the light of 675 nm at an intensity of 140 $\mu W/cm^2$. The inhibition proceeded by 85 % for 10 min of illumination while the same treatment in the dark casued no inhibition as seen from curve B.

The necessity for concurrent illumination of chloroplasts was confirmed from the following experiments. (1) Chloroplasts were pre-incubated with 100 μM NphSH for 1 h at 4 °C in darkness and then illuminated for 5 min with red light. The result indicated no increment of inhibition due to the pre-incubation as compared with the degree of inhibition determined without preincubation. (2) Chloroplasts were pre-illuminated for 10 min with red light in the absence of NphSH, and then 100 μM NpsSH was added to the chloroplast suspension in the dark at 1, 5 and 10 s

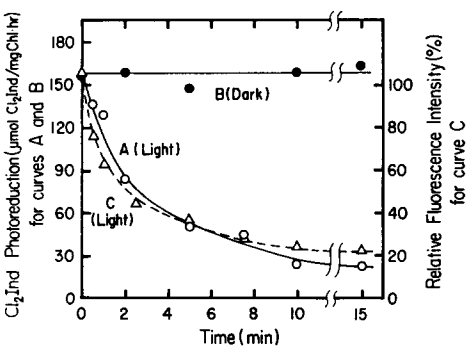


Fig. 1. Time courses of the inhibition of Cl_2Ind photoreduction and the decrease of fluorescence intensity at room temperature by the action of NphSH; Cl_2Ind photoreduction in the light (curve A, ○ — ○) and in the dark (curve B, ● — ●) and fluorescence intensity at 685 nm (curve C, △ — △). Chloroplasts (30 μg Chl/ml) were incubated with 100 μM NphSH under illumination with the monochromatic light of 675 nm at an intensity of 140 $\mu W/cm^2$ or in the dark.

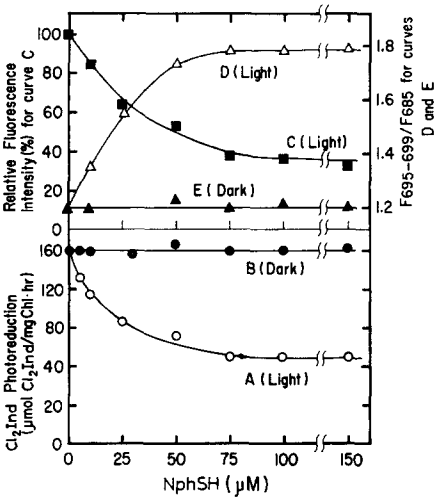


Fig. 2. Effects of NphSH concentration on the activity of Cl_2Ind photoreduction, the fluorescence intensity at room temperature and the ratio of F695–699/F685 in the light and in the dark; curves A (○ – ○) and B (● – ●) for the activity after the treatment in the light and that in the dark, respectively; curve C (■ – ■) for the fluorescence intensity (685 nm) at room temperature; curves D (△ – △) and E (▲ – ▲) for the ratio of F695–699/F685 at 77 °K after the treatment in the light and in the dark, respectively. Chloroplasts (30 μg Chl/ml) were treated with NphSH for 5 min in the red light (140 μW/cm²) and in the dark.

after the pre-illumination. The result obtained after 10 min of the dark treatment indicated no inhibition of the activity.

Curve A in Fig. 2 shows the residual activity measured as a function of NphSH concentration which was measured after 5 min of illumination with red light. The activity was inhibited by 70 % above 75 μM of NphSH whereas the treatment in the dark caused no inhibition even at the highest concentration of 150 μM (curve B).

The two curves on the left side of Fig. 3 show the degree of inhibition with

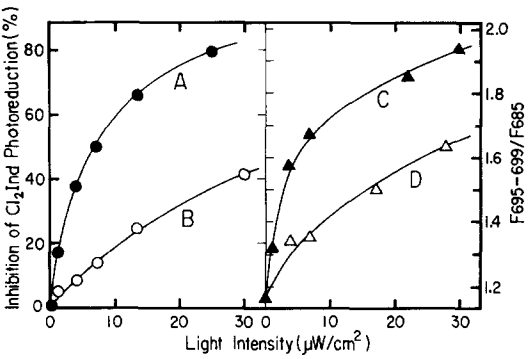


Fig. 3. Effects of light intensity on the degree of inhibition of Cl_2Ind photoreduction and the ratio of F695–699/F685 at 77 °K by the treatment with 100 μM NphSH for 15 min in monochromatic light (curves A and C at 680 nm and curves B and D at 550 nm). The concentration of chloroplasts was 30 μg Chl/ml.

TABLE II

EFFECT OF pH ON THE INHIBITION OF Cl_2Ind PHOTOREDUCTION BY NphSH

Chloroplasts (30 μg Chl/ml) in 50 mM phosphate buffer or 50 mM Tricine buffer containing 0.4 M sucrose and 10 mM NaCl were treated with 100 μM NphSH for 5 min in red light (140 $\mu W/cm^2$) or in the dark. The degree of inhibition was calculated from the activity data in the light and in the dark at the same pH. The activities were measured in Tris · HCl buffer (pH 7.4) as described in Methods.

pH	Phosphate buffer				Tricine buffer				
	5	6	7	7.4	7.4	8	9	9.5	10
Inhibition (%)	80	76	65	48	49	50	41	35	7

100 μM NphSH as a function of light intensity. Curve A was measured for the reaction mixtures illuminated for 15 min with the light of wavelength 680 nm, and curve B for the mixtures illuminated for the same period with the light of wavelength 550 nm. It is evident from these curves that the red light is more effective than the green light, and that approximate proportionality exists between activity and light intensity below 1 $\mu W/cm^2$ for the red light. NphSH dissolved in the medium for treatment showed an absorption band at 412 nm, so that NphSH in the reaction mixture to some extent absorbed the blue light. This precluded carrying out an inhibition experiment with the light in the blue region. The blue region of 440 nm was less effective than the red light, but this was partially due to absorption by NphSH.

Table II summarizes the effect of pH on the degree of inhibition which was measured after 5 min of the treatment with 100 μM NphSH in the red light at an intensity of 140 $\mu W/cm^2$. The activity of the reaction mixture treated in the dark at the same pH was taken as the control to calculate the degree of inhibition. The degree of inhibition was greater at acidic and neutral pH's and dropped abruptly above pH 9.5.

The effect on the fluorescence spectrum

As is well known, the sample suspension of spinach chloroplasts at room temperature showed a fluorescence band at 685 nm in the red region. The position of this band was not affected but its height was markedly lowered by the NphSH treatment in the light. The same treatment of the control in the dark did not affect the height or the band position. The intensity change during the treatment with 100 μM NphSH in the light (675-nm light, 140 $\mu W/cm^2$) is shown by curve C in Fig. 1, in which the ratio of the intensity after a certain period of treatment in the light to that after the same period of treatment in the dark was plotted against time. This curve for the intensity change is similar to curve A showing the process of the inhibition of Cl_2Ind photoreduction. The lowered fluorescence intensity was enhanced by the addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). For example, the intensity after the treatment with 100 μM NphSH for 5 min which was 36 % of the original height before the treatment was recovered by the addition of 20 μM DCMU to 61 % of the original height without DCMU.

The sample suspension of spinach chloroplasts at liquid nitrogen temper-

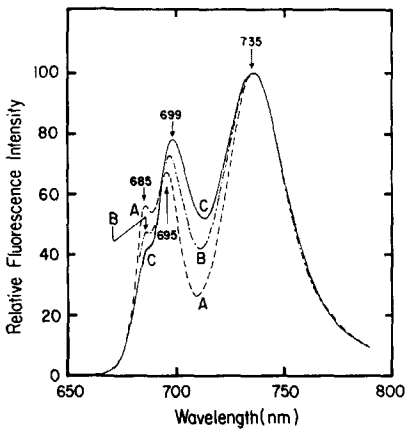


Fig. 4. Changes of fluorescence spectrum at 77 °K by the treatment with NphSH in the light. The height of F735 is made identical for the three curves of A, B and C; curve A for chloroplasts before the NphSH treatment and curves B and C for chloroplasts after the treatment with 100 μM NphSH in the red light (140 μW/cm^2) for 5 and 10 min, respectively. The figures with arrows on these curves show the maximum wavelength in nm. The chloroplast concentration in the reaction mixture was 17.5 μg Chl/ml.

ature (77 °K) showed three fluorescence bands at 685, 695 and 735 nm such as shown by curve A in Fig. 4; these bands or their heights are expressed in this paper by F685, F695 and F735, respectively. The relative heights of these three bands changed in an interesting manner onNphSH treatment in the light. Such changes observed after incubation with 100 μM NphSH in the red light for 5 and 10 min are shown by curves B and C, respectively, in the same figure where the height of F735 is made identical for the three curves of A, B and C. During incubation, the height of F685 relative to F695 or F735 decreased progressively and the maximum wavelength of the middle peak gradually shifted from 695 to 699 nm, while the F685 and F735 bands did not shift at all. The ratio, F695–699/F685, which was used as a measure of this relative height change, was 1.2, 1.5 and 1.9 for curves A, B and C measured after illumination for 0, 5 and 10 min, respectively, and was 2.7 after 20-min illumination. The treatment of the same sample for 30 min in the dark or the addition of NphSH in the dark after pre-illumination for 10 min did not bring about such a change of relative heights at 77 °K. Attempts to follow the change in height of each band independently were unsuccessful because the degree of band intensification by the low temperature fluorometry fluctuated considerably.

The fluorescence intensity at room temperature and the ratio of F695–699/F685 at 77 °K after the NphSH treatment in thelight are plotted as a function of NphSH concentration on curves C and D respectively, in Fig. 2. These curves level off above 75 μM NphSH, being similar to the activity drop shown by curve A in the same figure. The controls treated at various NphSH concentrations in the dark showed no change of the F695–699/F685 ratio as shown by curve E. Curves C and D in Fig. 3 show the changes of the ratio as a function of the light intensity at 680 and 550 nm, respectively. These curves are similar to curves A and B, respectively, which were obtained by activity measurements.

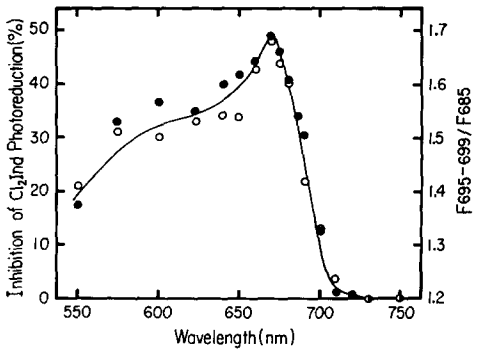


Fig. 5. Action spectra for the inhibition of Cl_2Ind photoreduction and for the change of fluorescence spectrum; ● — ●, for the inhibition of Cl_2Ind photoreduction; ○ — ○ for the change of F695–699/F685 ratio at 77 °K. The activity and the fluorescence spectrum were measured after illumination of the chloroplast suspension (30 μg Chl/ml) for 15 min with monochromatic light of an equal intensity of 6.5 $\mu W/cm^2$ in the presence of 100 μM NphSH, and the action spectra for the activity and the fluorescence ratio were calculated from the data. Corrections were made in the calculation to convert the data in equal intensities to those in equal quanta.

The action spectra for inhibition and fluorescence change

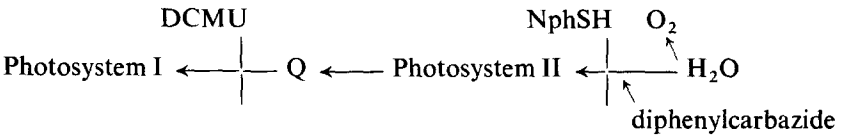
The light requirement prompted us to measure the action spectrum for the inhibitory effect of NphSH. The measurement was made by illuminating the sample for 15 min with monochromatic light at an intensity of 6.5 $\mu W/cm^2$ in the presence of 100 μM NphSH. According to the intensity curves in Fig. 3, this intensity is in the proportionality range for the measurement at 550 nm (curve B) but is out of the range at 680 nm (curve A). The degrees of inhibition measured after illumination at lower intensities, however, fluctuated considerably because of small readings of inhibition. This forced the authors to use the above intensity which is a compromise between these conditions for proportionality and accuracy. The degrees of inhibition thus measured are plotted with solid circles in Fig. 5. A similar experiment was carried out by measuring the ratio of F695–F699/F685 at 77 °K for the samples treated under the same conditions of light intensity and NphSH concentration, and the data are plotted with open circles in the same figure. As seen from this figure, these activity and fluorescence data followed the same curve when plotted in an appropriate relative scale. The curve shows a peak at 670 nm with a broad shoulder around 600 nm. The slightness of the effect of the far-red light above 700 nm suggests that the light absorbed by Photosystem I is not functional at all or much less functional than the light absorbed by Photosystem II. The flatness of the 670-nm band as compared with the red band in the absorption spectrum is likely to be due to the light intensity out of the proportionality range used for the measurement.

DISCUSSION

It was found in the present study that NphSH applied to chloroplasts in the light inhibits the Photosystem II activity and changes the fluorescence spectrum. The action spectra for these effects of NphSH showed a peak at 670 nm with a broad shoulder on its shorter wavelength side and a steep drop above 670 nm on the longer

wavelength side. These characteristics indicate that the light absorbed by Photosystem II is responsible for the inhibition and for the fluorescence change.

The Photosystem II activity lost by NphSH treatment in the light was not recovered by the addition of diphenylcarbazide, which is a reagent that donates electrons at a site between the reaction center of Photosystem II and the water-splitting system [16]. This indicates that the site of inhibition is on the reductive side of the site of electron donation by diphenylcarbazide. The fluorescence intensity at room temperature decreased in a similar time course (curve C in Fig. 1), and the decreased intensity was partially recovered by the addition of DCMU. A decrease of fluorescence intensity was found by Yamashita and Butler [17] by the inhibition of electron transport on the oxidation side of Photosystem II, and the enhancement of fluorescence by addition of DCMU to a chloroplast suspension was interpreted by Duysens [18] as due to the inhibition of re-oxidation of the reduced form of Q, a hypothetical compound on the reduction side of photosystem II. The fluorescence decrease by the NphSH treatment may, therefore, be ascribed to the inhibition on the oxidation side of Photosystem II. Summarizing these, the site of the inhibition by NphSH may be between the reaction center of Photosystem II and the electron-donating site of diphenylcarbazide;



Giaquinta et al. [9] showed that *p*-diazonium-benzene sulfonic acid inhibits the Photosystem II activity in the light. Recently, Arntzen et al [19] showed that the lactoperoxidase-catalyzed iodination of chloroplast membranes inhibits photophosphorylation, Photosystems I and II activities. They assumed that membrane-structural changes by the iodination of constituent amino acids (e.g. tyrosine and histidine residues) of proteins inhibit the Photosystem II activity. However, the addition of diphenyl-carbazide to the *p*-diazonium-benzene sulfonic acid-treated chloroplasts or iodinated chloroplasts partially restored the Photosystem II electron transport to Cl_2Ind. The mode of the NphSH inhibition seems, therefore, to be different from that of the inhibition by the acid treatment and iodination.

Tonomura et al. [20–23] demonstrated that NphSH binds to myosin in the presence of Mg^{2+} and ATP. They suggested that NphSH combines by a covalent bond to a carboxyl side chain of glutamic or aspartic acid on a protein molecule to form an acyl-thiol bond. The smaller degree of inhibition found at alkaline pH's (Table II) in the present experiment may be due to the instability of the acyl-thiol bond at the alkaline pH's [20].

The fluorescence change observed at 77 °K revealed that the change is not a simple process. The relative height, F695–699/F685, increased progressively and the middle band shifted from 695 to 699 nm during illumination for 10 min with red light (Fig. 4). A similar change of fluorescence spectrum was found by Satoh [24, 25], who treated chloroplasts with a mixture of a chaotropic reagent (urea or guanidine) and 1,10-phenanthroline. The shift of the 695-nm band to 700 nm found by the treatment was interpreted as being due to the appearance of a new band at a longer

wavelength around 700 nm. Satoh suggested that the mechanism was the alternation of the apoprotein for the fluorophore [24, 25]. The fluorescence bands at 685 and 695 nm were observed more strongly for the particles of Photosystem II than for the particles of Photosystem I [26–28]. It was postulated that the 695-nm band is emitted from the chlorophylls associated with the energy trap of Photosystem II [29–32]. Deduction, and the necessity of concurrent illumination of chloroplasts with NphSH for the inhibition and the fluorescence change together with these previous observations tempted the authors to postulate a light-induced conformational change of a protein in Photosystem II as the primary step of the mechanism for inhibition and fluorescence change. A site in the protein molecule responsible for electron transport from the electron-donating site of diphenylcarbazide to the reaction center of Photosystem II may be exposed by the conformational change to be modified with NphSH. The present result may indicate that a carboxyl side chain present in the site plays an important role in electron transport on the oxidation side of Photosystem II.

ACKNOWLEDGEMENT

The present study was supported by a research grant on "photosynthetic reaction centers" given by the Ministry of Education and by a grant for the study of "Life sciences" at The Institute of Physical and Chemical Research (Rikagaku Kenkyusho).

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