Biochimica et Biophysica Acta 809 (1985) 74-80
Elsevier

BBA 41814

Restoration by tetramethyl-*p*-phenylenediamine of photosynthesis in dibromothymoquinone-inhibited cells of the cyanobacterium *Synechococcus* sp.

Masaru Nanba and Sakae Katoh

Department of Pure and Applied Sciences, College of Arts and Sciences, University of Tokyo, Komaba, Meguro-ku, Tokyo 153 (Japan)

(Received April 22nd, 1985)

Key words: Photosynthesis; Electron transport; Cytochrome b_6-f complex; Electron flow inhibitor; Electron flow bypass; (*Synechococcus* sp.)

Inhibition of electron flow from water to methyl viologen by 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) was reversed by the addition of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) to cells of a thermophilic cyanobacterium *Synechococcus* sp. The restored activity is not a simple Photosystem I reaction because it was suppressed by 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU). TMPD was also found to restore photosynthetic oxygen evolution in the DBMIB-poisoned cells. That the restored oxygen evolution is coupled with CO_2 reduction was indicated by its sensitivity not only to electron-transport inhibitors such as DCMU and phenylmercuric acetate, but also to an inhibitor of CO_2 fixation (KCN), uncouplers (carbonyl cyanide *m*-chlorophenylhydrazone, methylamine and ammonium chloride) and an energy-transfer inhibitor (*N,N'*-dicyclohexylcarbodiimide). In the DBMIB-poisoned cells, TMPD accelerated only P-700 reduction, while leaving the reduction kinetics of cytochrome *c*-553 and cytochrome *f* inhibited, indicating that P-700 is the site of electron entry from TMPD to Photosystem I. It is concluded that TMPD restores photosynthetic electron transport by accepting electrons in the plastoquinone region and in turn donating them directly to P-700, and thereby bypassing cytochrome *c*-553 and DBMIB-blocked cytochrome b_6-f complexes.

Introduction

A plastoquinone antagonist, DBMIB, is a potent inhibitor of photosynthetic electron transport between Photosystem II and Photosystem I [1,2]. The inhibitor specifically blocks the oxidation of plastoquinone by binding to the cytochrome b_6-f complex at low concentrations [2,3], although it additionally suppresses the reduction of plastoquinone by Photosystem II at higher concentrations [4-6].

Trebst and Reimer [7-9] have shown that NADP photoreduction and concomitant oxygen evolution are restored by the addition of a catalytic amount of TMPD to the DBMIB-poisoned chloroplasts. They interpreted the reversal of the DBMIB inhibition by TMPD in terms of an electron bypass; TMPD (reduced form) is oxidized by Photosystem I after the DBMIB inhibition site, and in turn oxidized TMPD is reduced by Photosystem II before the inhibition site. Although

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea; PS, Photosystem; PMA, phenylmercuric acetate; DCCD, *N,N'*-dicyclohexylcarbodiimide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

TMPD does not carry protons across the membrane, the restored NADP photoreduction is coupled with ATP formation with a P/e_2 ratio similar to that of uninhibited chloroplasts [7–10].

The inhibition of NADP photoreduction by DCMU, which blocks electron flow from PS II to the plastoquinone pool, could not be reversed by TMPD. This indicates that the reduction site of TMPD is between the DBMIB- and the DCMU-inhibition site, i.e., the plastoquinone region [9]. TMPD is assumed to donate its electron to plastocyanin because no restoration by TMPD was observed in chloroplasts in which plastocyanin had been inactivated by $HgCl_2$ [9].

Reaction kinetics of P-700, cytochrome *c*-553, cytochrome *f* and the Rieske iron-sulfur center were recently resolved from flash-induced absorption changes in cells of the thermophilic cyanobacterium *Synechococcus* sp. by means of the computer subtraction [11,12]. Thus it is now possible to determine directly the site of electron donation from artificial electron donors to Photosystem I by measuring their effects on the reduction kinetics of individual electron carriers in *Synechococcus* cells.

In this communication, we report effects of TMPD on photosynthetic activities in DBMIB-inhibited *Synechococcus* cells. Spectrophotometric measurements were also carried out to determine the site of the electron donation from TMPD to Photosystem I.

Materials and Methods

The thermophilic cyanobacterium *Synechococcus* sp. was grown for 24 h at 55°C as described previously [13,14]. The cells were suspended in the fresh culture medium containing 25 mM Hepes-NaOH (pH 7.5) to give a final chlorophyll *a* concentration of about 10 μg/ml and kept under illumination with white light of 1000 lx at 22–24°C prior to measurements [11,12]. The starvation of cells were carried out by aerating the suspension at 55°C in the dark [14].

Absorption changes were measured with a single beam spectrophotometer as described previously [11,12]. Flashes with a half peak height duration of 5 μs were passed through a Toshiba VR-65 and a VR-66 filter and the photomultiplier was protected against the actinic light with two Corning 4-96 filters. Photoresponses of P-700, cytochrome *c*-553 and cytochrome *f* in the Soret band region were resolved by the computer subtraction [11]. To eliminate the fast electrochromic absorption changes, 5 μM gramicidin D and 50 mM KCl were added just before measurements [15]. All experiments were carried out at 55°C.

Photosynthetic O_2 evolution and methyl viologen photoreduction with water as electron donor were measured with a Clark-type oxygen electrode which had been calibrated to the oxygen concentration of distilled water in equilibrium with air at 55°C. Cells were illuminated with white light from a 650 W Ushio halogen lamp through a water filter of 6 cm thickness and a Hoya HA-50 heat absorbing filter. The light intensity was $3.3 \cdot 10^2\ W \cdot m^{-2}$. The reaction medium for photosynthetic O_2 evolution contained 5 mM $NaHCO_3$, and that for methyl viologen photoreduction with water as electron donor, 2 mM methyl viologen, 1 mM KCN and 20 mM methylamine. Chlorophyll *a* was determined as described by Mackinney [16].

Results

Reversal of DBMIB-inhibition of methyl viologen photoreduction by TMPD

Fig. 1 shows time-courses of methyl viologen photoreduction with water as electron donor in *Synechococcus* cells, which were monitored with an oxygen electrode. As reported previously [14], 10 μM DBMIB almost completely inhibited photosynthetic electron transport from water to methyl viologen (trace b). Trebst and Reimer [7–9] and Selman [10] have shown that the addition of TMPD or other phenylenediamines to the DBMIB-inhibited chloroplasts restores NADP photoreduction and coupled ATP formation (in the absence of ascorbate or ferricyanide, which will keep TMPD in the reduced or oxidized state, respectively). Trace c shows that methyl viologen photoreduction was largely restored by the addition of 25 μM TMPD to the DBMIB-poisoned cells. TMPD, in the presence of ascorbate, is known to serve as an efficient electron donor to Photosystem I [17,18]. The restored electron flow cannot, however, be ascribed to a TMPD-supported Photosystem I reaction because DCMU strongly suppressed the restored activity (trace d). It is con-

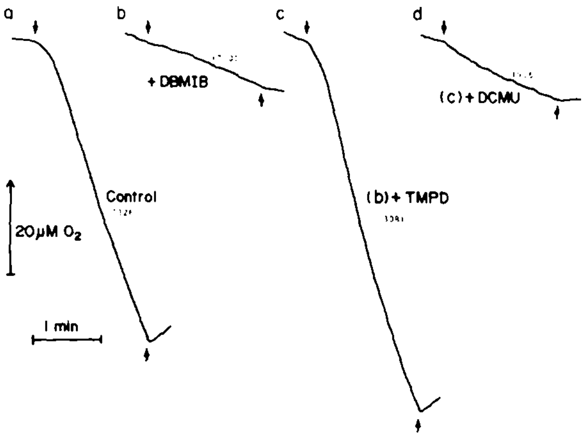

Fig. 1. Restoration of methyl viologen photoreduction by TMPD in DBMIB-inhibited cells. Oxygen uptake was determined with a Clark-type oxygen electrode. Cells were suspended in the growth medium supplemented with 25 mM Hepes-NaOH (pH 7.5)/2 mM methyl viologen/1 mM KCN/20 mM methylamine, and illuminated with white light of $3.3 \cdot 10^2$ $W \cdot m^{-2}$. Measurements were carried out at 55°C. Numbers in parentheses indicate the rates of O_2 uptake in µmol O_2/mg Chl per h. (a), No addition; (b), 10 µM DBMIB; (c), (b)+25 µM TMPD; (d), (c)+20 µM DCMU.

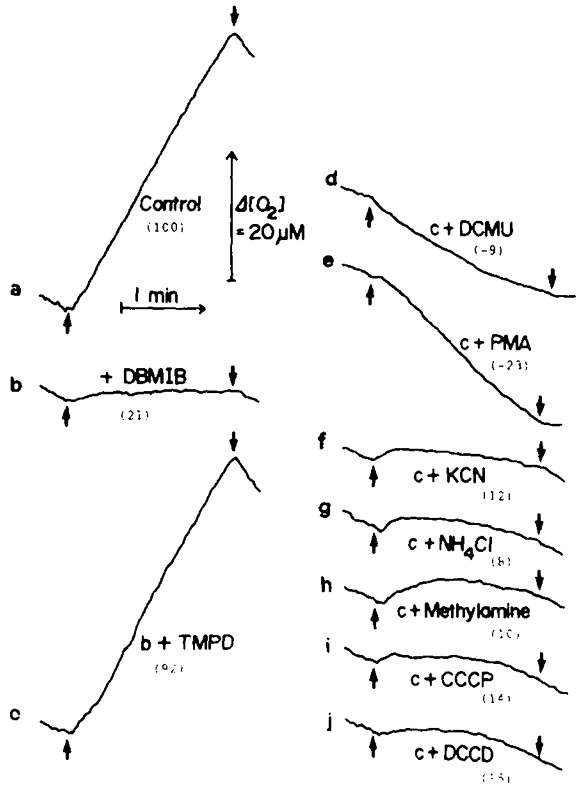

Fig. 2. Restoration of photosynthetic oxygen evolution by the addition of TMPD in DBMIB-inhibited cells and effects of various inhibitors on the restored oxygen evolution. Experimental conditions were the same as in Fig. 1, except that methyl viologen, KCN and methylamine were removed and 5 mM $NaHCO_3$ was added. Numbers in parentheses indicate the rates of O_2 evolution in percent of the control rate (175 µmol O_2/mg Chl per h). (a), No addition; (b), 10 µM DBMIB; (c), (b)+25 µM TMPD; (d), (c)+20 µM DCMU; (e), (c)+5 µM PMA; (f), (c)+1 mM KCN; (g), (c)+10 mM NH_4Cl; (h), (c)+20 mM methylamine; (i), (c)+5 µM CCCP; (j), (c)+50 µM DCCD.

cluded therefore that TMPD can readily penetrate into the cyanobacterial cells and release photosynthetic electron transport from the inhibition by DBMIB.

Reversal of DBMIB-inhibition of photosynthesis by TMPD

Photosynthetic electron transport restored by TMPD in DBMIB-poisoned chloroplasts is coupled with ATP formation [7–10]. It is difficult to determine whether or not TMPD restores photophosphorylation in intact cells of *Synechococcus*. However, the use of the cells with the plasmic membranes permeable to TMPD enabled us to examine effects of TMPD on photosynthetic oxygen evolution in the DBMIB-poisoned cells. Fig. 2 shows that photosynthetic oxygen evolution, which had been strongly inhibited by 10 µM DBMIB (trace b), was restored to a level corresponding to more than 90% of the control on addition of 25 µM TMPD to the inhibited cells (trace c). Cells from more than 20 cultures have been examined and the restoration of oxygen evolution was observed with about 70% of samples, the rest showing only the recovery of methyl viologen photoreduction by TMPD. The restored oxygen evolution proceeded linearly with time for 3–5 min, then abruptly came to a stop, in contrast to the activity of the unpoisoned cells which persisted for much longer times (data not shown).

When restored, oxygen evolution was sensitive to DCMU (trace d) and phenylmercuric acetate (trace e), an inhibitor of ferredoxin and ferredoxin-NADP oxidoreductase [19], indicating that both Photosystems I and II are involved in the activity restored by TMPD. The restored oxygen evolution was also inhibited by 1 mM KCN (trace f), which is known to be a potent inhibitor of CO_2 fixation [20]. This indicates that the restored oxygen evolution is coupled with CO_2 reduction rather than with reduction of some endogenous electron acceptors. The effect of KCN cannot be ascribed to the inactivation of plastocyanin in situ [21] because *Synechococcus* sp. has no plastocyanin [22]. Because CO_2 fixation requires both NADPH and ATP, the restored oxygen evolution, if coupled with CO_2 reduction, should be abolished by uncouplers or energy transfer inhibitors. This was found to be the case: traces g–j show that the restored activity was highly sensitive to uncouplers such as NH_4Cl, methylamine and carbonyl cyanide *m*-chlorophenylhydrazone and an inhibitor of ATP formation, *N,N′*-dicyclohexylcarbodiimide. It appears therefore that both NADP photoreduction and ATP synthesis proceed at rates sufficient to support CO_2 fixation, at least, for a few minutes on addition of TMPD to the DBMIB-inhibited cells.

The DBMIB-inhibition was reversed at relatively low concentrations of TMPD [8]: The maximum restoration of photosynthetic oxygen evolution was attained with 10–25 μM TMPD in the presence of 10 μM DBMIB (Fig. 3). Fig. 4 shows that the activity was fully restored by 25 μM TMPD at DBMIB concentrations below 2 μM, but the restoration was incomplete at higher inhibitor concentrations. Similar results were also obtained with methyl viologen photoreduction (triangles). This indicates that the restoration of photosynthesis is due to the reversal of the DBMIB-inhibited electron transport by TMPD. The incomplete restoration observed at high concentrations of DBMIB may be ascribed to an additional effect of the inhibitor on PS II electron transport [4–6].

Effects of TMPD on reduction kinetics of electron carriers

Trebst and Reimer [7,9] attributed the reversal of the DBMIB inhibition by TMPD to the formation of an electron pathway which bypasses the inhibition site. In order to examine this hypothesis, we studied effects of TMPD on flash-induced absorption changes of P-700, cytochrome *c*-553 and cytochrome *f* in the DBMIB-poisoned cells.

Fig. 5 shows that DBMIB strongly slowed down the reduction of flash-oxidized cytochrome *c*-553, cytochrome *f* and P-700 (traces d–f). The rapid reduction of a part of P-700 in the presence of DBMIB is ascribed to electron transport from the Rieske iron-sulfur center [12]. Magnitudes of P-700

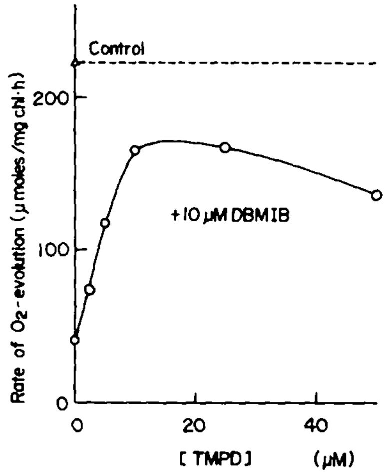

Fig. 3. Restoration of photosynthetic oxygen evolution in the DBMIB-inhibited cells as a function of TMPD concentration. Experimental conditions were the same as in Fig. 2c, except that TMPD concentration was varied.

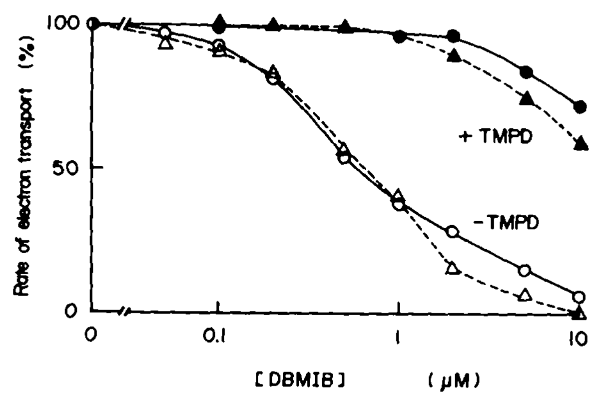

Fig. 4. Effects of DBMIB on the rates of photosynthetic oxygen evolution and methyl viologen photoreduction in the presence and absence of TMPD. Experimental conditions were the same as in Fig. 2 for photosynthetic oxygen evolution and in Fig. 1 for methyl viologen photoreduction. ○ and ●, photosynthetic oxygen evolution in the absence and presence of 25 μM TMPD, respectively; △ and ▲, methyl viologen photoreduction in the absence and presence of 25 μM TMPD, respectively. The 100% rates of photosynthetic oxygen evolution were 209 and 199 μmol O_2/mg Chl per h in the absence and presence of TMPD, respectively, and those of methyl viologen photoreduction were 254 and 350 μmol O_2/mg Chl per h in the absence and presence of TMPD, respectively.

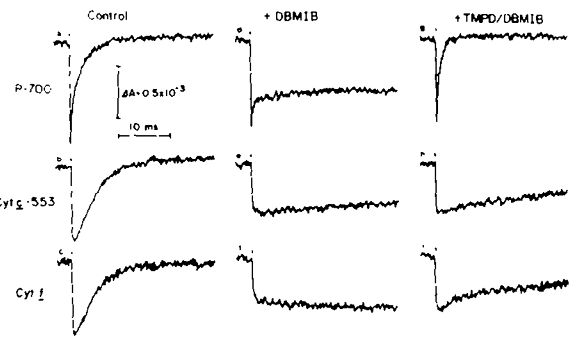

Fig. 5. Effects of TMPD on the reduction kinetics of P-700, cytochrome c-553 and cytochrome f in DBMIB-inhibited cells. Cells were suspended in fresh growth medium containing 25 mM Hepes-NaOH (pH 7.5), 5 μM gramicidin D and 50 mM KCl. Chlorophyll concentration was 5.3 μg/ml. Flash repetition rate was 0.5 Hz and 150 signals were averaged. Arrows mark flash illumination. (a), (b) and (c), no addition; (d), (e) and (f), 10 μM DBMIB was added; (g), (h) and (i), 10 μM DBMIB and 50 μM TMPD were added.

and cytochrome oxidation decreased to some extents in the presence of DBMIB, indicating incomplete rereduction of the electron carriers during the flash intervals of 2 s. Since this does not occur in the presence of ascorbate (see Ref. 11), we attribute the incomplete reduction to an oxidation of the plastoquinone pool which may be caused through the autooxidation of DBMIB at 55°C [23]. Ascorbate was not included in the reaction medium because TMPD serves only as an electron donor to PS I in the presence of the reductant [17,18].

It is seen that addition of TMPD to the DBMIB-poisoned cells markedly accelerated the reduction of P-700 (trace g). The acceleration was proportional to the concentration of TMPD added and the reduction kinetics of P-700 became monophasic in the presence of high concentrations of TMPD. In contrast, the reduction of both cytochrome c-553 and cytochrome f remained still largely inhibited: traces h and i show that addition of 50 μM TMPD induced only a slight acceleration of the cytochrome reduction. The results clearly indicate that TMPD reduces P-700, but not cytochromes c-553 and f. We conclude therefore that TMPD reconnects Photosystems I and II by accepting electrons from the plastoquinone region between the DCMU- and DBMIB-inhibition sites and in turn donating them to P-700, thereby bypassing cytochrome c-553 and the DBMIB-blocked cytochrome b_6-f complex.

Discussion

The present work shows that TMPD penetrates into the cells of the cyanobacterium *Synechococcus* sp. and releases photosynthetic electron transport from the inhibition by DMBIB. DBMIB blocks electron transport from plastoquinone to the Rieske iron-sulfur center by binding at a specific site of the cytochrome b_6-f complex [1–3,12]. The reactivation of electron transport by TMPD is not a simple reversal of the DBMIB-inhibition, or a release of the inhibitor from the cytochrome b_6-f complex, because reduction of cytochromes c-553 and f remains inhibited after the addition of TMPD. Evidently, TMPD opens an electron pathway from Photosystem II to Photosystem I in the cells with electron transport inhibited in the plastoquinone-cytochrome f region. Thus the results provided direct evidence for the view that TMPD restores electron transport by generating a bypass between the two photosystems [7–9].

In chloroplasts, TMPD is considered to give electrons to Photosystem I via plastocyanin because the TMPD-supported electron flow is sensitive to $HgCl_2$, which specifically inactivates plastocyanin [9]. However, a direct reduction of P-700 by TMPD in digitonin-Photosystem I particles, which must have been deprived of plastocyanin, was reported [25]. Our results conclusively demonstrate that TMPD provides electrons directly to P-700, but not to cytochrome c-553, which replaces plastocyanin functionally in the cyanobacterium. Since there is no reason to consider that cytochrome c-553 is inaccessible to TMPD, this should be ascribed to a difference in the reactivity with TMPD between the cytochrome and P-700.

The use of the intact cells with the cell membrane permeable to TMPD led to an interesting finding that O_2 evolution coupled with CO_2 reduction is restored by TMPD in the DBMIB-poisoned cells. This is evidence that not only NADPH but also ATP are produced in the cells with cytochrome b_6-f complexes inhibited. The finding poses, however, a problem as to the role of the cytochrome b_6-f complex in photosynthesis. The basic mechanism of electron transfer through the cytochrome b_6-f complex is the Q-cycle, which is considered to allow the uptake of one extra proton for each electron transferred to P-700 [26–29]. Because TMPD is not a proton carrier and hence delivers only electrons from plastoquinol to P-700 at the lumenal side of the thylakoid membranes [7–10], no additional proton-uptake will be induced by the addition of TMPD. The occurrence of photosynthesis in the DBMIB-inhibited cells implies, therefore, that an electrogenic reaction in the cytochrome b_6-f complex is not important for the Calvin cycle to operate, or even that no additional proton translocation takes place in the cytochrome b_6-f complex region. Interestingly, the P/e_2 ratios of the TMPD-restored non-cyclic photophosphorylation in chloroplasts were reported to be almost the same as that of the uninhibited chloroplasts [7,9,10].

There still remain considerable ambiguities in the TMPD-restored oxygen evolution, however. The restoration of photosynthesis depends on states of the cells employed because about 30% of the cell cultures examined failed to respond to the addition of TMPD. The restored oxygen evolution lasted only for 3–5 min, presumably reflecting the development of conditions unfavorable for the CO_2 fixation during the illumination. At present, therefore, we cannot exclude a possibility that the cells have some other means (oxidative or substrate-level phosphorylation, or storage of high-energy compounds such as polyphosphate) to supplement ATP to the CO_2-reduction cycle for a short period of time when the cytochrome b_6-f complex is bypassed. We expect that experiments along this line will provide important information on the relationship between the CO_2 fixation and ATP producing processes in the cyanobacterial cells.

A TMPD-bypass of respiratory electron transport in rat liver mitochondria, which is blocked by antimycin A, has recently been reported [30]. The respiratory electron transport in *Synechococcus* cells is unique in that it shares the plastoquinone pool with photosynthetic electron transport [14,23,31]. Thus the TMPD bypass will provide an interesting approach to the function of the cytochrome b_6-f complex not only in photosynthetic but also in respiratory electron transport of the cyanobacterium.

Acknowledgement

The present work was supported by grants for Scientific Research from the Ministry of Education, Science and Culture, Japan.

References

1 Trebst, A., Harth, E. and Draber, W. (1970) Z. Naturforsch. 25b, 1157–1169
2 Böhme, H., Reimer, S. and Trebst, A. (1971) Z. Naturforsch. 26b, 341–352
3 Hurt, E. and Hauska, G. (1981) Eur. J. Biochem. 117, 591–599
4 De Kouchkovsky, Y. and De Kouchkovsky, F. (1974) Biochim. Biophys. Acta 368, 113–124
5 Guikema, J.A. and Yocum, C.F. (1978) Arch. Biochem. Biophys. 189, 508–515
6 Bowes, J.A. and Crofts, A.R. (1981) Arch. Biochem. Biophys. 209, 682–686
7 Trebst, A. and Reimer, S. (1973) Z. Naturforsch. 28c, 710–716
8 Trebst, A. and Reimer, S. (1973) Biochim. Biophys. Acta 325, 546–557
9 Trebst, A. and Reimer, S. (1977) in Photosynthetic Organelles, Structure and Function (Miyachi, S., Katoh, S., Fujita, Y. and Shibata, K., eds.), pp. 201–209, Center for Academic Publications Japan, Tokyo
10 Selman, B.R. (1976) J. Bioenerg. Biomembranes 8, 143–156
11 Nanba, M. and Katoh, S. (1983) Biochim. Biophys. Acta 725, 272–279
12 Nanba, M. and Katoh, S. (1984) Biochim. Biophys. Acta 767, 396–403
13 Yamaoka, T., Satoh, K. and Katoh, S. (1978) Plant Cell Physiol. 19, 943–954
14 Hirano, M., Satoh, K. and Katoh, S. (1980) Photosynth. Res. 1, 149–162
15 Hirano, M. and Katoh, S. (1981) Photochem. Photobiol. 34, 637–643
16 Mackinney, G. (1941) J. Biol. Chem. 140, 315–32217
17 Trebst, A. (1964) Z. Naturforsch. 19b, 418–434
18 Izawa, S. (1980) Methods Enzymol. 69, 413–434
19 Honeycutt, R.C. and Krogmann, D.W. (1972) Plant Physiol. 49, 376–380
20 Whishnick, M. and Lane, M.D. (1969) J. Biol. Chem. 244, 55–59
21 Ouitrakul, R. and Izawa, S. (1973) Biochim. Biophys. Acta 305, 105–118
22 Aoki, M., Hirano, M., Takahashi, Y. and Katoh, S. (1983) Plant Cell Physiol. 24, 517–525
23 Aoki, M. and Katoh, S. (1983) Plant Cell Physiol. 24, 1379–1386
24 Kimimura, M. and Katoh, S. (1972) Biochim. Biophys. Acta 283, 279–292
25 Hiyama, T. and Ke, B. (1972) Biochim. Biophys. Acta 267, 160–171

26 Cox, R.P. and Olsen, L.F. (1982) in Electron Transport and Photophosphorylation (Barber, J. ed.), pp. 49–79, Elsevier, Amsterdam
27 Bendall, D.S. (1982) Biochim. Biophys. Acta 683, 119–151
28 Hauska, G., Hurt, E., Babellini, N. and Lockau, W. (1983) Biochim. Biophys. Acta 726, 97–133
29 Rich, P.R. (1984) Biochim. Biophys. Acta 768, 53–79
30 Alexandre, A. and Lehninger, A.L. (1984) Biochim. Biophys. Acta 767, 120–129
31 Aoki, M. and Katoh, S. (1982) Biochim. Biophys. Acta 682, 307–314