Biochimica et Biophysica Acta, 440 (1976) 600–608

BBA 47157

LIGHT-DEPENDENT INHIBITORY ACTION OF *p*-NITROTHIOPHENOL ON PHOTOSYSTEM II IN RELATION TO THE REDOX STATE OF ELECTRON CARRIERS

YOSHICHIKA KOBAYASHI, YORINAO INOUE and KAZUO SHIBATA

Laboratory of Plant Physiology, The Institute of Physical and Chemical Research (Rikagaku Kenkyusho), Wako-shi, Saitama (Japan)

(Received February 27th, 1976)

SUMMARY

The photosystem-II activity of chloroplasts was inhibited by the treatment with *p*-nitrothiophenol (NphSH) in the light, and the inhibition was accompanied by a change of the fluorescence spectrum. Aromatic mercaptans examined were active in causing this inhibition and fluorescence change. These effects of *p*-nitrothiophenol were highly accelerated by blocking the electron transport on the oxidation side of photosystem II by carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) or Tris · HCl or heat pre-treatment, whereas these were suppressed by blocking the transport on the reduction side by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). It was deduced that the site of NphSH action in the electron transport chain is closer to the reaction center of photosystem II that the blocking site of CCCP or Tris · HCl or heat, and that such a site in photosystem II is exposed to be modified with NphSH when electron carriers on the oxidation side of photosystem II are oxidized by illumination.

INTRODUCTION

Useful informations about events in chloroplast membranes during illumination have been obtained by observing conformational changes of biopolymers in the membranes [1, 2]. Chemical modifications of proteins in chloroplasts in the light revealed that some proteins bound to thylakoid membranes respond to illumination to be modified with reagents [3–7]. The treatment of chloroplasts with *p*-nitrothiophenol (NphSH) in the light was found in a previous study [8] to cause specific inhibition of the photosystem-II activity measured as oxygen evolution or 2,6-dichlorophenolindophenol (Cl_2Ind) photoreduction. The inhibition was accompanied by a change of fluorescence spectrum measured at liquid nitrogen temperature.

Abbreviations: NphSH, *p*-nitrothiophenol; Cl_2Ind, 2,6-dichlorophenolindophenol; CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DABS, *p*-diazonium benzene sulfonic acid.

During the illumination of chloroplasts with NphSH, the height of the 685-nm fluorescence band (F685) relative to the 695-nm or 735-nm band (F695 or F735) decreased progressively, and the maximum wavelength of the middle peak gradually shifted from 695 nm to 700 nm. The increase of the ratio, F695-700/F685, during the illumination was used in the previous experiment as a measure of this fluorescence change. The action spectra for these effects of NphSH indicated that the light absorbed by photosystem II was responsible for these activity and fluorescence changes.

The present study was undertaken to see the correlation of the effects of NphSH to the redox state of electron carriers in the chain. The redox states of electron carriers were changed by use of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) as inhibitors or by Tris- and heat-treatments to block the electron transport on either the reduction or oxidation side of photosystem II.

MATERIALS AND METHODS

Chloroplasts were isolated from spinach leaves and suspended in the isolation medium (50 mM Tris · HCl, pH 7.4, 0.4 M sucrose and 10 mM NaCl). NphSH purchased from Tokyo Kasei Co. was purified before use [9]. A solution (4.5 ml) of NphSH in the same medium was added to the chloroplast suspension containing 170 μg of chlorophyll in 0.5 ml, and the reaction mixture was incubated in the light or in the dark at 20 °C for a desired period [8]. Monochromatic light (670 nm, 140 $\mu W/cm^2$) from a Baush and Lomb grating monochromator equipped with a 45-watt halogen lamp was used to illuminate the reaction mixture. After a certain period of incubation of the reaction mixture, the chloroplasts were sedimented by centrifugation at 10 000 $\times g$ for 5 min, and the sediment was resuspended in the isolation medium under a dim green safety light and subjected to various measurements. The activity of Cl_2Ind photoreduction and the fluorescence spectrum at 77° K were measured in the same manner as reported previously [8]. The reaction mixture (3 ml) for the Cl_2Ind photoreduction contained 150 μM Cl_2Ind and chloroplasts containing 50 μg of chlorophyll. The concentration of chlorophylls was determined according to the method of MacKinney [10]. The same treatment with 100 μM NphSH was made on (a) intact chloroplasts suspended in the isolation medium containing CCCP or DCMU, (b) chloroplasts pre-treated with Tris · HCl or by heat, and (c) particles of photosystems I and II. The pre-treatment with Tris · HCl (0.8 M, pH 8.0) was made for 20 min at 0° C [11]. The heat pre-treatment was made for 5 min at 55 °C in the isolation medium. Particles of photosystems I and II were isolated from spinach chloroplasts with 0.5 % digitonin according to the procedure of Anderson and Boardman [12].

The absorbance changes of C550, carotenoid and cytochrome *f* were measured by dual-wavelength spectrophotometry with a Shimadzu recording spectrophotometry model UV-300. The sample was illuminated with actinic red light ($\geqslant$ 670 nm, 8 mW/cm^2) through a Toshiba VR-67 filter. A Corning-9782 filter was placed in front of the photomultiplier to block the actinic light.

Similar treatments were made with a number of other reagents related to NphSH. The reagents examined and their concentrations are listed in Table I.

RESULTS

Specificity of thiophenols as reagent

Before studying the action of NphSH, various aromatic and aliphatic mercaptans and some other related compounds were examined to determine the structural requirement for a reagent to cause the activity and fluorescence changes found in the previous study [8]. The results obtained with 16 different reagents are summarized in Table I where the percent inhibition of the Cl_2Ind-photoreduction activity and the F695-700/F685 ratio after the treatment are listed for each reagent. The concentrations of these reagents were 1 mM except for thiocresol, thiophenol and *p*-chloromercuribenzoic acid examined at 10 μM. The treatment with a reagent was made for 10 min in the light. The activity and the ratio after the same period of treatment in the dark were 133 ± 12 μmol · mg chlorophyll · h and 1.16 ± 0.08, respectively, except in the case of thiocresol and thiophenol.

All of the four aromatic mercaptans examined were effective in causing the inhibition and the fluorescence change, among which thiocresol and thiophenol were much stronger than NphSH and thiosalicylic acid. Thiocresol and thiophenol inhibited the activity at the much lower concentration of 10 μM in the light and the fluorescence ratio after the treatment with these reagents were much greater than those obtained with the other two aromatic mercaptans at 1 mM. The treatment with these stronger reagents at 10 μM raised the fluorescence ratio appreciably even in the dark (1.37 and 1.77 with thiocresol and thiophenol, respectively), but the activity was not much affected. The treatment with these stronger reagents at a higher concentration of 1 mM in the dark affected the activity and fluorescence more strongly.

Aromatic phenols or acids devoid of sulfhydryl groups such as *p*-nitrothiophenol, *p*-nitrophenol, *p*-nitrobenzoic acid, *p*-aminobenzoic acid and *m*-nitrobenzene-

TABLE I

THE EFFECTS OF MERCAPTANS AND RELATED COMPOUNDS ON THE Cl_2Ind-PHOTOREDUCTION ACTIVITY AND THE FLUORESCENCE-INTENSITY RATIO, F695-700/F685, BETWEEN 695-700 AND 685 nm.

Chemicals	Inhibition (%)	F695-700/F685
p-Nitrothiophenol (1 mM)*	72	1.89
Thiosalicylic acid (1 mM)	62	1.80
Thiocresol (10 μM)	75	7.26
Thiophenol (10 μM)	47	16.30
5,5′-Dithiobis (2-nitrobenzoic acid)* (1 mM)	20	1.36
Inactive reagents	$\pm$ 5	1.16 ± 0.08

Aliphatic mercaptans (L-cysteine* and thioglycolic acid* at 1 mM)
Aromatic derivatives without SH group (*p*-nitrosophenol, *p*-nitrophenol, *p*-nitrobenzoic acid, *p*-aminobenzoic acid and sodium-*m*-nitrobenzenesulfonate at 1 mM)
Reagents for SH modification (*N*-ethylmaleimide* at 1 mM and *p*-chloromercuribenzoic acid* at 10 μM)
Reagents for COOH modification (2-ethyl-5-*m*-sulfophenylisoxazolium hydroxide* and *N,N′*-dicyclohexylcarbodiimide* at 1 mM)

* These reagents were dissolved in the isolation medium and the other were dissolved in a small volume of ethanol (5 % in the reaction mixture).

sulfonic acid at 1 mM were ineffective in causing these changes either in the light or in the dark. Aliphatic mercaptans such as L-cysteine and thioglycolic acid were also ineffective. 5,5'-Dithiobis(2-nitrobenzoic acid), Ellman's reagent for the assay of sulfhydryl groups in proteins, is effective to cause these changes. However, it was confirmed that the changes were caused by 5-thio-2-nitrobenzoic acid formed by the reduction of this reagent by the photoreaction of photosystem I. In fact, photosystem-II particles did not undergo such a fluorescence change on the treatment with this reagent. These comparative data indicate that the sulfhydryl residue on the benzene ring is essential to cause the inhibition or the fluorescence change. *N*-ethylmaleimide and *p*-chloromercuribenzoic acid which are reagents for the assay of sulfhydryl groups, were ineffective. *p*-Chloromercuribenzoic acid was examined at 10 μM because of solubility limitation. Two other reagents examined were 2-ethyl-5-*m*-sulfophenylisoxazolium hydroxide (Woodward's reagent) and *N,N'*-dicyclohexylcarbodiimide for modification of carboxyl groups. These reagents were also ineffective.

The effect of NphSH on photosystem I

The light-minus-dark difference spectrum was measured with intact and NphSH-treated chloroplasts in the presence of ascorbate to see the photo-oxidation of cytochrome *f* in photosystem I. The spectrum obtained for the chloroplasts treated with 100 μM NphSH for 10 min in the light showed a peak of cytochrome *f* at 554 nm which was identical in position and height with the peak obtained for intact chloroplasts. The photo-oxidation of P700 was also measured on isolated photosystem-I particles before and after the treatment with NphSH in the light, and the result showed no effect of the NphSH treatment on the photo-oxidation.

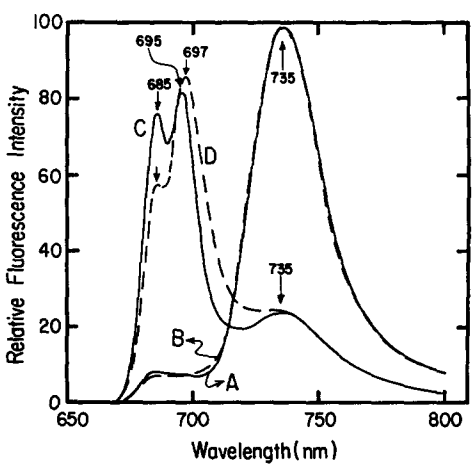

Fig. 1. The changes of fluorescence spectra at 77 °K caused by the treatment of the particles of photosystems I and II with 100 μM NphSH in the red light (670 nm, 140 μW/cm^2) for 5 min; curve A, photosystem-I particles before the NphSH treatment and curve B, after the treatment; curve C, photosystem-II particles before the treatment and curve D, after the treatment. The height of the 735-nm band is taken to be identical for these curves. The figures with arrows on the curves indicate the maximum wavelength in nm.

Curve A in Fig. 1 is the fluorescence spectrum at 77 °K of photosystem-I particles. Treatment of the particles with 100 μM NphSH for 5 min in the light did not alter the major band of photosystem-I particles at 735 nm. Two weak bands at shorter wavelengths were changed to some extent (curve B) but this may be regarded as being due to contamination by photosystem-II particles.

The effect of NphSH on photosystem II

Illumination of chloroplasts in the presence of CCCP [13] or after the treatment with 0.8 M Tris · HCl [11] bleaches carotenoids. Pre-treatment of intact chloroplasts with 100 μM NphSH for 10 min in the light, however, suppressed the bleaching, and the addition of 100 μM CCCP to NphSH-treated chloroplasts did not induce the bleaching.

Fig. 2 shows the time course of light-induced absorbance change of C550, which has been proposed to be a primary electron acceptor in photosystem II or an indicator of its acceptance [14, 15]. Curve A was obtained for intact chloroplasts, and curve C for the chloroplasts pre-treated with 100 μM NphSH for 10 min in the light. As seen from these curves, the pre-treatment with NphSH suppressed the absorbance change of C550. The degree of suppression measured repeatedly ranged from 35 to 50 %. The addition of 20 μM DCMU to these two types of chloroplasts did not affect the extent of this absorbance change, although the rate of dark recovery was decreased appreciably (curves B and D in Fig. 2). This observation agrees with that of Knaff and Arnon [16].

It is known that Tris-treated chloroplasts under illumination are not capable of reducing Cl_2Ind with water as electron donor but are capable of reducing it with diphenylcarbazide as the donor [17]. The treatment of intact chloroplasts with 100 μM NphSH in the light caused a marked drop of the activity with water as the donor, and the addition of 100 μM diphenylcarbazide did not restore the activity as shown in the upper part of Table II. The activity of Tris-treated chloroplasts with diphenyl-

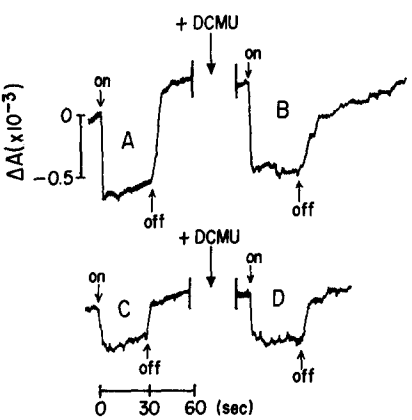

Fig. 2. The time course of light-induced absorbance change at 550 nm measured in the presence of 2 mM potassium ferrycyanide with the reference wavelength at 540 nm; curve A, intact chloroplasts, and curve C, for the chloroplasts pre-treated with 100 μM NphSH in the light (670 nm, 140 μW/cm^2) for 10 min. Curves B and D were measured in the presence of 20 μM DCMU for these two types of chloroplasts, respectively. The chloroplast concentration was 85 μg chlorophyll/ml.

carbazide was approximately one half the activity of intact chloroplasts with water as the donor, and this activity of Tris-treated chloroplasts was decreased more rapidly by the NphSH treatment in the light than the activity of intact chloroplasts, as seen from the data obtained after 1 min of treatment.

The effect of DCMU on the NphSH treatment was examined in the following manner. Chloroplasts were treated in the light for 10 min with 100 μM NphSH in the presence and in the absence of 10 μM DCMU and then washed 5 times to remove bound DCMU. As the control, intact chloroplasts were incubated for the same period in the light without NphSH in the presence and in the absence of DCMU. As seen from the result shown in the lower part of Table II, the incubation without NphSH in the presence of DCMU followed by washing decreased the activity slightly. The activity after NphSH treatment in the presence of DCMU was almost equal to the activity of the control chloroplasts treated without NphSH in the presence of DCMU, whereas the treatment with NphSH in the absence of DCMU decreased the activity greatly. It was thus shown that the inhibition of photosystem-II activity by NphSH is retarded by blocking the electron transport on the reduction side of photosystem II.

Curve C in Fig. 1 is the fluorescence spectrum of isolated photosystem-II particles. This spectrum was changed to curve D by the treatment with 100 μM NphSH for 5 min in the light. The height of F685 relative to F695 or F735 decreased, and the peak at 695 nm shifted to 697 nm, as found previously for whole chloroplasts [8]. The ratio of F695-697/F685 increased from 1.11 to 1.50 during illumination

TABLE II

The effects of Tris treatment and DCMU on the inhibition of the photosystem-II activity (Cl_2Ind photoreduction in μmol · mg chlorophyll · h) by NphSH. Intact or Tris-treated chloroplasts (17 μg chlorophyll/ml) were incubated with 100 μM NphSH in the light (670 nm, 140 μW/cm^2) or in the dark. The chloroplasts treated with 10 μM DCMU were washed 5 times to remove DCMU. The concentration of diphenylcarbazide (DPC) used as electron donor was 100 μM.

Sample chloroplasts	Treatment	Time (min)	Activity	Electron donor
Intact	NphSH			
	(dark)	10	119	Water
	(light)	1	104	Water
	(light)	5	62	Water
	(light)	10	29	Water
	(light)	10	27	DPC
Tris-treated	NphSH			
	(dark)	10	50	DPC
	(light)	1	30	DPC
	(light)	5	18	DPC
	(light)	10	13	DPC
Intact	None (light)+washing	10	85	Water
	DCMU (light)+washing	10	61	Water
	NphSH (light)+washing	10	32	Water
	(NphSH+DCMU) (light) +washing	10	59	Water

TABLE III

THE EFFECTS OF DCMU, CCCP AND DIPHENYLCARBAZIDE (DPC) ON THE CHANGE OF THE FLUORESCENCE RATIO, F695-700/F685, BY THE NphSH TREATMENT

Intact, Tris-treated or heat-treated chloroplasts (17 μg chlorophyll/ml) were incubated with 100 μM NphSH for 10 min in red light (670 nm, 140 μW/cm^2) or in the dark in the presence or absence of 20 μM DCMU, 100 μM CCCP or 100 μM diphenylcarbazide.

Sample chloroplasts	Addition on the NphSH treatment	F695-700/F685	
		Dark	Light
Intact	None	1.16	1.95
	DCMU	1.17	1.29
	CCCP	1.18	4.05
Tris-treated	None	1.16	3.50
	DCMU	1.16	1.75
	DPC	1.19	1.90
Heat-treated	None	1.19	2.20

for 5 min. The treatment under the same conditions in the dark did not cause any change of spectrum. The presence of DCMU during the NphSH treatment suppressed this fluorescence change. For example, the NphSH treatment under the same condition but in the presence of 20 μM DCMU changed the ratio slightly from 1.13 to 1.19, and the 695-nm band did not shift at all on the treatment.

Various chloroplast preparations were treated with 100 μM NphSH for 10 min in the light, and the ratio was measured after the treatment. The increase of ratio found with intact chloroplasts as the control was completely suppressed by the presence of 20 μM DCMU, as shown in Table III. Contrary to this effect of DCMU, the treatment with Tris · HCl prior to the NphSH treatment accelerated the change strikingly. Even higher acceleration was induced by the presence of 100 μM CCCP during the NphSH treatment, and slight acceleration was produced by heat pretreatment. These accelerations were more or less suppressed by the presence of DCMU during the NphSH treatment. A similar extent of suppression was observed in the presence of diphenylcarbazide during the NphSH treatment of Tris-treated chloroplasts. The comparative data shown in Table III indicate that the ratio change was largely dependent on the redox state or the oxidation side of photosystem II.

DISCUSSION

It was confirmed in the preliminary observation shown in Table I that aromatic mercaptans such as NphSH, thiosalicylic acid, thiocresol and thiophenol exert a specific effect on the photosystem-II activity and the fluorescence spectrum. It is known that NphSH combines by a covalent bond with a carboxyl side chain in protein to form an acylthiol bond [18, 19], so that the modification of carboxyl groups in some protein(s) of photosystem II may have caused the specific effect. The possibility that the aromatic mercaptans bind to sulfhydryl groups in chloroplast proteins could be excluded because reagents such as 5,5'-dithiobis(2-nitrobenzoic acid), *N*-ethylmaleimide and *p*-chloromercuribenzoic acid for the modifica-

tion of sulfhydryl groups in proteins showed no effect on both activity and fluorescence, while *N*-ethylmaleimide combines with the coupling factor in chloroplasts under illumination [7]. The positive effect of 5,5'-dithiobis(2-nitrobenzoic acid) was found to be due to its photo-decomposed product formed by the action of photosystem I.

The photo-oxidation of cytochrome *f* and P700 both in photosystem I were not affected by the pre-treatment of chloroplasts with NphSH in the light, but the photobleaching of carotenoids in the presence of CCCP, the absorbance change of C550 and the photoreduction of Cl_2Ind in the presence of diphenylcarbazide, all these caused by the photoreaction of photosystem II, were suppressed by the NphSH treatment in the light. The site of inhibition by NphSH seems, therefore, closer to the reaction center of photosystem II than any of the sites responsible for causing the carotenoid bleaching, the inhibition by CCCP or Tris treatment and the electron donation by diphenylcarbazide.

The activity of Cl_2Ind photoreduction was inhibited by the treatment with NphSH, but the inhibition was retarded when the treatment was made in the presence of DCMU. The changes of the fluorescence ratio by the NphSH treatment of intact and Tris-treated chloroplasts and photosystem-II particles were also suppressed by the presence of DCMU. By contrast, the fluorescence change was accelerated by blocking the electron transport on the oxidation side by CCCP, Tris or heat treatment, and this effect of Tris treatment was suppressed to some extent by the presence of diphenylcarbazide which donates electrons on the oxidation side. The electron carriers on the oxidation side of photosystem II close to its reaction center may be less oxidized by the presence of DCMU or by the electron donation from diphenylcarbazide, but are more oxidized when CCCP, Tris or heat treatment blocks the electron transport between the electron carriers and water. It is inferred from these facts that a protein in photosystem II is modified with NphSH when electron carriers on the oxidation side of its reaction center are oxidized by illumination.

CCCP has been classified by Renger et al. [20] as one of the ADRY reagents which are known to make a shunt between the plastoquinone pool and a positive hole located on the oxidation side in photosystem II. Another possible interpretation is that the acceleration of the NphSH effect by CCCP is caused somehow by the cyclic electron flow thus artificially induced in photosystem II.

Giaquinta et al. [19] found that *p*-diazonium benzene sulfonic acid (DABS) inhibits the photosystem-II activity in the light, and that the illuminated chloroplasts bound 3-fold more DABS than did chloroplasts treated similarly in the dark. With this reagent, however, both DCMU and Tris treatments abolished the light-induced increment of bound DABS. They suggested that a change of the membrane conformation caused an increase of binding potentiated by the electron flow through the region between the primary electron acceptor, Q, for photosystem II and plastoquinone. It is interesting to note that both effects of NphSH and DABS are dependent on the redox state of electron carriers, but the former dependent on the oxidation side while the latter on the reduction side of the reaction center 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).

REFERENCES

1 Ryrie, I. J. and Jagendorf, A. T. (1971) J. Biol. Chem. 246, 3771–3774
2 Ryrie, I. J. and Jagendorf, A. T. (1972) J. Biol. Chem. 247, 4453–4459
3 Giaquinta, R. T., Dilley, R. A. and Anderson, B. J. (1973) Biochem. Biophys. Res. Commun. 52, 1410–1417
4 Giaquinta, R. T., Dilley, R. A., Selman, B. R. and Anderson, B. J. (1974) Arch. Biochem. Biophys. 162, 200–209
5 Selman, B. R., Giaquinta, R. T. and Dilley, R. A. (1974) Arch. Biochem. Biophys. 162, 210–214
6 McCarty, R. E., Pittman, P. R. and Tsuchiya, Y. (1972) J. Biol. Chem. 247, 3048–3051
7 McCarty, R. E. and Fagan, J. (1973) Biochemistry 12, 1503–1507
8 Kobayashi, Y., Inoue, Y. and Shibata, K. (1976) Biochim. Biophys. Acta 423, 80–90
9 Price, C. C. and Stacy, G. W. (1946) J. Am. Chem. Soc. 68, 498–501
10 MacKinney, G. (1940) J. Biol. Chem. 132, 91–109
11 Yamashita, T. and Butler, W. L. (1965) Plant Physiol. 44, 1342–1346
12 Anderson, J. M. and Boardman, N. K. (1965) Biochim. Biophys. Acta 112, 403–421
13 Yamashita, K., Konishi, K., Itho, M. and Shibata, K. (1969) Biochim. Biophys. Acta 172, 511–524
14 Okayama, S. and Butler, W. L. (1972) Plant Physiol. 49, 769–774
15 Katoh, S. and Kimimura, M. (1974) Biochim. Biophys. Acta 333, 71–84
16 Knaff, D. B. and Arnon, D. I. (1969) Proc. Natl. Acad. Sci. U.S. 64, 715–722
17 Vernon, L. P. and Shaw, E. R. (1969) Plant Physiol. 44, 1645–1649
18 Kitagawa, S., Chiang, K. and Tonomura, Y. (1963) Biochim. Biophys. Acta 82, 83–91
19 Kubo, S., Kinoshita, N. and Tonomura, Y. (1966) J. Biochem. 60, 476–479
20 Renger, G., Bouges-Bocquet, B. and Buchel, K. (1973) Bioenergetics 4, 491–505
21 Giaquinta, R. and Dilley, R. A. (1974) Proceedings Third International Congress on Photosynthesis, Rehovot (Avron, M., ed.), pp. 883–895, Elsevier, Amsterdam