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The sites of electron donation of Photosystem I to methyl viologen

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Effectiveness of methyl viologen as an electron acceptor was studied by oxygen polarography and millisecond time range flash photolysis spectroscopy at room temperature on three types of chloroplast preparation deficient in $NADP^+$ photoreduction activity. $HgCl_2$-treated chloroplasts which had completely lost Fe-S center B and also $NADP^+$ photoreduction activity were still partially active in methyl viologen photoreduction. Compared with untreated chloroplasts, V_{max} of the oxygen uptake in this preparation was almost halved and its apparent K_m for methyl viologen was about 10 times greater. Photosystem I particles extracted with digitonin from the treated chloroplasts showed, in the absence of methyl viologen, a flash-induced absorption transient at 430 nm whose magnitude and decaying time were very similar to those of the particles extracted from untreated chloroplasts. However, the former required a concentration of methyl viologen for stabilization of $P\text{-}700^+$ more than 10 times higher than control particles. The shape of the difference spectrum of the faster decaying component in the presence of methyl viologen was similar to P-430. Our conclusions are: (1) this spectral component represents the redox of Fe-S center A, and chloroplasts can transfer electrons from center X to center A even when their center B is destroyed; (2) center B is the main site of electron donation to methyl viologen, and center A can donate electrons to methyl viologen although with a lower affinity. Chloroplasts anaerobically photoinactivated under strongly reducing conditions in which electron transport between A_0 and center X was impaired showed very low oxygen uptake activity which was almost insensitive to methyl viologen. Dependence of oxygen uptake on methyl viologen concentration by aerobically photoinactivated chloroplasts in which three Fe-S centers were partially destroyed somewhat resembled that of $HgCl_2$-treated chloroplasts.

Introduction

Light excitation of Photosystem I (PS I) induces a charge separation between the primary electron donor P-700 and primary electron acceptor A_0. This initial charge separation is stabilized by electron transfer to acceptors A_1 and the three Fe-S centers (X, B and A) [1,2]. A visible spectral component P-430 is assigned to be Fe-S centers A and B, and A_2 to be Fe-S center X [1,2], although there is a different view about this [3]. Arrangement of the three Fe-S centers in the PS I electron-transport chain is controversial [1,2]: Nugent et al. [4] and Golbeck and Warden [5] proposed a branched chain model in which both centers B and A can be directly reduced by center X; Bonnerjea and Evans [6] proposed that PS I contained two centers X which constitute linear or parallel electron transport; and Malkin [7] proposed a linear electron-transport model which Inoue et al. [8] support.

Methyl viologen-induced oxygen uptake is frequently used to assess the PS I activity of chloroplasts (for a review, see Ref. 9); nevertheless, the exact site(s) of methyl viologen reduction is uncertain. Takahashi and his co-workers [10,11] prepared PS I core particles from *Synechococcus* sp. with sodium dodecyl sulfate (SDS) which lacked P-430 and A_2, and observed charge stabilization in these particles by methyl viologen [12]. However, the concentration of methyl viologen required for the stabilization was rather high; thus, they concluded that the main site of electron donation to methyl viologen in intact chloroplasts was P-430, but that it was A_1 in SDS-treated PS I core particles. Malkin [7] reported that treatment of chloroplasts with diazobenzene sulfonate decreased considerably center B, but did

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Abbreviations: PS I, Photosystem I; DCIP, 2,6-dichlorophenolindophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; SDS, sodium dodecyl sulfate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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not change the oxygen uptake activity mediated by 25 μM methyl viologen. Golbeck and Cornelius [13] concluded from flash-photolysis experiments that 33 μM methyl viologen could not accept electrons from PS I particles which lacked centers A and B by the treatment with lithium dodecyl sulfate.

Recently, we prepared three types of chloroplast preparations in which the inhibition sites of electron transport on the acceptor side of PS I are defined: (1) incubation with $HgCl_2$ results in selective destruction of center B as evidenced by the absence of its ESR signal at cryogenetic temperatures even though the chloroplasts had been illuminated in a dithionite-containing medium at room temperatures and frozen in liquid nitrogen under continuous illumination during freezing, which brought about the reduction of the center X [14]; (2) illumination of chloroplasts with high intensity light, under strongly reducing conditions in the presence of dithionite, results in inhibition of electron flow between A_0 and center X [15]; and (3) illumination of chloroplasts under aerobic conditions results in partial destruction of all of the three Fe-S centers [8].

In this paper, we will report the effects of methyl viologen on photoinduced oxygen uptake and on flash-induced absorption changes in the millisecond time range in these three chloroplast preparations. We have attempted to identify the sites of electron donation of PS I to methyl viologen and to gain some insight into the arrangement of Fe-S centers in the electron transport chain.

Materials and Methods

Preparation of chloroplasts. Chloroplasts were isolated from market spinach by blending the leaves in a Waring blender and differential centrifugation. Isolated chloroplasts were osmotically shocked in 15 mM Tris-HCl (pH 7.8)/10 mM NaCl, collected by centrifugation at $10000 \times g$ for 10 min as described by Inoue et al. [8], and used in subsequent experiments. Chlorophyll concentration was determined by the method of Arnon [16].

Destructive treatments of chloroplast PS I. Treatment of chloroplasts with $HgCl_2$ was performed essentially as described by Kojima et al. [14]. Briefly, chloroplasts were incubated in 50 mM Na,K-phosphate buffer (pH 7.8)/0.4 M sucrose with variable concentrations of $HgCl_2$ at a chlorophyll concentration of 0.2 mg/ml at 0 °C. After 1 h, EDTA was added to the incubation medium to a final concentration of 1 mM and the chloroplasts were washed twice by centrifugation at $10000 \times g$ for 10 min with 50 mM Tris-HCl (pH 7.8)/0.4 M sucrose/10 mM NaCl/1 mM EDTA. Photoinhibition of isolated chloroplasts was performed at 25 °C with actinic light from 300 W incandescent lamps from each side under anaerobic and aerobic conditions as described by Inoue et al. (Refs. 15 and 8, respectively). The light intensity measured with an Integrating Quantum - Radiometer - Photometer (Model LI-188 Li-Cor., Inc.) was about 3 $mE \cdot m^{-2} \cdot s^{-1}$ at the surface of the flasks. For anaerobic photoinhibition, the gas phase was N_2 and the incubation medium was 50 mM glycine/50 mM 2-amino-2-methyl-1,3-propanediol-NaOH buffer (pH 9.3)/10 mM NaCl/2.5 mM $MgCl_2$/10 μM DCMU/20 mM sodium dithionite at a chlorophyll concentration of 0.1 mg/ml. For aerobic photoinhibition, the gas phase was air and the incubation medium was 50 mM Tris-HCl (pH 7.8)/10 mM NaCl at a chlorophyll concentration of 0.1 mg/ml. After these treatments, the photoinhibited chloroplasts were washed twice by centrifugation at $10000 \times g$ for 10 min with 50 mM Tris-HCl (pH 7.8)/0.4 M sucrose/10 mM NaCl. Each preparation was finally suspended in 50 mM Tris-HCl (pH 7.8)/0.4 M sucrose/10 mM NaCl at an original chlorophyll concentration of 3 mg/ml, and either used immediately or stored at −70 °C until use.

Preparation of PS I particles. PS I particles were extracted from the above-treated chloroplasts with digitonin essentially as described by Ohki et al. [17]. Briefly, chloroplasts were suspended in 50 mM sodium phosphate buffer (pH 7.4)/5 mM $MgCl_2$/0.5% (w/v) digitonin at a chlorophyll concentration of 0.3 mg/ml. After incubation at 0 °C for 30 min, the mixture was centrifuged at $40000 \times g$ for 40 min at 4 °C and the supernatant containing PS I particles was concentrated with Centricon 30 (Amicon Corp.).

Measurements of photochemical activities. Methyl viologen-mediated photoinduced oxygen uptake was measured in a thermostated glass cell at 25 °C with a Clark-type oxygen electrode (YSI 4004, Yellow Springs Instrument Co., Inc.). The actinic light from a 650 W slide projector was passed through a 5 cm layer of aqueous $CuSO_4$ solution. The intensity of light was about 4.8 $mE \cdot m^{-2} \cdot s^{-1}$ at the cell surface.

Photoreduction of $NADP^+$ was measured at 340 nm with a spectrophotometer (Model 557, Hitachi) equipped with a crossillumination actinic projector as described by Inoue et al. [8]. The reaction mixture contained 40 mM Tris-HCl (pH 8.0)/35 mM NaCl/5 mM sodium ascorbate/0.1 mM DCIP/0.5 mM $NADP^+$/10 μM DCMU/1 μM FCCP/1 μM Antimycin A/5 μM ferredoxin, a saturating amount of ferredoxin-$NADP^+$ reductase, and chloroplasts at a chlorophyll concentration of 10 μg/ml. Prior to measurement of the activity, the air-tight cuvette containing the above mixture was evacuated and flushed with N_2 gas.

Unless otherwise indicated, activities of the treated chloroplasts were expressed on the basis of chlorophyll contained in them before treatments.

P-700 content of chloroplasts was determined with a double-wavelength spectrophotometer (Model 557, Hitachi) by either (A) a photochemical or (B) a chem-

ical method. (A) The continuous light-induced oxidation of P-700 was followed at 430 nm as described by Sakurai and San Pietro [18] except that the reference wavelength was 450 nm and 0.1% (w/v) Triton X-100 was included in the medium. (B) Ferricyanide (1 mM)-oxidized minus sodium ascorbate (few grains of solid)-reduced difference absorbance was determined at 700 nm with a reference wavelength at 730 nm [19]. P-700 content of PS I particles was determined by the light-induced absorbance change at 430 nm as described by Sakurai and San Pietro [18] by using an extinction coefficient of 45 $mM^{-1} \cdot cm^{-1}$ as determined by Hiyama and Ke [20]. Flash-induced absorption transients in the millisecond time range were measured with a laboratory-constructed single-beam spectrophotometer system as described by Inoue et al. [15]. In brief, absorption transients induced by a xenon flash (1.5 μs half-duration, Micro Pulse Fiber Illuminator Model 457F, K.K. Ewig Shoukai) were detected by a photomultiplier (R374, Hamamatsu Photonics), amplified (427 Current Amplifier, Keithley), sampled with a transient converter (TCG-4000S, Riken Denshi), and averaged by a microcomputer (MB6890, Hitachi).

ESR measurement. ESR spectra at liquid helium temperatures were obtained with an ESR spectrometer (ER200, Bruker) as described by Hiyama et al. [21]. Fe-S center A was determined at $g = 1.94$ [22], center B at $g = 1.92$ [23] and center X at $g = 1.76$ [24]. Before these measurements, each sample was mixed with 40 mM sodium dithionite at pH 10.0 under argon, illuminated, and frozen in liquid nitrogen under continuous illumination as described by Hiyama et al. [21].

Results

Properties of $HgCl_2$-treated chloroplasts

When chloroplasts were treated with $HgCl_2$ [14], Fe-S center was destroyed and $NADP^+$ photoreduction activity was inhibited; in contrast neither Fe-S center A nor P-700 were affected (Fig. 1A). $NADP^+$ photoreduction activity was almost completely inhibited and Fe-S center B was totally destroyed by 60 μM $HgCl_2$. Fig. 1B shows the photoinduced oxygen uptake by these chloroplast preparations in the presence and absence of methyl viologen. Increasing concentration of $HgCl_2$, up to 60 μM, effectively decreased photoinduced oxygen uptake activities, but its further increase up to 150 μM was almost without effect both in the presence and absence of methyl viologen. The broken line in Fig. 1B is the oxygen uptake by control chloroplasts which had been heated at 90 °C for 5 min and were completely devoid of chemically oxidizable P-700. The rate of this oxygen uptake was independent of methyl viologen (see Fig. 3), increased with light intensity, and was not saturated even by a strong light of 4.8 $mE \cdot m^{-2} \cdot s^{-1}$ (data not shown). The rather high activity of heated

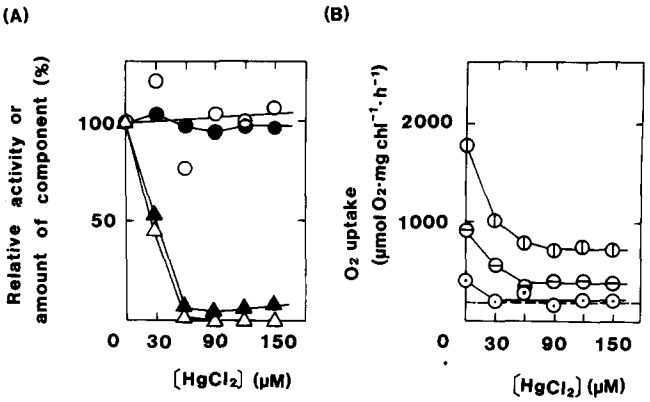

Fig. 1. Effects of $HgCl_2$ concentration on Fe-S centers, P-700, and $NADP^+$ photoreduction. Broken chloroplasts at 0.2 mg/ml were treated with the indicated concentrations of $HgCl_2$ as described in Materials and Methods. (A) center A ($g = 1.94$) (○), center B ($g = 1.92$) (△), chemically oxidizable P-700 (●) and $NADP^+$ photoreduction activity (▲). ESR conditions: temperature, 20 K; microwave frequency, 9.6 GHz; microwave power, 20 mW; scan time, 100 s; magnetic field, 3520 ± 500 G; modulation amplitude, 10 G. $NADP^+$ photoreduction rate of the control chloroplasts was 160 μmol per mg chlorophyll (Chl) per h. (B) Oxygen uptake at 0 μM (⊙), 1 μM (⊖) and 100 μM (Φ) methyl viologen. The broken line shows the activity of boiled untreated chloroplasts.

chloroplasts in this experiment was probably due to a relatively strong actinic light used in measurements, and we conclude that this oxygen uptake was due to bulk chlorophylls.

Effects of DCIP concentration on oxygen uptake

Fig. 2 shows the effects of reduced DCIP, an electron donor, on photo-induced oxygen uptake by chloroplasts in the presence of 100 μM methyl viologen and 15 mM ascorbate. The affinity of control chloroplasts for DCIP was much higher than that of $HgCl_2$-treated chloroplasts, the apparent K_m of the former being about 30 μM, and that of the latter a few hundred μM. At 1.5 mM DCIP, the oxygen uptake activity of $HgCl_2$-treated chloroplasts was about 60% of that of control chloroplasts.

Effects of methyl viologen concentration on oxygen uptake

Photoinduced oxygen uptake activities of chloroplasts at 1 mM DCIP and 15 mM ascorbate were measured over a wide concentration range of methyl viologen (Fig. 3). Control chloroplasts had a higher affinity for methyl viologen and higher oxygen uptake activity than $HgCl_2$-treated chloroplasts. Boiled control chloroplasts showed a considerable oxygen uptake activity, which was unaffected by methyl viologen. The difference in affinity between control and $HgCl_2$-treated chloroplasts was more pronounced at low methyl viologen concentrations (below 0.3 μM) where the activity in $HgCl_2$-treated chloroplasts was scarcely enhanced

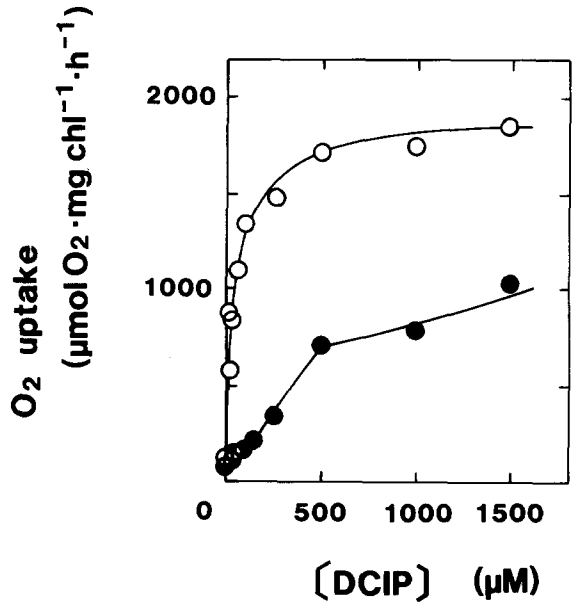

Fig. 2. Effects of DCIP concentration on the oxygen uptake by control chloroplasts (○) and (150 μM) $HgCl_2$-treated chloroplasts. (●). Reaction mixtures contained 50 mM Tris-HCl (pH 7.8)/1 mM NaN_3/15 mM sodium ascorbate/100 μM methyl viologen/10 μM DCMU/1 μM FCCP/chloroplasts at a chlorophyll (Chl) concentration of 4 μg/ml and indicated concentrations of DCIP.

by methyl viologen. At 1 mM methyl viologen, the activity of $HgCl_2$-treated chloroplasts was about half of that of control chloroplasts.

Effects of methyl viologen on flash-induced absorption transients

In order to study the differential affinity for methyl viologen further, PS I particles were prepared with digitonin from control and $HgCl_2$-treated chloroplasts, and flash-induced absorption transients in millisecond time range were compared at the same concentrations

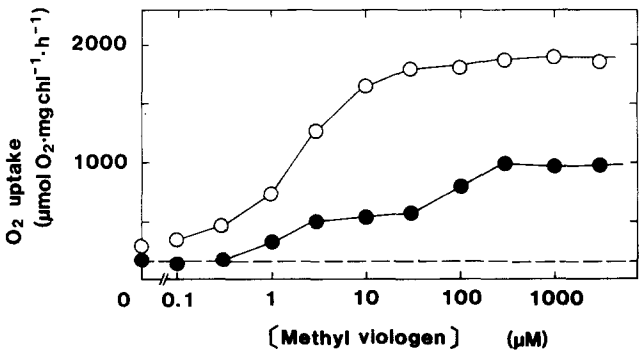

Fig. 3. Effects of methyl viologen on the oxygen uptake of control chloroplasts (○) and $HgCl_2$-treated chloroplasts (●). The broken line shows the activity of boiled untreated chloroplasts. Broken chloroplasts were treated with 60 μM $HgCl_2$ as described in Materials and Methods. Reaction mixtures contained 50 mM Tris-HCl (pH 7.8)/1 mM NaN_3/15 mM sodium ascorbate/1 mM DCIP/10 μM DCMU/1 μM FCCP/chloroplasts at a chlorophyll (Chl) concentration of 4 μg/ml and indicated concentrations of methyl viologen.

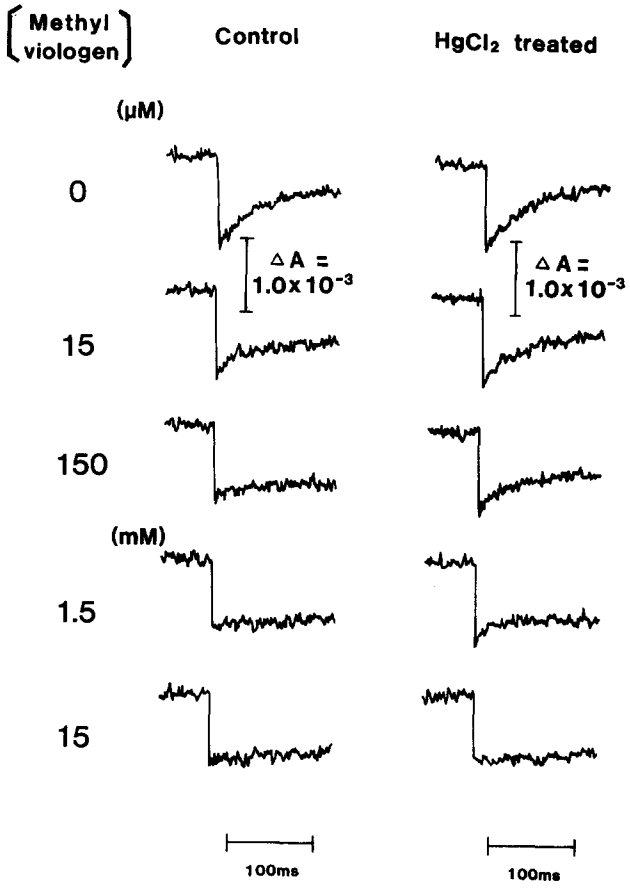

Fig. 4. Effects of methyl viologen on flash-induced absorption changes. PS I particles were extracted from control (left) and from (500 μM) $HgCl_2$-treated (right) chloroplasts, in which 9% of $NADP^+$ photoreduction activity remaining. Measuring wavelength was 430 nm. Reaction mixture contained 50 mM Tris-HCl (pH 7.8)/200 mM NaCl/8 mM sodium ascorbate/40 μM TMPD/30 nM continuous light-oxidizable P-700/indicated concentrations of methyl viologen. 32 flashes were fired at an interval of 12 s and signals were averaged with a microcomputer.

of continuous-light inducible $P\text{-}700^+$ (Fig. 4). In control PS I particles, the decay of the flash-induced absorbance change at 430 nm in the absence of methyl viologen was principally ascribed to re-reduction of $P\text{-}700^+$ by $P\text{-}430^-$ [25,26]. In the absence of methyl viologen, both the extent and the decay rate of A_{430} of $HgCl_2$-treated particles were very similar to those of control particles though the former particles could not reduce $NADP^+$: in three separate experiments, both the half times of the decay of A_{430} in control particles and in treated particles in the absence of methyl viologen was about 40–55 ms. Methyl viologen competes with $P\text{-}700^+$ for $P\text{-}430^-$, thus accelerating the decay of $P\text{-}430^-$ and delaying the reduction of $P\text{-}700^+$ which now accepts electrons from TMPD and ascorbate with a half-time of more than 200 ms. In control PS I particles, the extent of the slowly decaying component, $P\text{-}700^+$, was increased by methyl viologen, and its half-maximal level was already attained at 15 μM methyl viologen. The

affinity of treated particles for methyl viologen was much lower than that of control particles, and the effects of 150 μM methyl viologen on the former were still lower than those of 15 μM methyl viologen on the latter. The effects of methyl viologen on the faster decaying component, P-430$^-$ (Fig. 4), was also in accord with the explanation described.

The extent of the flash-induced absorbance change at 430 nm of PS I particles extracted from $HgCl_2$-treated chloroplasts in the presence of 1.5 mM methyl viologen was almost the same as in the absence of methyl viologen. At 15 mM methyl viologen the faster-decaying component apparently disappeared due to very fast-re-oxidation by methyl viologen which could not be traced by our measuring system (Fig. 4). The decay of the absorbance change in the presence of methyl viologen consisted of reoxidation of the faster decaying component plus re-reduction of P-700$^+$. The difference absorption spectrum of the slowly decaying component (P-700$^+$) in $HgCl_2$-treated particles was obtained by extrapolating its curve at each wavelengths in the presence of 1.5 mM methyl viologen to time zero (Fig. 5). The slowly decaying spectral component in the presence of 1.5 mM methyl viologen did not represent the oxidation of bulk chlorophylls, because it was reversible with a half-time of about 80 ms at 290 μM TMPD, which was shortened by raising the latter concentration (data not shown). We obtained the difference spectrum of the

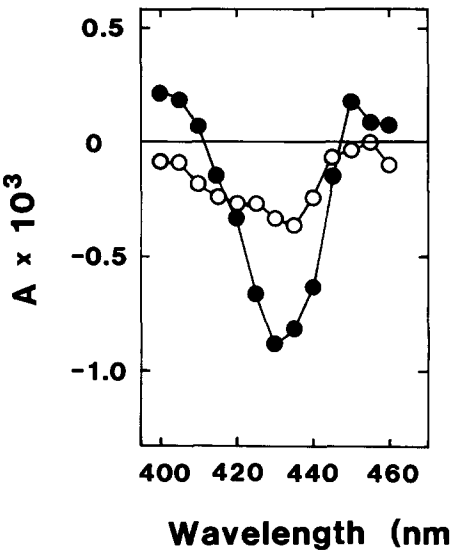

Fig. 5. Difference spectra of P-700 (●) and of the methyl viologen-reactive component (○) in PS I particles extracted from $HgCl_2$-treated chloroplasts. Experimental conditions were the same as in Fig. 4 except for methyl viologen concentrations and measuring wavelength. The former spectrum was obtained by extrapolating the more slowly decaying curve in the presence of 1.5 mM methyl viologen to time zero. The latter spectrum was obtained by subtracting the above-P700 change from the maximal absorbance change at each wavelength obtained in the absence of methyl viologen. 25–32 flashes were fired at an interval of 12 s and signals were averaged with a microcomputer.

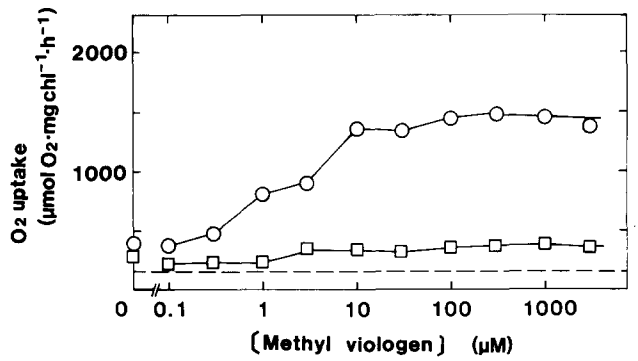

Fig. 6. Effects of methyl viologen on oxygen uptake by control chloroplasts (○) and anaerobically photoinhibited chloroplasts (□). Chloroplasts were treated as described in Materials and Methods. Experimental conditions for oxygen uptake measurements were as in Fig. 3.

faster decaying component of the same particles by subtracting the above obtained P-700 difference absorbance from the flash-induced maximal absorbance change in the absence of methyl viologen at each wavelength (Fig. 5). The spectral shape of the faster decaying component was very similar to P-430 described by Hiyama and Ke [25,26]. The ratio of the absorbance changes at 430–435 nm of P-430/P-700 of the treated particles (0.41) was very similar to that of the untreated particles (0.43).

Effects of methyl viologen on anaerobically photoinhibited chloroplasts

When chloroplasts were illuminated by strong light for 120 min under N_2 in the presence of 20 mM sodium dithionite, the NADP$^+$ photoreduction activity decreased to 4.4% of that of control chloroplasts. As characterized by Inoue et al., the continuous light-oxidizable P-700 was much less susceptible to this photoinhibition [15], 87% of it remaining after 2 h of illumination. Fe-S centers were also resistant to the photoinhibition treatment; ESR measurements at cryogenic temperatures indicated that 70% of center A, 91% of center B, and 117% of center X remained. These anaerobically photoinhibited chloroplasts had some photoinduced oxygen uptake activity, which was only slightly enhanced by methyl viologen (Fig. 6). Flash-induced absorption transients of PS I particles extracted from control and photoinhibited chloroplasts are shown in Fig. 7. Normalized by the continuous-light oxidizable P-700 in extracted particles, the extent of the absorption transient at 430 nm in the absence of methyl viologen and that affected by methyl viologen in the treated particles were considerably smaller than those in control particles (Fig. 7). These results indicate that in a portion of the treated particles the reduced acceptor was quickly oxidized by P-700$^+$, which could not be followed by our measuring system. The above acceptor could not donate

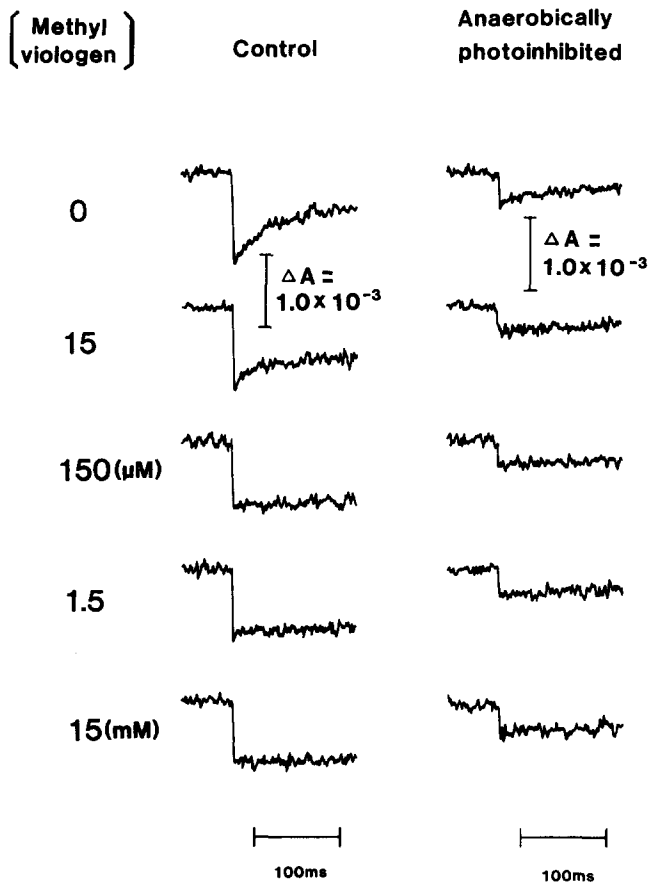

Fig. 7. Effects of methyl viologen on flash-induced absorption changes in PS I particles extracted from control (left) and from anaerobically photoinhibited (right) chloroplasts. Treatment of chloroplasts and preparation of PS I particles were as described in Materials and Methods. Experimental conditions for absorption kinetics measurements were as in Fig. 4.

its electron to methyl viologen, because the extent of the absorbance change in treated particles was not increased by high concentrations of methyl viologen. Although the actinic flash used was lower than the saturation level, the decreased extent of absorption transient was not solely due to the decrease of antenna pigments as indicated by Inoue et al. in double reciprocal plot analysis [15]. In this experiments, the maximal flash-induced absorption transient deduced in the same way in the millisecond time range of control and anaerobically photoinhibited particles was 1.3-fold and 1.6-fold, respectively, of the absorption change shown in Fig. 7. The deduced maximal change in the photoinhibited particles was about 50% of that of control particles.

Effects of methyl viologen on aerobically photoinhibited chloroplasts

When chloroplasts were illuminated with strong light under aerobic conditions, their $NADP^+$ photoreduction activity was inhibited, which was explainable by the destruction of the three kinds of Fe-S centers to varying extents [8]. The dependence of photoinduced oxygen uptake on methyl viologen of aerobically photoinhibited chloroplasts somewhat resembled that of $HgCl_2$-treated chloroplasts (Fig. 8). In these chloroplasts, $NADP^+$ photoreduction activity decreased to 31% of the control chloroplasts after 30 min irradiation, while 78% of P-700 survived. It seems that the preparation was composed of at least three functionally different chloroplast populations. The first type was intact and could reduce $NADP^+$ (31%); the second was inactive in P-700 photochemistry (22%); and third was active in P-700 photooxidation but inactive in $NADP^+$ photoreduction (47%). The third should probably be composed of heterogeneous subpopulations; one-center-, two-centers- and three-centers-destroyed chloroplasts. If we denote the observed photoinduced oxygen uptake activities of control chloroplasts as R_{cont} and of treated chloroplasts as R_{obs}, and those of boiled control chloroplasts as R_{boil}, the activity of the third type of chloroplast population on photochemically active P-700 basis (R_{cal}) would be

$$R_{obs} = 0.31R_{cont} + 0.22R_{boil} + 0.47R_{cal}$$

which makes

$$R_{cal} = \frac{R_{obs} - 0.31R_{cont} - 0.22R_{boil}}{0.47}$$

The calculation showed that the third type had a definite methyl viologen photoreduction activity, although the activity and the apparent affinity for methyl viologen of the third chloroplast population were much lower than those of control chloroplasts (Fig. 8).

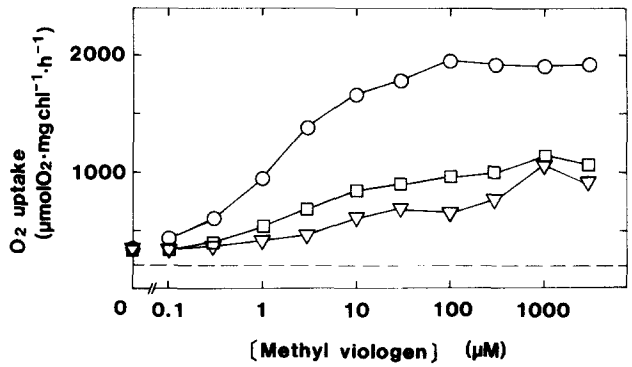

Fig. 8. Effects of methyl viologen on oxygen uptake activity in control and aerobically photoinhibited chloroplast preparations. Chloroplasts were treated as described in Materials and Methods. Control chloroplasts (○), aerobically photoinhibited chloroplast preparation (□), photoinhibited chloroplast population deficient at least one of the Fe-S centers calculated as indicated in the text (▽). Experimental conditions for oxygen uptake measurements were as in Fig. 3.

Discussion

When chloroplasts were incubated with $HgCl_2$, Fe-S center B was selectively destroyed, which was paralleled by the decrease of $NADP^+$ photoreduction activity (Fig. 1A). As reported by Kojima et al. [14] and partially confirmed in the present paper, neither center A nor center X was appreciably decreased by $HgCl_2$-treatment (Fig. 1A and B; see also Fig. 5 in Ref. 14). When $HgCl_2$-treated particles were frozen in the dark, illuminated for 30 s at 20 K and ESR spectra measured, we normally observed that 40–60% of center A detectable by dithionite reduction was photoreduced in contrast with nearly 100% photoreduction in untreated particles (unpublished observation).

$HgCl_2$-treated chloroplasts which had almost completely lost $NADP^+$ photoreduction activity were still active in methyl viologen photoreduction although the maximal activity at 1 mM DCIP was about half that of untreated chloroplasts (Fig. 3). These results suggest that either some PS I electron acceptor prior to center B can donate electrons to methyl viologen or center X can donates electron to center A which then donates it to methyl viologen. In flash photolysis experiments in the millisecond time range, the A_{430} change of PS I particles obtained with digitonin from $HgCl_2$-treated chloroplasts was, in the absence of methyl viologen, almost the same in magnitude and in decay time as those exhibited by the control PS I particles and the former particles showed an affinity for methyl viologen more than 10 times lower than that of the latter particles (Fig. 4). The flash-induced absorption transients of this low affinity component showed an Fe-S like spectrum (Fig. 5) almost identical with that of P-430 [25,26]. Hiyama and Ke [25,26] ascribed the A_{430} change with a half-time of 40–50 ms to its re-oxidation by $P\text{-}700^+$. The half-time of re-oxidation of X^- (or A_2^-) was reported to be about 1 ms or less in PS I particles [13,27,28]. Golbeck and Cornelius [13] reported that PS I particles which were devoid of centers A and B by treatment with lithium dodecyl sulfate were insensitive to 33 μM methyl viologen. Takahashi and Katoh [12] reported that PS I core particles prepared with SDS which lacked A_2 and P-430 had very low affinity for methyl viologen. Itoh et al. [29] reported that diethyl ether-extracted PS I particles which lacked A_1 cannot photoreduce benzyl viologen. These observations indicate that neither A_0, A_1 nor A_2 can efficiently donate electrons to methyl viologen. We tentatively conclude that the component with a decay half-time of about 40–55 ms and with a low affinity for methyl viologen is center A. The fact that control preparations showed an affinity for methyl viologen about 10 times higher than $HgCl_2$-treated preparations (Figs. 3 and 4) suggests either that the main site of electron donation to methyl viologen is center B or that the site is center A whose reactivity is greatly decreased by the destruction of center B. Malkin [7] reported that diazobenzene sulfonate-treated chloroplasts in which center B was largely decreased were as active as untreated chloroplasts in methyl viologen photoreduction, which is in contradiction with our results.

In anaerobically photoinhibited chloroplasts in which continuous light-oxidizable P-700 was only slightly decreased, methyl viologen photoreduction activity decreased almost in parallel with $NADP^+$ photoreduction activity, and after 120 min illumination, methyl viologen as high as 3 mM could not accept electrons from this preparation (Fig. 6). From flash-induced A_{430} measurements in the millisecond time range we concluded that the preparation was composed of three PS I populations: (1) the first was intact remaining; (2) the second was active in flash-induced P-700 but inactive in $NADP^+$ photoreduction; and (3) the third showed transients undetectable in this time range. The occurrence of the second population is evident because $NADP^+$ photoreduction activity of the treated PS I particles was about 4.4% and A_{430} transients in the absence of methyl viologen were about 50% of those of control particles. It seems that this population was devoid of Fe-S absorption change in flash experiments and insensitive to methyl viologen because its addition did not increase the flash-induced maximal absorption changes (Fig. 7). The electron acceptor pertaining to this spectral population is not certain at present. These results indicate that chloroplasts in which electron flow between A_0 and center X was inhibited cannot efficiently donate electrons to methyl viologen, and support our conclusion that centers B and A are the sites of electron donation to methyl viologen.

The results with $HgCl_2$-treatment (Fig. 3) and aerobic photoinactivation (Fig. 8) clearly indicate that the assessment of the intactness of the whole PS I activity by measuring methyl viologen photoreduction is sometimes erroneous, although it may be almost valid with anaerobically photoinhibited chloroplasts (Fig. 6). Cornic and Maginiac-Maslow [30] reported without explanation that the activity from DCIP to $NADP^+$ in broken spinach chloroplasts was more sensitive to photoinhibition than that from DCIP to methyl viologen. These discrepancies can be explained by differences in the degree of intactness required for the two activities as shown in Fig. 8 and discussed above.

As indicated in Fig. 2, the affinity of $HgCl_2$-treated chloroplasts to DCIP was lower than that of the control chloroplasts. These observations can be explained as a result of the destruction of plastocyanin, which is no less sensitive than center B. In order to confirm the above idea, PS I particles devoid of plastocyanin were prepared from $HgCl_2$-treated and control chloroplasts, and their flash-induced A_{430} kinetics were measured. We found that both preparation had very similar rate constants of re-reduction of $P\text{-}700^+$ by reduced DCIP

and TMPD ($k = 1.9 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ for DCIP, and $k = 3.0 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ for TMPD) in the presence of sufficient concentrations of methyl viologen (data not shown). These results indicate that the lower affinity for DCIP of $HgCl_2$-treated chloroplasts (Fig. 2) might be due to the destruction of intact plastocyanin.

A molar ratio of $HgCl_2$ to chlorophyll just enough for almost complete destruction of center B varied with each chloroplast preparation, and was often lower than 1.0 which Kimimura and Katoh [31] recommended for specific inactivation of plastocyanin in chloroplasts; in four osmotically shocked preparations treated as described in Materials and Methods, we found that the $HgCl_2$/chlorophyll ratio just enough for almost complete destruction of center B was 0.3–0.5 (mol/mol). It was about 1.0 in one chloroplast preparation which was prepared without an osmotic shock. Kimimura and Katoh already noticed that there was another site in PS I which is less susceptible to $HgCl_2$ than plastocyanin, and Kojima et al. [14,32] later indicated that the latter susceptible site is center B. As described above, the difference in the susceptibility between the two is not very large (Figs. 1 and 2). These results call for attention against the imprudent use of $HgCl_2$ as a specific inhibitor of plastocyanin. When using $HgCl_2$ as an inhibitor of plastocyanin, one must be careful to ascertain the intactness of center B either by ESR spectroscopy or by measuring the $NADP^+$ photoreduction activity under saturated concentrations of plastocyanin or DCIP plus ascorbate.

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