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Restoration of the high-potential form of cytochrome *b*559 of photosystem II occurs via a two-step mechanism under illumination in the presence of manganese ions

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Abstract

Spinach photosystem II membranes that had been depleted of the Mn cluster contained four forms of cytochrome (Cyt) *b*559, namely, high-potential (HP), HP′, intermediate-potential (IP) and low-potential (LP) forms that exhibited the redox potentials of +400, +310, +170 and +35 mV, respectively, in potentiometric titration. When the membranes were illuminated with flashing light in the presence of 0.1 mM Mn^{2+}, the IP form was converted to the HP′ form by two flashes and then the HP′ form was converted to the HP form by an additional flash. The quantum efficiency of the first conversion appeared to be quite high since the conversion was almost complete after two flashes. By contrast, the second conversion proceeded with low quantum efficiency and 40 flashes were required for completion. The effects of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) suggested that the first conversion did not require electron transfer from Q_A to Q_B while the second conversion had an absolute requirement for it. It was also suggested that the first conversion involved the reduction of the heme of Cyt *b*559, probably by Q_A^-, and we propose that direct reduction by Q_A^- induces a shift in the redox potential of the heme. The second conversion was also accompanied by the reduction of heme but it appeared that this conversion did not necessarily involve the reduction. The effects of DCMU on the reduction of heme suggested that the heme became reducible by Q_B^- after the first conversion had been completed. This observation implies that the efficiency of electron transfer from Q_A to Q_B increased upon the conversion of the IP form to the HP′ form, and we propose that restoration of the high-potential forms of Cyt *b*559 itself acts to make the acceptor side of photosystem II functional.

Keywords: Cytochrome *b*559; Manganese; Photosystem II; Potentiometric titration; Redox potential

1. Introduction

Cytochrome (Cyt) *b*559 is an integral component of photosystem II (PSII) and it is tightly bound to the PSII reaction center complex, which consists of two homologous proteins, the D1 and D2 proteins [1]. The cytochrome consists of two protein subunits, α and β, and it is considered that the heme iron forms cross-links with single histidine residues in each subunit [2]. From the orientation in the thyla-

Abbreviations: Chl, chlorophyll; Cyt, cytochrome; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; E_m, midpoint potential; HP, high-potential; IP, intermediate-potential; LP, low-potential; MES, 2-(*N*-morpholino) ethanesulfonic acid; PSII, photosystem II; Q_A and Q_B, primary and secondary quinone acceptors, respectively, of photosystem II; Tris, tris(hydroxymethyl)aminomethane; VLP, very-low-potential

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koid membrane of each subunit and the spectroscopic characterization of the heme inside the membrane, it has been suggested that the heme is located close to the stromal surface of the membrane [3–5]. Although Cyt *b*559 is necessary for the biogenesis and assembly of PSII [6,7], its function in the assembled PSII remains obscure. It has been suggested that Cyt *b*559 might act to protect PSII from damage caused by excess light energy, either by mediating a cyclic electron-transport reaction around PSII (for review, see [7]) or by oxidizing the reduced form of the primary electron acceptor of PSII, pheophytin *a*, to suppress the overreduction of Q_A [8].

Cyt *b*559 is unusual in that its redox potential is variable, and it can exist in three forms, each of which has a different redox potential, namely, the high-potential (HP), low-potential (LP) and very-low-potential (VLP) forms. The redox potentials of the HP, LP and VLP forms are about +370, +60 and $\leq$0 mV, respectively [9]. The presence of an intermediate-potential (IP) form, with a redox potential at pH 6.5–7.0 that ranges from +170 to +240 mV, has also been reported [10–13]. It has been proposed that the low-potential forms (probably the VLP form) are located in the stromal thylakoids [14], possibly in association with inactive PSII [15].

More than half of Cyt *b*559 is generally in the HP form in materials in which PSII contains a Mn cluster and is active in the O_2 evolution, both in vivo [16] and in vitro [7,9]. By contrast, in materials in which PSII lacks in the Mn cluster and is inactive in the O_2 evolution, almost all of the Cyt *b*559 is in the LP form in vivo [16,17] and in isolated thylakoids [18]. For many years, it was assumed that the HP form of Cyt *b*559 was required for the O_2 evolution. However, recent studies using isolated PSII membranes have demonstrated that Cyt *b*559 can remain in the HP form even after the O_2 evolution has been completely suppressed by removal of the Mn cluster [19–21].

The LP form of Cyt *b*559 can be converted to the HP form under conditions for photoactivation of O_2 evolution (reconstitution of the Mn cluster) both in vivo [16] and in vitro [18,21]. Our previous studies in vitro using thylakoids and PSII membranes have demonstrated that, when PSII that has been depleted of the Mn cluster is illuminated in the presence of exogenous Mn^{2+} (photoactivation conditions), the HP form is restored prior to the reconstitution of the Mn cluster [18,21]. Moreover, this conversion to the HP form requires two photoreactions in PSII [18,21]. It has also been proposed that Mn^{2+} merely acts as an electron donor to PSII during the restoration of the HP form, and that Q_A^-, generated by an electron transport reaction through PSII, is involved in the process [21]. However, the mechanism responsible for the restoration of the HP form has not yet been fully characterized.

In this study, we reexamined in detail the restoration of the HP form of Cyt *b*559 during illumination in the presence of Mn^{2+}. We found that it is not the LP form but, rather, the IP form that is converted to the HP form and, moreover, that the conversion proceeds via an intermediary-potential form. Furthermore, it is suggested that the conversion involves the reduction of the heme of Cyt *b*559. A possible mechanism for the restoration of the HP form and its physiological role in PSII are discussed.

2. Materials and methods

PSII membranes were prepared from spinach leaves and stored at −80°C as described previously [21]. The total amount of Cyt *b*559 in these membranes was 1.9–2.1 per 220 Chl (mol/mol). To remove the Mn cluster and the three extrinsic proteins from PSII, the membranes were suspended in 0.8 M Tris-HCl (pH 9.1)/5 mM EDTA at 0.5 mg Chl/ml and incubated in darkness for 2 h. To remove the extrinsic proteins of 23 and 18 kDa, the membranes were treated with 1.5 M NaCl for 30 min in darkness [22]. The treated membranes were collected by centrifugation at $35\,000 \times g$ for 20 min and washed twice with 20 mM NaCl/0.4 M sucrose/50 mM MES-NaOH (pH 6.5) by resuspension (0.3 mg Chl/ml) and centrifugation. Membranes were finally suspended in the same medium and stored at −80°C until use. All procedures were performed at 0–4°C. Handling of the treated membranes was performed under dim light. Chl concentration was determined as described by Arnon [23]. Depletion of the Mn cluster in the Tris-treated membranes and depletion of the extrinsic proteins in the Tris-treated and NaCl-treated membranes were confirmed by atomic absorption analysis and SDS-polyacrylamide gel electrophoresis, respectively.

Illumination of PSII membranes in the presence of Mn^{2+} was performed at 25°C in a medium that contained 1 M NaCl, as described previously [21]. In the case of the Tris-treated membranes, membranes were subjected to preillumination treatment as follows. They were suspended in 1 M NaCl/0.4 M sucrose/50 mM MES-NaOH (pH 6.5; high-salt medium) at 250 μg Chl/ml, illuminated for 30 s at 25°C with continuous light from a white fluorescent lamp at 33 μE m^{-2} s^{-1} and then incubated for a further 5 min in darkness. This preillumination treatment in the presence of 1 M NaCl decreased the level of the HP form by about 0.2–0.5/220 Chl (mol/mol) (see [21]). The Tris-treated or NaCl-treated membranes, suspended in the high-salt medium at 250 μg Chl/ml, were supplemented with 0.1 mM Mn^{2+} and 50 mM Ca^{2+} and then illuminated by a series of flashes at 1-s intervals [21]. When experiments were performed under anaerobic conditions, the suspension of PSII membranes was supplemented with 5 mM glucose, 0.1 mg/ml glucose oxidase (from *Aspergillus niger*; Wako, Osaka, Japan) and 0.1 mg/ml catalase (from bovine liver; Wako) and incubated in darkness for 5 min prior to illumination.

Levels of Cyt *b*559 with various redox potentials were determined from 'reduced-minus-oxidized' difference spectra [21]. The suspension of PSII membranes (250 μg Chl/ml) was diluted 5-fold with the high-salt medium that contained 0.1 mM Mn^{2+} and 50 mM Ca^{2+} prior to the analysis. For the reduction of Cyt *b*559, the sample was supplemented sequentially with 20 and 400 μM hydroquinone, 5 mM sodium ascorbate and a few mg of sodium dithionite (see Section 3). After each addition, the absorbance spectrum was recorded and the difference spectrum of Cyt *b*559 was obtained, with the absorbance spectrum recorded in the presence of 80 μM potassium ferricyanide taken as the reference spectrum. Absorbances at 559 and 570 nm were determined after subtracting the baseline from the difference spectrum, and the amount of Cyt *b*559 in the reduced form was calculated using a difference extinction coefficient [$\varepsilon^{\Delta}_{(559-570\ nm)}$] of 15 mM^{-1} cm^{-1} [9]. The deviation among results of replicate experiments was less than 5%.

Potentiometric titration of Cyt *b*559 was performed at 25°C as follows. PSII membranes were suspended in the high-salt medium at 100 μg Chl/ml, the suspension was supplemented with glucose, glucose oxidase and catalase to create anaerobic conditions, as described above, and then further supplemented with the redox mediators, namely, 25 μM potassium ferricyanide (E_{m7} = +430 mV) and 20 μM each hydroquinone (E_{m7} = +280 mV), *N*-methylphenazonium methosulfate (E_{m7} = +80 mV), 5-hydroxy-1,4-naphthoquinone (E_{m7} = +33 mV), 2-hydroxy-1,4-naphthoquinone (E_{m7} = −140 mV) and sodium anthraquinone-2-sulfonate (E_{m7} = −225 mV). For oxidative titration, all the Cyt *b*559 was first reduced by decreasing the ambient redox potential of the suspension to about −100 mV with a small volume of 0.5 M sodium dithionite in 10 mM NaOH. Then, the ambient potential was increased gradually with small volumes of 25, 50, 100, 200 or 500 mM potassium ferricyanide. For reductive titration, all the Cyt *b*559 was first oxidized by increasing the ambient potential to about +500 mV with a small volume of 0.2 M potassium ferricyanide and then the ambient potential was gradually decreased with small volumes of 1–400 mM hydroquinone, 0.1–1 M sodium ascorbate or 0.1–0.5 M sodium dithionite. The ambient redox potential of the suspension was measured at 25°C with a Pt/Ag-AgCl electrode (TOA, Tokyo, Japan) that was connected to a pH meter. The electrode was calibrated by measuring the potential of a saturated solution of quinhydrone. The absorbance spectrum was recorded after the ambient potential had reached a constant value: the potential became stable within 10 min after addition of the reductant or oxidant and remained constant for at least 2 h at every potential examined. Levels and E_m values of Cyt *b*559 in various potential forms were obtained by fitting the titrations to a linear combination of multiple $n = 1$ Nernst equation curves using a nonlinear curve-fitting program, Igor (Wavemetrics, USA) as reported by Iwasaki et al. [12].

3. Results

It has been reported that the LP form of Cyt *b*559 is converted to the HP form upon photoactivation of O_2 evolution in vivo and in vitro [16,18,21]. In the cited studies, the different forms of Cyt *b*559 were quantified after sequential addition of hydroquinone (E_{m7} = +280 mV), ascorbate (E_{m7} = +60 mV) and di-

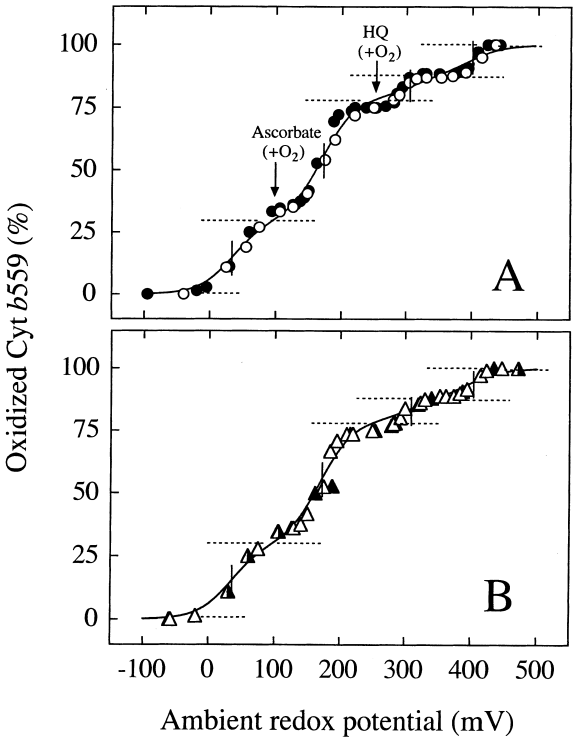

Fig. 1. Potentiometric titration of Cyt *b*559 in Tris-treated PSII membranes. Tris-treated PSII membranes were suspended in 1 M NaCl/0.4 M sucrose/50 mM Mes-NaOH (pH 6.5; high-salt medium), illuminated with continuous light for 30 s to lower the level of the HP form of Cyt *b*559, and incubated in darkness for 5 min (preillumination treatment). Then, the membranes were subjected to oxidative or reductive titration in the high-salt medium under anaerobic conditions. (A) Oxidative titration (●) followed by reductive titration (○). Ambient redox potentials in the presence of 2 mM hydroquinone (HQ) or 5 mM ascorbate under aerobic conditions are also shown. (B) Reductive titration (△) followed by oxidative titration (▲). Solid lines represent the fitting curves that consist of four $n=1$ Nernst curves with different redox potentials obtained from oxidative (A) and reductive (B) titrations. The total level of Cyt *b*559 was 1.92/220 Chl (mol/mol).

thionite ($E_{m7} < -1$ V) under aerobic conditions, and fractions of the cytochrome reduced by each addition were taken as the HP, LP and VLP forms, respectively, according to a previously described method [24]. Under these conditions, however, the IP form cannot be distinguished from the LP form. In addition, it is known that the presence of oxygen molecules affects the ambient redox potential of a solution. Therefore, in the present study, we first established experimental conditions that were suitable for quantification of the various different forms of Cyt *b*559 under aerobic conditions.

Fig. 1 shows the results of potentiometric titration of Cyt *b*559 in Tris-treated PSII membranes under anaerobic conditions. The titration was performed in four different ways, namely, oxidative titration and the following reductive titration (Fig. 1A), and reductive titration and the following oxidative titration (Fig. 1B). All the four titrations gave almost identical curves and four forms of Cyt *b*559 with different redox potentials were detected. Their E_m values were +400 (HP form), +310, +170 (IP form) and +35 mV (LP form). The VLP form was not detected in the PSII membranes that we used (Fig. 1).

The form of Cyt *b*559 with an E_m value of +310 mV has not previously been described. As shown in Fig. 2, spectral features of this form were identical to those of the HP form of Cyt *b*559: the wavelength of the absorption maximum and the half-band width were the same in both cases. Thus, it is likely that the redox component with an E_m value of +310 mV is one of different forms of Cyt *b*559. This form is designated hereafter as the HP′ form.

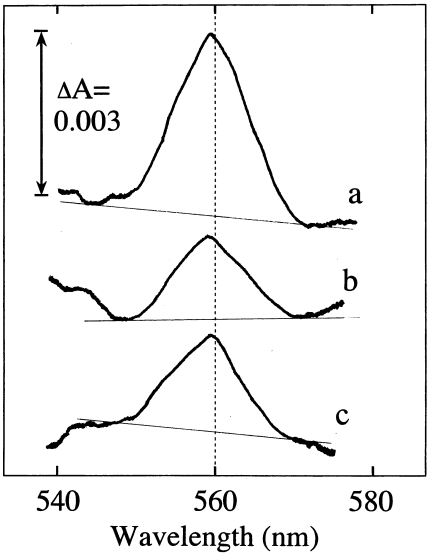

Fig. 2. Reduced-minus-oxidized spectra of the HP and HP′ forms of Cyt *b*559. Difference spectra obtained during reductive titration in Fig. 1B were presented. All the Cyt *b*559 was first oxidized with potassium ferricyanide and then the ambient potential (E_h) was gradually decreased to designated potentials with hydroquinone. (a) The HP+HP′ forms ($E_h = +282$ mV), (b) the HP form ($E_h = +330$ mV), (c) the HP′ form (spectrum a−spectrum b).

We determined the ambient redox potentials of the membrane suspension under aerobic conditions by potentiometry. Ambient redox potentials were about +250, +120 mV and −1 V after sequential additions of 2 mM hydroquinone, 5 mM ascorbate and a few mg of dithionite, respectively (see Fig. 1A). These reagents had been used to quantify the HP, LP and VLP forms in previous studies. Our results indicated that, under aerobic conditions, 2 mM hydroquinone reduced the HP and HP′ forms and 5 mM ascorbate reduced the IP form but not the LP form. We demonstrated previously that the Cyt *b*559 that is reducible by 5 mM ascorbate is converted to a form that is reducible by 2 mM hydroquinone upon photoactivation of O_2 evolution [18,21]. Therefore, our observations indicated that the form of Cyt *b*559 that was converted to the HP form upon photoactivation was the IP form but not the LP form.

We performed titrations with hydroquinone to quantify the HP and HP′ forms separately under aerobic conditions (Fig. 3A). In the Tris-treated PSII membranes used in this experiment, about 0.4 Cyt *b*559 per 220 Chl (mol/mol) was reducible by 2 mM hydroquinone, while 0.8 and 0.8/220 Chl were reducible by ascorbate (IP form) and dithionite (LP form), respectively. In the absence of hydroquinone, almost all the Cyt *b*559 was present in the oxidized form. With increasing concentrations of hydroquinone, the amount of Cyt *b*559 in the reduced form increased. Under the conditions of this experiment, the level of Cyt *b*559 in the reduced form reached a stable maximum level within 10 min after the addition of hydroquinone, an indication that the ambient potential had reached equilibrium. It is likely that added hydroquinone and dissolved oxygen molecules would act as redox mediators under these conditions. The titration gave a curve that consisted of two successive sigmoid curves, with plateaus at 10–30 μM and 300–1000 μM hydroquinone, respectively. This result suggests that two forms of the cytochrome with different redox potentials could be reduced by hydroquinone. As judged from the ambient potentials at mid-points of the two sigmoid curves (+390 and +300 mV, respectively), the two sigmoid curves appeared to represent reduction of the HP form and the HP′ form, respectively. The concentrations of hydroquinone at which the HP and the HP′ forms of Cyt *b*559 were completely reduced were 20 and 400 μM, respectively.

Fig. 3B shows the results of titration with hydroquinone of Cyt *b*559 in Tris-treated PSII membranes after illumination with 40 flashes in the presence of 0.1 mM Mn^{2+}. The titration gave a curve similar to that obtained from membranes prior to illumination. Since the concentrations of hydroquinone that gave the mid-points of the two sigmoid curves were almost the same in both cases, it appeared that the redox potentials of the HP and HP′ forms were unaffected by illumination in the presence of Mn^{2+}.

In the experiments described below, we used the

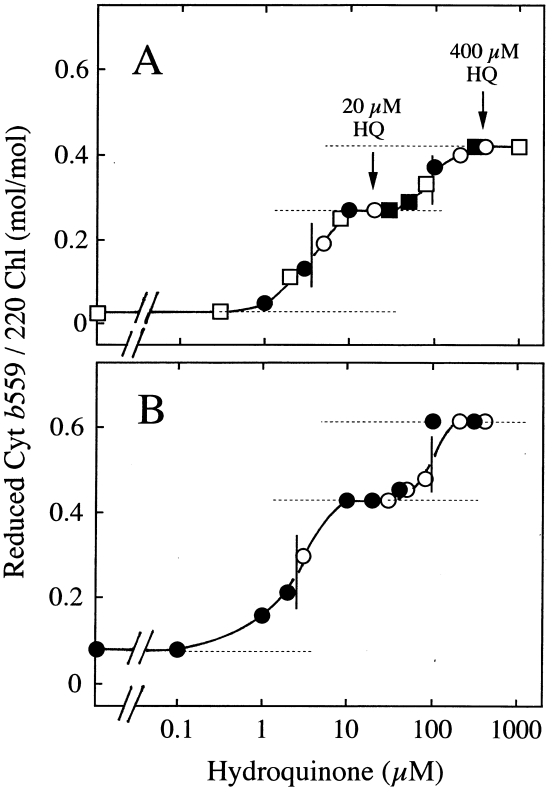

Fig. 3. Titration with hydroquinone (HQ) of Cyt *b*559 in Tris-treated PSII membranes under aerobic conditions. A suspension of Tris-treated PSII membranes that had been subjected to pre-illumination treatment was supplemented with 0.1 mM Mn^{2+} and 50 mM Ca^{2+}. After incubation in darkness for 10 min, the suspension was illuminated with 40 flashes at intervals of 1 s. For titration, the suspension was supplemented with the designated concentrations of hydroquinone. (A) Tris-treated PSII membranes before illumination. (B) Tris-treated PSII membranes after illumination in the presence of Mn^{2+}. Different symbols represent results from different titration sequences.

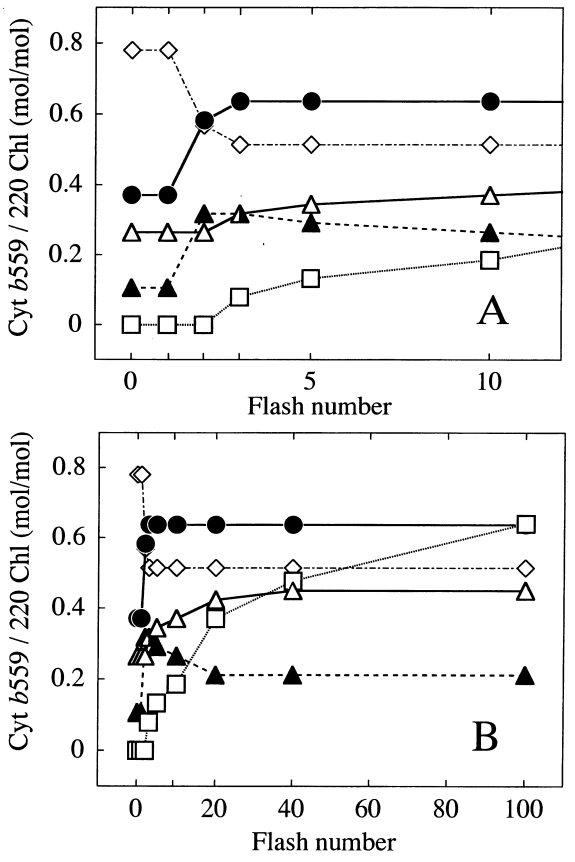

Fig. 4. Restoration of the high-potential forms of Cyt *b*559 in Tris-treated PSII membranes by illumination in the presence of Mn^{2+}. Tris-treated PSII membranes that had been subjected to preillumination treatment were illuminated with the designated number of flashes in the presence of 0.1 mM Mn^{2+} and 50 mM Ca^{2+}. The levels of the HP, HP′ and IP forms of Cyt *b*559 were determined from the levels of the cytochrome that had been reduced by 20 μM and 400 μM hydroquinone, and 5 mM ascorbate, respectively. (△) The HP form; (▲) the HP′ form; (●) the HP+HP′ forms; (◇) the IP form; (□) Cyt *b*559 that remained in the reduced form during the 2 min that followed illumination.

sequential addition of 20 and 400 μM hydroquinone to quantify the HP and HP′ forms separately under aerobic conditions. The levels of the IP and LP forms were determined after addition of 5 mM ascorbate and a few mg of dithionite, respectively.

Fig. 4 shows the changes in the levels of the HP and HP′ forms of Cyt *b*559 during illumination with flashes in the presence of Mn^{2+}. As reported previously [21], the total level of the HP and HP′ forms together, which corresponded to the level of the HP form determined in our previous study, was unchanged by a single flash, but it increased considerably after two flashes and reached a maximum after three flashes. By quantifying levels as described above, we found that the level of the HP′ form was increased by two flashes while the level of the HP form remained unchanged. The level of the IP form decreased concomitantly with the increase in the level of the HP′ form. These results suggested that the IP form of Cyt *b*559 had been converted to the HP′ form by two flashes. The quantum efficiency of this conversion appeared to be quite high since the level of the HP′ form reached a maximum after two flashes. After the third flash, the level of the HP′ form did not change significantly but the level of the HP form increased slightly. With subsequent flashes, the level of the HP form increased gradually, reaching a maximum after 40 flashes, with a concomitant decrease in the level of the HP′ form. Since the total level of the HP and HP′ forms together remained constant after the third flash, it seems likely that the HP′ form was converted to the HP form by subsequent flashes. By contrast to the initial conversion of the IP form to the HP′ form, the conversion of the HP′ form to the HP form was induced by a single flash, but the quantum efficiency of the conversion appeared to be low since the maximum level of the HP form was only reached after 40 flashes. The level of the LP form remained unchanged throughout the illumination (data not shown). These observations suggested that the IP form of Cyt *b*559 was converted to the HP form via an intermediary state, namely, the HP′ form.

Fig. 4 also shows changes in the level of Cyt *b*559 that remained in the reduced form for 2 min after illumination. The level of Cyt *b*559 in the reduced form increased gradually after the third flash, reaching a maximum at 100 flashes. The maximum level of the reduced Cyt *b*559 was equal to the total level of the HP and HP′ forms together.

Fig. 5 shows the effects of DCMU, an inhibitor of the electron transport from Q_A to Q_B, on the restoration of the high-potential forms of Cyt *b*559. When DCMU was added prior to illumination in the presence of Mn^{2+}, restoration of the HP form was completely suppressed but restoration of the HP′ form, the intermediary state, was detected (Fig. 5A). However, restoration of the HP′ form was also affected by DCMU. The level of the HP′ form was increased

by two flashes, as observed in the absence of DCMU, but the extent of the increase was about half of that observed in the absence of DCMU (see Fig. 4). With subsequent flashes, the level of the HP′ form increased, reaching a maximum after five flashes and then declining. The maximum level of the HP′ form that was restored by illumination in the presence of DCMU was almost the same as that in the absence of DCMU. Therefore, it appeared that DCMU might lower the quantum efficiency of the conversion of the IP form to the HP′ form. To examine exclusively the effects of DCMU on the conversion of the HP′ form to the HP form, we performed an experiment in which DCMU was added to a sample that had been exposed to two preliminary flashes in the absence of DCMU. As shown in panel c in Fig. 5B, the restoration of the HP form after the third flash was completely suppressed by the addition of DCMU. It seems unlikely that the HP′ form that had been restored by the first two flashes had decayed to the IP form during the time required for the addition of DCMU (about 1 min) since incubation for 1 min after the second flash, in the absence of DCMU, did not affect the restoration of the HP form after the third flash (panel b in Fig. 5B). Our observations clearly indicated that DCMU specifically suppressed the conversion of the HP′ form to the HP form, and they suggested that the electron transport from Q_A to Q_B might be required for this conversion. Reduction of Cyt *b*559 during illumination was also completely suppressed by DCMU (Fig. 5A).

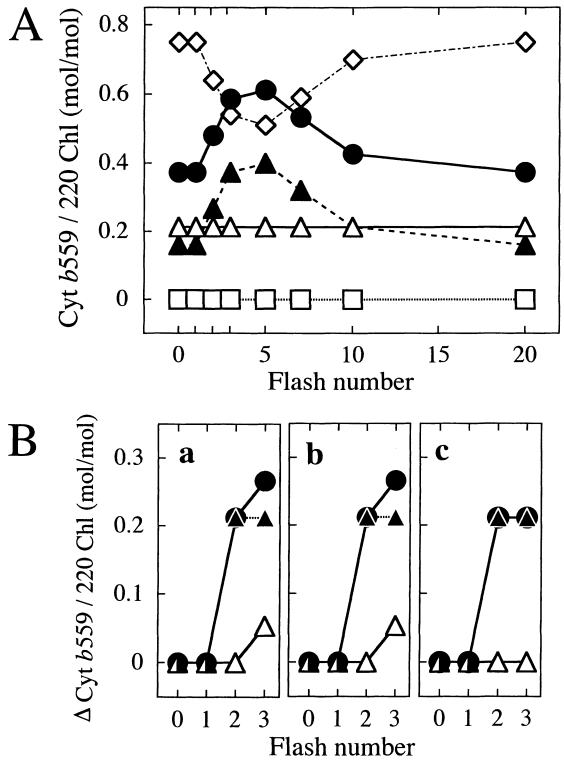

Fig. 5. Effects of DCMU on restoration of the high-potential forms of Cyt *b*559. A suspension of Tris-treated PSII membranes that had been subjected to preillumination treatment was supplemented with 0.1 mM Mn^{2+} and 50 mM Ca^{2+} and incubated in darkness for 10 min. Then the suspension was illuminated with flashes in the presence or absence of 10 μM DCMU. Addition of DCMU did not affect the level of the various forms of Cyt *b*559 prior to illumination. (A) Effects of DCMU that was added to the suspension prior to illumination. (B) Effects of DCMU that was added to the suspension after the second flash. (a) No DCMU was added. (b) No DCMU was added but the suspension was kept in darkness for one min after the second flash. (c) DCMU was added to the suspension after the second flash. (△) The HP form; (▲) the HP′ form; (●) the HP+HP′ forms; (◇) the IP form; (□) Cyt *b*559 that remained in the reduced form during the 2 min that followed illumination. Increases caused by illumination are plotted in (B).

In a previous study [21], we demonstrated that, for restoration of the HP form of Cyt *b*559, Mn^{2+} merely acts as an electron donor to PSII. We examined whether electron donation from the functional Mn cluster could also support the restoration of the HP form. NaCl-treated PSII membranes that had been depleted of the extrinsic 23- and 18-kDa proteins but retained the Mn cluster were illuminated in the presence of Mn^{2+}. As shown in Fig. 6, the levels of the HP and HP′ forms remained unchanged during exposure to as many as 100 flashes. The same result was obtained when NaCl-treated PSII membranes were illuminated in the absence of Mn^{2+} (data not shown). These observations indicated that electron donation by the Mn cluster did not support restoration of the HP form of Cyt *b*559. Illumination of NaCl-treated PSII membranes also resulted in reduction of the HP and HP′ forms of Cyt *b*559 (Fig. 6).

In the case of Cyt *c* from the horse heart, reduction of the heme shifts the equilibrium from the denatured and low-potential form toward the native and high-potential form [25]. During the restoration of the high-potential forms of Cyt *b*559, heme was

reduced upon illumination in the presence of Mn^{2+}, but no correlation between the restoration of the high-potential forms and the reduction of heme was observed (Figs. 4 and 5; [21]). Since the ambient redox potential of the membrane suspension in the presence of Mn^{2+} under aerobic conditions was about +420 mV, the reduced Cyt *b*559, even in the HP form, might have been reoxidized by ambient oxygen molecules. In fact, we observed that about 75% of Cyt *b*559 that had been reduced by exposure to 10 flashes in the presence of Mn^{2+} was reoxidized within 30 s in darkness under aerobic conditions [21]. Even when all the HP and HP′ forms were reduced by exposure to 100 flashes (see Fig. 4), they were completely reoxidized within 10 min (data not shown). Under our experimental conditions, it took about 2 min to record the absorbance spectrum of Cyt *b*559 after illumination. Thus, it is quite possible that Cyt *b*559 once reduced by a few flashes was immediately reoxidized and hardly detected under aerobic conditions.

Under anaerobic conditions, the ambient redox potential of the suspension was around +350 mV, and it was almost impossible to distinguish between the HP and HP′ forms by titration with hydroquinone. Therefore, we determined the total level of the HP and HP′ forms together by adjusting the ambient potential of the suspension to +260 mV by addition of 50 μM hydroquinone.

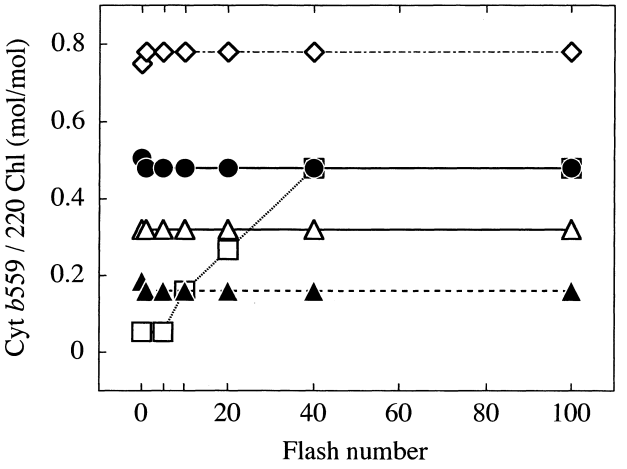

Fig. 6. Effects of illumination in the presence of Mn^{2+} on Cyt *b*559 in NaCl-treated PSII membranes. NaCl-treated PSII membranes that retained the Mn cluster were suspended in the high-salt medium and illuminated with the designated number of flashes in the presence of 0.1 mM Mn^{2+} and 50 mM Ca^{2+}. (△) The HP form; (▲) the HP′ form; (●) the HP+HP′ forms; (◇) the IP form; (□) Cyt *b*559 that remained in the reduced form after illumination.

When Tris-treated PSII membranes were transferred to anaerobic conditions, the level of the HP and HP′ forms together increased from 0.4 to 0.7/220 Chl (mol/mol) even in darkness (see Fig. 7). A similar increase in the level of the HP and HP′ forms together under anaerobic conditions was also observed in NaCl-treated PSII membranes. Illumination of Tris-treated PSII membranes in the presence of Mn^{2+} under anaerobic conditions further increased the level of the HP and HP′ forms together as observed under aerobic conditions: the level was unchanged after a single flash but increased after the second flash, reaching a maximum after the third flash (Fig. 7A). The level of Cyt *b*559 that remained in the reduced form after illumination was much higher than the level under aerobic conditions and the level exhibited a different dependence on the flash number. An increase in the level of reduced Cyt *b*559 was first detected after the third flash under aerobic conditions (see Fig. 4) while such an increase was detected after the second flash under anaerobic conditions.

To our surprise, under anaerobic conditions, the increases in the level of reduced Cyt *b*559 after the second and third flashes were exactly the same as the extents of restoration of the HP and HP′ forms together after the same respective numbers of flashes. If only the HP′ form had been restored by the second and third flashes, our results would imply that conversion of the IP form to the HP′ form is accompanied by the reduction of heme. To examine this possibility, we performed the same experiment in the presence of DCMU, which had specifically suppressed conversion of the HP′ form to the HP form (see Fig. 5). As shown in Fig. 7A, the increase in the level of reduced Cyt *b*559 was identical to the extent of restoration of the HP′ form. Thus, it appeared that the conversion of the IP form to the HP′ form involved the reduction of heme. With respect to the further conversion of the HP′ form to the HP form, it was unclear whether any reduction of heme was involved since the reduction of heme occurred after the third flash, independently of restoration of the HP form, as observed in the case of NaCl-treated PSII membranes (Fig. 7B).

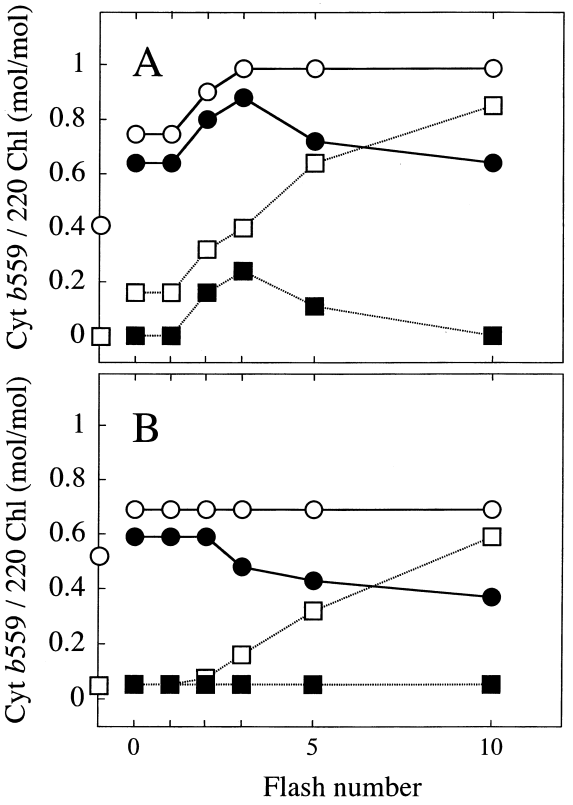

Fig. 7. Reduction of Cyt *b*559 by illumination in the presence of Mn^{2+} under anaerobic conditions. Tris-treated PSII membranes that had been preilluminated or NaCl-treated PSII membranes were incubated in the high-salt medium that contained 0.1 mM Mn^{2+} and 50 mM Ca^{2+} in darkness for 10 min. Then, the suspension was supplemented with glucose, glucose oxidase, and catalase to create anaerobic conditions, incubated in darkness for 5 min, and finally illuminated with the designated number of flashes in the presence (closed symbols) or absence (open symbols) of 10 μM DCMU. (A) Tris-treated PSII membranes. (B) NaCl-treated PSII membranes. (○, ●) The HP+HP′ forms; (□, ■) Cyt *b*559 that remained in the reduced form during the 2 min that followed illumination. Symbols on the ordinate represent the levels under aerobic conditions prior to transfer to anaerobic conditions.

4. Discussion

4.1. Restoration of the HP form of Cyt b559 proceeds via an intermediary state

We demonstrated previously that Cyt *b*559 reducible by ascorbate is converted to a form with a higher redox potential reducible by hydroquinone when Tris-treated PSII membranes are illuminated in the presence of Mn^{2+} [21]. The present study revealed that it is not the LP form (E_m = +35 mV) but, rather, the IP form (E_m = +170 mV) of Cyt *b*559 that is converted to the HP form (E_m = +400 mV) by illumination and, moreover, that the restoration of the HP form proceeds via an intermediary state, namely, the HP′ form (E_m = +310 mV).

The presence of a form of Cyt *b*559 with an E_m value slightly lower than that of the HP form has previously been demonstrated only in NH_2OH-treated PSII membranes depleted of the Mn cluster upon illumination with strong light [12]. It was reported that the HP form (E_m = +435 mV) was first converted to a transient and intermediary form (E_m = +350 mV) before being converted to forms with lower redox potentials upon illumination. It is unlikely that the HP′ form of Cyt *b*559 is present only in PSII depleted of the Mn cluster since we also detected the HP′ form in NaCl-treated PSII membranes that retained the Mn cluster by titration with hydroquinone (data not shown). It is unlikely either that the HP′ form is an artifact obtained during potentiometric titration.

It is known that the higher-potential forms of Cyt *b*559 are unstable in the oxidized form and can be converted to forms with lower redox potentials during potentiometric titration when titration is performed in a reductive direction [13]. As shown in Fig. 1, however, potentiometric titrations both in oxidative and reductive directions gave identical curves and, moreover, no hysteresis was observed during the reductive titration following the oxidative titration and vice versa. It is unlikely either that the detection of the HP′ form resulted from poor redox buffering under our titration conditions since hydroquinone (E_m = +280 mV) would sufficiently buffer redox potential in a potential range for titration of the HP′ form. Rather, the redox buffering appeared to be poor in a potential range for titration of the IP form. Since a form of Cyt *b*559 with a similar redox potential (E_m = +170–+240 mV) has previously been reported by many research groups using different sets of redox mediators [10–13], the IP form could not be an artifact, though its redox potential determined in this study (+170 mV) might not be accurate.

Taken together, we consider that the HP′ form is actually one of forms of Cyt *b*559 with different redox potentials, or it represents some state of Cyt *b*559 distinct from other three potential forms. We

presume that the failure to detect the HP′ form in previous studies might have been due to the limits of the resolution of previous titrations of Cyt *b*559. Since the difference in the E_m value between the HP and HP′ forms is relatively small (90 mV), it is difficult to discriminate each form as a single component during titration.

In untreated PSII membranes that contained the Mn cluster and all the three extrinsic proteins, the HP′ form was not detected by potentiometric titration [26]. This observation implies that the HP′ form of Cyt *b*559 exists only in PSII that has been affected by removal of the extrinsic proteins and/or treatments to remove the proteins.

Restoration of the HP form appears to proceed via two steps, namely, conversion of the IP form to the HP′ form and subsequent conversion of the HP′ form to the HP form. These two steps differ from one another in some respects. The first conversion was induced by two flashes at high quantum efficiency, while the subsequent conversion was inducible by a single flash but the quantum efficiency was low (Fig. 4). The effects of DCMU on the two steps were also different. DCMU merely lowered the quantum efficiency of the first conversion but it suppressed the second conversion completely (Fig. 5). Thus, it is likely that the first conversion does not involve electron transfer from Q_A to Q_B while the second conversion is absolutely dependent on it. It seems clear, therefore, that the two conversions involve different mechanisms.

The Tris-treated PSII membranes used in this study were completely depleted of the three extrinsic proteins of PSII, and thus, it is proposed that restoration of high-potential forms of Cyt *b*559 can occur in the absence of these proteins.

4.2. Involvement of the reduction of the heme in the restoration of the HP form of Cyt b559

We examined previously whether restoration of the HP form of Cyt *b*559 involved the reduction of heme. However, under aerobic conditions, the extent of the reduction was always smaller than that of the restoration because of reoxidation of the reduced heme by ambient oxygen molecules (Figs. 4 and 5; [21]). We performed experiments under anaerobic conditions and found that the first conversion, from the IP form to the HP′ form, which occurred by illumination with two flashes, would involve the reduction of heme (Fig. 7A). One might consider that the reduction of heme is of thermodynamic necessity if its midpoint potential is increased, and that it could not be a cause but a result of the restoration of the HP′ form. However, this would not be the case. As seen in Fig. 7A, the increase in the level of reduced Cyt *b*559 was exactly the same as the extent of restoration of the HP′ form after the second and third flashes. Thus, it appeared that a fraction of the IP form that was going to be converted to the HP′ form was selectively reduced with high quantum efficiency, while the HP and HP′ forms that had originally been present before illumination were reduced with low quantum efficiency. Taking these into consideration, we propose that the reduction of heme of Cyt *b*559 induces the conversion of the IP form to the HP′ form, as in the case of Cyt *c* [25].

Chemical reduction of heme by a reductant, such as ascorbate, in darkness is insufficient to restore the high-potential forms of Cyt *b*559 [21]. Therefore, it seems likely that heme has to be reduced by some redox component(s) of PSII for the conversion of the IP form to the HP′ form. The most probable candidate, in this case, for the reductant of heme is Q_A in its reduced form (Q_A^-) since both the reduction and the conversion occurred in the presence of DCMU (Fig. 5). This hypothesis is supported by our previous observations that artificial benzoquinone acceptors that efficiently reoxidize Q_A^-, namely, 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone (DBMIB) and 2,6-dichloro-1,4-benzoquinone, completely suppressed the conversion [21].

The reduction of heme also occurred even after the conversion of the IP form to the HP′ form had been completed with three flashes, and it continued until the HP and HP′ forms had been fully reduced (Figs. 4 and 7). We also observed the reduction of heme in NaCl-treated PSII membranes under illumination (Figs. 6 and 7). In both cases, the reduction was completely suppressed by DCMU. Therefore, the reductant of heme in these cases was probably Q_B or a plastoquinone molecule in the reduced form, as demonstrated previously in thylakoids [27,28] and PSII membranes [10].

When more than five flashes were given in the presence of DCMU, the level of the HP′ form de-

creased gradually to the level before illumination, with a concomitant decrease in the level of reduced heme (Fig. 7A). Since the decrease in the level of reduced heme occurred even under anaerobic conditions, the oxidant of the heme in these cases could not have been ambient oxygen molecules. The reoxidation of heme might be ascribed to the cyclic electron flow around PSII (for review, see [7]). If this hypothesis is correct, the concomitant decreases in the level of the HP′ form and in the level of reduced heme suggest that the reoxidation of heme by some redox component(s) at the donor side of PSII might convert the HP′ form back to the IP form. This scenario is in sharp contrast to the reoxidation of heme by ambient oxygen molecules, which does not affect the redox potential of Cyt *b*559.

4.3. The mechanism of restoration of the HP form of Cyt b559

As discussed above, it seems likely that reduction of the heme of Cyt *b*559 in the IP form, probably by Q_A^-, induces the conversion of the IP form to the HP′ form. Although this conversion requires two photoreactions in PSII, we observed the conversion itself and the reduction of heme only after the second photoreaction (Fig. 7). Thus, it is possible that a single photoreaction might be sufficient for the conversion and that the first photoreaction in PSII might not participate in the conversion.

The effects of sequential addition of DCMU and benzoquinone acceptors led us to propose previously that the non-heme iron at the acceptor side of PSII must stay in the Fe(II) state for restoration of the HP form of Cyt *b*559 [21]. In general, the non-heme iron is in the Fe(II) state both in light and in darkness [29]. However, when the non-heme iron is in the Fe(III) state in darkness for some reason, illumination with a single flash leads to reduction of the non-heme iron through Q_A^- [29]. Some fraction of the non-heme iron appears to exist in the Fe(III) state in PSII membranes depleted of the Mn cluster by treatment with NH_2OH [20]. Therefore, it might be possible that the non-heme iron in the Tris-treated PSII membranes used in the present study stayed in the Fe(III) state and, consequently, that the first photoreaction in PSII resulted in the reduction of the non-heme iron but not of the heme of Cyt *b*559. In fact, we observed that, when Tris-treated PSII membranes were illuminated with a single flash prior to the addition of Mn^{2+}, the high-potential forms of Cyt *b*559 reducible by hydroquinone (the HP and HP′ forms) were restored by a single flash in the presence of Mn^{2+} (data not shown).

The second conversion, from the HP′ form to the HP form, required a single photoreaction in PSII but the quantum efficiency was low. Although it is still unclear whether this conversion involves the reduction of heme, it does require electron transport from Q_A to Q_B. In view of the low quantum efficiency of the conversion, it is possible that the ambient potential of the microenvironment around the heme of Cyt *b*559 might gradually be lowered during illumination with repetitive flashes so that the equilibrium between the HP′ form and the HP form shifts toward the HP form.

With respect to the reductant of the heme of Cyt *b*559, it was Q_A^- during illumination with the first few flashes while it was replaced by Q_B^- (or reduced plastoquinone) during subsequent flashes. This change in the reductant might be ascribable to the change in the redox potential of Q_A. It has been reported that, upon removal of the Mn cluster, Q_A is converted from an active form with a low redox potential ($E_m = -80$ mV) to an inactive form with a high redox potential ($E_m = +55$ mV) that is unable to transfer electrons to Q_B [30]. This inactive Q_A can be recovered to the active form during or after the reconstitution of the Mn cluster [30]. If Q_A were to be converted to the active form during illumination in the presence of Mn^{2+}, the conversion would occur after the first few flashes. Under the illumination conditions in the present study, reconstitution of the Mn cluster occurred only after the sixth flash [21]. Thus, it is possible that active Q_A might have been restored before reconstitution of the Mn cluster.

With respect to the events at the donor side of PSII, we demonstrated previously that Mn^{2+} merely acts as an electron donor to PSII and that other artificial electron donors can also support restoration of the HP form of Cyt *b*559 [21]. The present study revealed that some redox component(s) at the donor side of PSII, which is exposed to the outer aqueous phase upon removal of the Mn cluster, is involved in the restoration. It has been proposed that a redox-active histidine residue(s) at the donor side of PSII is

photooxidized in PSII depleted of the Mn cluster and that the oxidized histidine subsequently oxidizes exogenous Mn^{2+} with high quantum efficiency [31,32]. It is possible that electron transfer reaction(s) between the oxidized histidine(s) and the exogenous donor might be required for restoration of the high-potential forms of Cyt *b*559. This hypothesis is consistent with the observation that chemical reduction of heme by an exogenous reductant does not lead to the restoration [21].

The restoration of high-potential forms of Cyt *b*559 occurred even in darkness when PSII membranes depleted of the Mn cluster were exposed to anaerobic conditions (Fig. 7A). Similar restoration under anaerobic conditions was observed previously in PSII membranes depleted of the Mn cluster by treatment with NH_2OH [33]. In contrast to the light-dependent restoration of the high-potential forms, the restoration under anaerobic conditions occurred even in NaCl-treated PSII membranes that retained the Mn cluster (Fig. 7B). Therefore, a different mechanism appears to operate in the restoration under anaerobic conditions.

As discussed above, we propose that the restoration of the HP′ form under illumination involves electron transfer reaction(s) from the exogenous donor to Q_A and the subsequent reduction of the heme by Q_A^-. To understand the precise mechanism of the restoration, however, further study is required.

It has been proposed that Cyt *b*559 exists in the HP form when the heme is located inside an extremely hydrophobic microdomain that is shielded from water molecules by protein segments [34]. According to this hypothesis, the heme of Cyt *b*559 in its low-potential forms is exposed to the outer aqueous phase. These postulates imply that the conformation of the microdomain of the heme is altered significantly upon the conversion of the various forms of Cyt *b*559. Upon conversion of Cyt *b*559 from the IP form to the HP′ form, this change in conformation should occur with quite high quantum efficiency. If restoration of active Q_A were to occur concomitantly with the restoration of the HP′ form, as discussed above, it is likely that the entire conformation at the acceptor side of PSII would be altered considerably upon this conversion.

This hypothesis implies that a change in the conformation of Cyt *b*559 itself might lead to a change

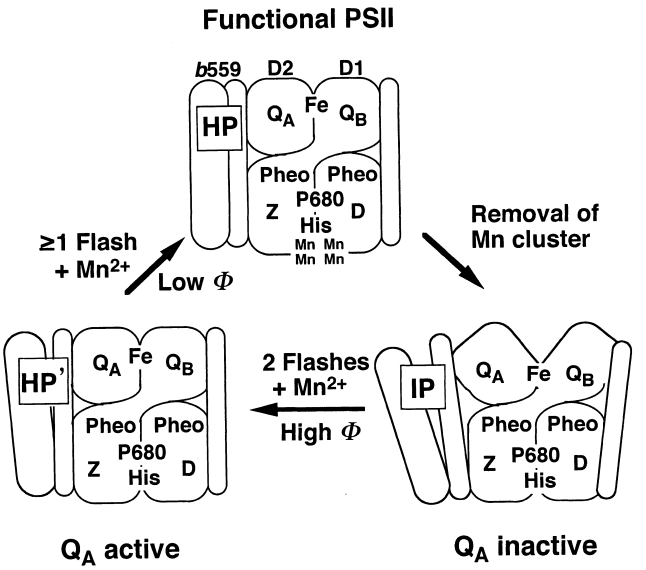

Fig. 8. Schematic diagram showing the events that occur in the PSII reaction center complex by removal of the Mn cluster and subsequent illumination in the presence of Mn^{2+}.

in the entire structure of the reaction center complex of PSII so as to make the acceptor side of PSII functional. Our preliminary experiments indicated that the reduction of heme of Cyt *b*559 after illumination with two flashes proceeded very slowly with a half time of not less than several seconds (data not shown). Although the time required for the conversion of the IP form to the HP′ form could not be determined yet, it is expected that the conversion would proceed as slowly as the reduction of heme. Such a slow conversion is not so unlikely if a change in the entire conformation at the acceptor side of PSII is involved in the process. Once the acceptor side has become functional, reconstitution can occur of the Mn cluster, a reaction that proceeds with low quantum efficiency. Conversely, it is also possible that, when the high-potential forms of Cyt *b*559 are converted to the IP form upon removal of the Mn cluster, Q_A becomes unable to transfer electrons to Q_B, thereby increasing the probability of charge recombination reactions inside the PSII reaction center and preventing photoinhibition of PSII, as proposed previously [30]. A schematic diagram of this hypothesis is shown in Fig. 8.

In this study we used PSII membranes to investigate the precise mechanism of restoration of the HP form of Cyt *b*559. However, the extent of the inter-

conversion between the IP form and the high-potential forms is much smaller in PSII membranes than in thylakoids. In PSII membranes and thylakoids, respectively, upon removal of the Mn cluster by treatment with Tris, 30–40% and 80% of the high-potential forms are converted to the IP form, and 30–50% and 50–80% of the IP form are reconverted to the high-potential forms by photoactivation treatment [21]. If these differences are due to differences in the integrity of PSII and/or the thylakoid membranes, it is likely that, in vivo, all the IP and high-potential forms of Cyt *b*559 can be interconverted, depending on the presence or absence of the Mn cluster. We propose, furthermore, the working hypothesis that the interconversion of the various forms of Cyt *b*559 with low and high redox potentials acts as a molecular switch to regulate the function of PSII.

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