Study on the Mechanism of Restoration of the High-Potential Form of Cytochrome c559 during Photoactivation of Photosynthetic Oxygen Evolution

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ABBREVIATIONS

ASC	sodium ascorbate
BSA	bovine serum albumin
Chl	chlorophyll
Cyt	cytochrome
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone
DCBQ	2,6-dichloro-1,4-benzoquinone
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DCPIP	2,6-dichlorophenolindophenol
Dith	sodium dithionite
DPC	1,5-diphenylcarbazide
EDTA	ethylenediaminetetraacetic acid
FeCN	potassium ferricyanide
Hepes	*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)
HP	high-potential
HQ	hydroquinone
LP	low-potential
Mes	2-(*N*-morpholino)ethanesulfonic acid
P680	primary electron donor of photosystem II
PSI and PSII	photosystem I and photosystem II
Q_A and Q_B	primary and secondary quinone electron acceptors, respectively, of photosystem II
SDS	sodium dodecylsulfate
Tris	tris(hydroxymethyl)aminomethane
VLP	very-low-potnetial
Y_Z	redox-active tyrosine-161 of the D1 protein

ABSTRACT

Cytochrome *b*559 (Cyt *b*559) is one of the *b*-type cytochromes present in the thylakoid membrane. This cytochrome is an intrinsic membrane protein and tightly bound to the photochemical reaction center complex of photosystem II (PSII). Cyt *b*559 is considered to be an essential component of PSII because of its location inside PSII. However, the function of Cyt *b*559 remains to be unequivocally resolved. One unique characteristics of Cyt *b*559 is that its redox potential is variable, and it exists in at least three forms with different redox potentials, namely, the high-potential (HP; Eo'= +370 mV), the low-potential (LP; Eo'= +60 mV) and the very low-potential (VLP; Eo'$\leq$ 0 mV) forms. The different potential forms of Cyt *b*559 are partly interconvertible. In materials in which PSII contains the Mn cluster and active in O_2 evolution, more than half of Cyt *b*559 is present in the HP form. The HP form can be converted to the LP form by treatments which deplete the Mn cluster to inactivate O_2 evolution. Conversely, the HP form of Cyt *b*559 can be restored from the LP form by reconstitution of the Mn cluster under illumination to reactivate O_2 evolution [photo(re)activation of O_2 evolution]. However, the mechanism of the interconversion between the HP and LP forms of Cyt *b*559, and also the physiological role of the interconversion have not been fully understood yet.

In this study, the changes in the redox potential of Cyt *b*559 upon inactivation and photo(re)activation of O_2 evolution were first investigated in detail using isolated thylakoids. Next, the mechanism of the restoration of the HP form of Cyt *b*559 from the LP form upon the photo(re)activation was investigated using PSII-enriched membrane fragments prepared from thylakoids to obtain some insight into the physiological role of the restoration of the

HP form of Cyt *b*559.

Isolated spinach thylakoids used in this study contained about two molecules of Cyt *b*559 per 400 chlorophyll (Chl) molecules, a PSII unit. Among them, 0.5 to 0.7 molecule of Cyt *b*559 was present in the HP form. When the thylakoids were treated with 0.8 M Tris (pH 9.1) to deplete the Mn cluster, the HP form of Cyt *b*559 was almost completely converted to the LP form, concomitantly with inactivation of O_2 evolution. When the Tris-treated thylakoids were illuminated with weak light for 20 min in the presence of Mn^{2+} and Ca^{2+} [photo(re)activation treatment], 50-80% of the LP form of Cyt *b*559 was restored to the HP form, concomitantly with recovery of the O_2-evolving activity. Mn^{2+} and illumination, which are required for the photo-(re)activation of O_2 evolution, were also necessary for the restoration of the HP form. However, the restoration of the HP form was found to precede the recovery of O_2-evolving activity. This observation suggests that the restoration of the HP form is a primary process of the photo(re)activation of O_2 evolution which occurs prior to the reconstitution of the Mn cluster. It was also found that inhibitors of electron transport from PSII to PSI, namely, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone (DBMIB) completely suppressed the recovery of O_2-evolving activity while they did not at all affect the restoration of the HP form. These results suggest that the restoration of the HP form of Cyt *b*559 and the reconstitution of the Mn cluster proceed via different reaction mechanisms. Uncouplers such as nigericin and gramicidin-S inhibited both the restoration of the HP form and the recovery of O_2-evolving activity. This result might imply the possibility that the pH gradient across the thylakoid membrane would participate in the restoration of the HP form.

To investigate the mechanism of the restoration of the HP form of

Cyt *b*559 during the photo(re)activation of O_2 evolution, PSII membranes were used, which had been prepared by mild treatment of thylakoids with Triton X-100. The PSII membranes contain PSII but practically lack in other electron transport components such as Cyt *f* and Cyt *b*563. In addition, the active site for O_2 evolution in PSII membranes is exposed to the outer aqueous phase. Therefore, kinetical analyses are much easier and exogenously added reagents can directly be reacted with the active site in PSII membranes.

As in the case of isolated thylakoids, the HP form of Cyt *b*559 was converted to the LP form upon inactivation of O_2 evolution by treatment with Tris, and the LP form was converted to the HP form upon the subsequent photo(re)activation of O_2 evolution, although the extent of interconversion between the HP and the LP forms was smaller in PSII membranes than in thylakoids. Ca^{2+} was not essential for the restoration of the HP form but was necessary for retention of the HP form after illumination. When the photo-(re)activation was achieved with flashing light, the restoration of the HP form and the recovery of O_2-evolving activity exhibited quite different dependences on flash number. The restoration of the HP form occurred after 2 flashes, while the reactivation of O_2 evolution required more than 6 flashes. The levels of Mn^{2+} required for the restoration of the HP form and for the reactivation of O_2 evolution were also different. The concentrations of Mn^{2+} that gave the half-maximum restoration were 5 and 17 μM for the HP form and the O_2 evolution, respectively. It was also found that the HP form could be restored when the PSII membranes were illuminated in the presence of artificial electron donors instead of Mn^{2+}, such as NH_2OH, 1,5-diphenylcarbazide and semicarbazide. These observations suggest that the restoration of the HP form of Cyt *b*559 is a process that is independent of the reconstitution of the Mn cluster and that the electron donation by Mn^{2+} to PSII is

responsible for the restoration of the HP form. The restoration of the HP form by illumination of Tris-treated PSII membranes in the presence of Mn^{2+} was not affected by DCMU, an inhibitor of electron transport from Q_A to Q_B in PSII. This result suggests that electron transport from Q_A to Q_B is not required for the restoration of the HP form. By contrast, the restoration of the HP form was completely suppressed by artificial electron acceptors which bind to the Q_B site and reoxidize Q_A^-, namely, DBMIB and 2,6-dichloro-1,4-benzoquinone. These results suggest that Q_A^- formed by photochemical reaction in PSII is involved in the process of the restoration of the HP form.

It is known that the lifetime of Q_A^- is greatly extended by exogenous electron donors. Therefore, it is likely that the electron donors support the restoration of the HP form by stabilizing Q_A^-. It has been demonstrated recently that, in the case of horse heart Cyt *c*, the reduction of the heme shifts the equilibrium from the denatured and low-potential form toward to the native and high-potential form. In PSII, the heme of Cyt *b*559 and Q_A are both located at the stromal side, relatively close to each other. Therefore, it is quite possible that the LP form of Cyt *b*559 is reduced by Q_A^- generated by a photoreaction in PSII during the course of the restoration of the HP form.

When PSII is newly assembled *in vivo*, the protein components of PSII including Cyt *b*559, are newly synthesized, inserted into the thylakoid membrane, and assembled into PSII which lacks in the Mn cluster. Then, the Mn cluster is constituted by photochemical reactions in the nascent PSII itself. It is known that Cyt *b*559 is present in the LP form before the Mn cluster is constituted but is converted to the HP form during constitution of the Mn cluster. This study has revealed that electron donors to PSII are necessary for the conversion of Cyt *b*559 from the LP to the HP form. *In vivo*, Mn^{2+} present in the thylakoid lumen at a micromolar level would presumably be

a sole substance for the electron donor to PSII. Thus, it is likely that, when PSII is assembled *in vivo*, the LP form of Cyt *b*559 is first converted to the HP form through electron donation by Mn^{2+}, and subsequently, the Mn cluster is constituted by photooxidation of Mn^{2+}. It is considered that the conversion of Cyt *b*559 from the LP to the HP form is a primary process of the photoactivation of the nascent PSII *in vivo*.

GENERAL INTRODUCTION

Photosynthesis is a process in which solar energy is captured and converted into biologically usable chemical energy in the form of NAD(P)H and ATP, which is subsequently utilized by a sequence of enzymatic reactions to convert CO_2 to organic molecules. The energy conversion reactions to form NAD(P)H and ATP occur in the photosynthetic membrane.

Light is first absorbed by pigments (chlorophylls, carotenoids and phycobilins), and then the absorbed light energy is transferred to a pair of chlorophyll molecules with a specialized structure, the reaction center chlorophyll. When the reaction center chlorophyll is excited by the light energy, a charge separation reaction occurs between the reaction center chlorophyll (primary electron donor, D) and a neighboring pigment molecule (primary electron acceptor, A), to give rise to the state D^+–A^-. The oxidizing and reducing powers of D^+ and A^-, respectively, are transferred to redox active molecules in sequence, so that an electron is transferred from the terminal electron donor to the terminal electron acceptor.

In higher plants, the charge separation reaction occurs in two different supramolecular protein complexes embedded in the thylakoid membrane, namely, photosystem II (PSII) and photosystem I (PSI) (Vermass and Ikeuchi, 1991; Fig. 1). The overall function of the PSII complex is to transfer electrons from H_2O to plastoquinone (PQ) molecules (Vermass and Ikeuchi, 1991). Oxidation of H_2O by PSII gives rise to molecular oxygen, while reduced plastoquinone (plastoquinol) serves as an electron donor to the cytochrome (Cyt) *b*563/*f* complex, which then donates electrons to the PSI complex via a water soluble electron carrier, plastocyanin (PC), located in the thylakoid lumen. Upon excitation of the reaction center chlorophyll of PSI by light,

electrons are transported from plastocyanin to $NADP^+$, via two electron carriers, ferredoxin and ferredoxin-$NADP^+$ reductase (FNR) to give rise to NADPH (Chitnis and Nelson, 1991). This linear electron transport reaction from H_2O to $NADP^+$ generates the proton concentration gradient across the thylakoid membrane, and this gradient is used for the formation of ATP by the ATP synthase complex (Jagendorf and McCarty, 1991). ATP and NADPH generated in this way are used for CO_2 fixation (Arnon, 1987).

Cytochrome is a heme protein that carries out redox reaction with its heme iron, $Fe(II) \rightleftharpoons Fe(III) + e$. In the thylakoid membrane, three different cytochromes, namely, Cyt *b*563 (also designated as Cyt b_6), Cyt *f* and Cyt *b*559 are present. Two molecules of Cyt *b*563 and one molecule of Cyt *f* constitute the Cyt *b*563/*f* complex together with the Rieske iron-sulfer protein (Hurt and Hauska, 1982; Widger and Cramer, 1991), and they are involved in the photosynthetic electron transport. By contrast, Cyt *b*559 which is bound to the PSII complex does not directly participate in the electron transport reaction (Cramer et al., 1986; Cramer et al., 1993).

Photosystem II

The PSII complex of higher plants is a supramolecular protein complex composed of more than twenty different integral proteins and three extrinsic proteins (Fig. 2; Vermass and Ikeuchi, 1991). The reaction center complex of PSII is considered to be formed by two homologous proteins, the D1 and D2 proteins. These two proteins bind all the components involved in the charge separation and the subsequent electron transfer reactions (Trebst, 1986; Michel and Deisenhofer, 1988).

After light absorption, an electron is transferred from an excited state of the reaction center chlorophyll (P680; Doring et al., 1969) to the

Fig. 1. The schematic illustration of the photosynthetic electron transport reactions and the ATP synthesis in the thylakoid membrane of higher plants. Arrows indicate the direction of transport of electrons. Fd: ferredoxin; FNR: ferredoxin-NADP reductase; PC, plastocyanin; PQ: plastoquinone.

Fig. 2. A Schematic model for the PSII complex embedded in the thylakoid membrane of higher plants. Arrows indicate the direction of transport of electrons. The reaction center complex of PSII consists of the D1 and D2 proteins, and binds all components involved in the charge separation and the subsequent electron transport reactions, namely, the reaction center chlorophyll (P680), the primary electron acceptor pheophytin (Pheo), quinone acceptors (Q_A and Q_B), the non-heme iron (Fe), the secondary donor Y_Z (Z) and the Mn cluster for oxidation of H_2O. LHC represents light-harvesting chlorophyll complex. CP47 and CP43 represent chlorophyll binding proteins which serve as an antenna associated with the reaction center complex. 33, 23 and 18 represent the extrinsic proteins that bind to the PSII complex and regulate the reactivity of the Mn cluster (modified from Vermass and Ikeuchi, 1991).

primary electron acceptor, a pheophytin *a* molecule (Klimov et al., 1977). An electron on pheophytin is transferred via a plastoquinone molecule Q_A (van Gorkom, 1974), which is tightly bound to the D2 protein, to the other plastoquinone molecule Q_B (Crofts and Wraight, 1983), which is loosely bound to the D1 protein. Q_B is a two-electron carrier and, after double-reduced, it is converted to the protonated form Q_BH_2, and then leaves its binding site on the D1 protein. At the oxidizing side of PSII, the cation radical $P680^+$ is formed by releasing an electron from the excited state of P680. Then, $P680^+$ oxidizes a tyrosine residue of the D1 protein Y_Z (Debus, 1988), which in turn oxidizes a cluster of four Mn atoms, the Mn cluster. The Mn cluster accumulates the four oxidizing equivalents that are required for the oxidation of two molecules of H_2O to evolve an O_2 molecule (Brudvig et al., 1989; Debus, 1992). Cl^- and Ca^{2+} are required for the proper function of the Mn cluster (Brudvig et al., 1989; Debus, 1992).

To perform the light-driven electron transport reaction from H_2O to plastoquinone, at least four proteins are required in addition to the D1 and D2 proteins that are CP47, CP43, Cyt *b*559 (Cramer et al., 1986; Cramer et al., 1993) and extrinsic 33-kDa protein. CP47 and CP43 are integral membrane proteins which bind chlorophylls and serve as an antenna associated with the reaction center complex. The extrinsic protein of 33 kDa stabilizes the Mn cluster, a catalytic center of O_2 evolution (Murata and Miyao, 1985).

In addition to the 33-kDa protein, two other extrinsic proteins of 23 and 18 kDa bind to the PSII complex and act to regulate the reactivity of the Mn cluster (Murata and Miyao, 1985). The 23-kDa protein acts as a concentrator of Ca^{2+} and Cl^-, essential cofactors for O_2 evolution, to decrease the concentrations of these ions required for O_2 evolution. The 18-kDa protein acts as a concentrator of Cl^- and is necessary for the O_2

evolution when Cl^- concentration is below 3 mM Cl^- (Murata and Miyao, 1985). The 23-kDa and 18-kDa proteins can be selectively removed from the PSII complex without affecting the Mn cluster by treatment of the complex with 1 M NaCl (Miyao and Murata, 1985). The 33-kDa protein can also be removed without affecting the Mn cluster by treatment of the complex with 1 M $CaCl_2$ or 3 M urea with concentrated Cl^- (Murata and Miyao, 1985). Removal of the three extrinsic proteins decreases O_2-evolving activity but the lost activity can be restored by reconstitution of these proteins (Murata and Miyao, 1985).

The Mn cluster can be dissociated from the PSII complex by treatment of isolated thylakoids and PSII membranes with NH_2OH (Tamura and Cheniae, 1987; Miyao and Inoue, 1991), alkaline Tris (Yamashita and Tomita, 1976; Yamamoto et al., 1981; Kuwabara and Murata, 1982) to inactivate O_2 evolution. Treatment with Tris also dissociates the three extrinsic proteins of 33, 23, and 18 kDa from the PSII complex (Yamamoto et al., 1981; Kuwabara and Murata, 1982). Conversely, the Mn cluster can be reconstituted in the PSII complex by incubating materials depleted of the Mn cluster in the presence of Mn^{2+} under illumination to restore O_2 evolution. This light-dependent reconstitution of the Mn cluster is called photoactivation or photoreactivation of O_2 evolution. This process involves at least two successive photoreactions in PSII and a dark reaction in between (Radmer and Cheniae, 1977). The two photoreactions have been proposed to involve photooxidation of Mn^{2+} by PSII to yield Mn^{3+} (Tamura and Cheniae, 1987; Miller and Brudvig, 1990).

Cyt b*559*

The reaction center complex of PSII that consists of a heterodimer of the D1 and D2 proteins is considered to have a structure homologous to the reaction center of photosynthetic purple bacteria such as *Rhodopseudomonas*

viridis (Trebst, 1986; Michel and Deisenhofer, 1988) and *Rhodobacter sphaeroides* (Feher et al., 1989). However, it differs from the bacterial complex in some respects. One major difference is that the PSII reaction center complex generates a high oxidizing potential of about 1.1 V to oxidize H_2O and evolve O_2 molecules (Vermass and Ikeuchi, 1991). Another difference is that a *b*-type cytochrome, Cyt *b*559, is tightly bound to the PSII reaction center complex (Nanba and Satoh, 1987).

Cyt *b*559 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 (Fig. 3; Widger et al., 1985, Cramer et al., 1993). From the orientation in the thylakoid membrane of each subunit, it has been suggested that the heme is located close to the stromal surface of the membrane (Fig. 3; Tae et al., 1988; Tae and Cramer, 1994). It is unclear whether a unit of PSII reaction center complex binds one or two molecules of Cyt *b*559. In isolated thylakoids, it has been reported that two hemes of Cyt *b*559 are present per a PSII unit (Whitmarsh and Ort, 1984; Thompson et al., 1989). In the case of isolated PSII membranes, by contrast, conflicting results have been reported, namely, one heme (Ghanotakis et al., 1984; Miyazaki et al., 1989; Buser et al., 1992) or two hemes (Lam et al., 1983; Murata et al., 1984) per a PSII unit. The physiological role of Cyt *b*559 also remains to be unequivocally resolved, and many possible roles for this cytochrome have been proposed (for reviews, see Cramer et al., 1986; Cramer et al., 1993). It has been suggested (1) that Cyt *b*559 participates in the light-dependent reconstitution of the Mn cluster (Cramer et al., 1986); (2) that it protects PSII from photo-inhibition by mediating a cyclic electron-transport reaction around PSII (Thompson and Brudvig, 1988) or by oxidizing the reduced form of the primary electron acceptor, pheophytin, to suppress the overreduction of Q_A (Poulson

Fig. 3. A tentative model for the structure of Cyt *b*559 in the thylakoid membrane. α: α subunit β: β subunit (modified from Cramer et al., 1993).

et al., 1995); (3) that it participates in a redox-linked proton pump that contributes to the proton electrochemical gradient across the thylakoid membrane (Butler and Matsuda, 1983; Arnon and Tang, 1988); and (4) that it acts as an anchor for the protein components during the assembly of the PSII reaction center complex (Pakrasi et al., 1991; Tae et al., 1992).

One unique characteristic of Cyt *b*559 is that its redox potential is variable, and it exists in three forms with different redox potentials, 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 mV, +60 mV and $\leq$ 0 mV, respectively (Cramer and Whitmarsh, 1977). An intermediate-potential form (Eo'= +230 mV) has also been reported (Thompson et al., 1989). In general, about half or more of Cyt *b*559 exists in the HP form in materials in which PSII contains the Mn cluster and is active in O_2 evolution: about half of Cyt *b*559 is in the HP form *in vivo* (Shinohara et al., 1992), in intact chloroplasts (Heber et al., 1976) and in isolated thylakoids (Thompson et al., 1989; Mizusawa et al., 1995), while, in isolated PSII membranes, the proportion of the HP form ranges from 50 to 90% of the total depending on the preparation (Thompson et al., 1989; Ortega et al., 1988; McNamara et al., 1995).

The different potential forms of Cyt *b*559 are partly interconvertible. The HP form can be converted to lower-potential forms *in vitro* when the O_2 evolution is inactivated by treatment with concentrated Tris, $CaCl_2$ or NaCl, which depletes the Mn cluster and/or the extrinsic proteins that participate in the O_2 evolution (Briantais et al., 1985; Ghanotakis et al., 1986; Thompson et al., 1989). This phenomenon is also observed *in vivo* : almost all Cyt *b*559 exists in the LP and/or VLP form in materials in which PSII lacks in the Mn cluster and is inactive in O_2 evolution, as in the LF1 mutant cells of

Scenedesmus (Metz et al., 1980), in wheat seedlings grown under intermittent illumination (Ono et al., 1984), and in dark-grown pine cotyledons (Shinohara et al., 1992). Conversely, the HP form of Cyt *b*559 can be restored when O_2 evolution is reactivated by reconstitution of the Mn cluster in PSII *in vivo* (Shinohara et al., 1992). The restoration of the HP form upon reconstitution of the Mn cluster *in vitro* has not been reported yet.

From the correlation between the presence of the HP form and the capacity for evolving oxygen, it would appear that the binding of the extrinsic proteins and/or the Mn cluster to PSII might be necessary for retention of the HP form of Cyt *b*559. However, recent studies have demonstrated that Cyt *b*559 can remain in the HP form under some certain conditions, even after the extrinsic proteins and/or the Mn cluster have been disintegrated from PSII (Tamura and Cheniae, 1987; Miller and Brudvig, 1990; McNamara and Gounaris, 1995). Thus, unidentified factors appear to regulate the redox potential of Cyt *b*559.

The Aim of This Study.

The aim of this study is to elucidate the mechanism of interconversion of the different redox potential forms of Cyt *b*559. I employed the techniques to inactivate and reactivate O_2 evolution, namely, the treatment with Tris and the photo(re)activation treatment to change the redox potential of Cyt *b*559 *in vitro*. In CHAPTER I, the changes in the high and low redox potential (HP and LP) forms of Cyt *b*559 during the Tris and subsequent photo-(re)activation treatments were investigated in detail using isolated thylakoids. In CHAPTER II, the mechanism of the restoration of the HP form of Cyt *b*559 during the photo(re)activation treatment was investigated using PSII membranes which had been prepared by mild treatment of intact thylakoids with

a non-ionic detergent, Triton X-100. In this PSII preparation, the active site for O_2 evolution is exposed to the outer aqueous phase (Berthold et al., 1981; Kuwabara and Murata, 1982). Therefore, it is possible in PSII membranes, but not in thylakoids, to control binding of the extrinsic proteins to the PSII complex. In addition, the PSII membranes practically lack in the PSI complex, the Cyt *b*563/*f* complex and the ATP synthase complex (Berthold et al., 1981; Kuwabara and Murata, 1982; Murata et al., 1984). Thus, kinetical analyses are much easier and exogenously added reagents can directly be reacted with the active site. In "GENERAL DISCUSSION", on the basis of studies in CHAPTERS I and II and also observations by other researchers, a possible molecular mechanism of the interconversion between the HP and LP forms of Cyt *b*559 is discussed. The physiological significance of the restoration of the HP form of Cyt *b*559 in the course of the photo(re)activation of O_2 evolution is also discussed.

CHAPTER I

CHANGES IN REDOX POTENTIAL OF CYTOCHROME *b*559 UPON INACTIVATION AND REACTIVATION OF THE O_2 EVOLUTION IN ISOLATED THYLAKOIDS

SUMMARY

Interconversion between the HP and LP forms of Cyt *b*559 that occurs upon inactivation and reactivation of O_2 evolution was investigated using isolated spinach thylakoids. Isolated spinach thylakoids used in this study contained about two molecules of Cyt *b*559 per 400 chlorophyll (Chl) molecules, a PSII unit. Among them, 0.5 to 0.7 molecule of Cyt *b*559 was present in the HP form. When the thylakoids were treated with 0.8 M Tris (pH 9.1) to deplete the Mn cluster, the HP form of Cyt *b*559 was almost completely converted to the LP form, concomitantly with inactivation of O_2 evolution. When the Tris-treated thylakoids were illuminated with weak light for 20 min in the presence of Mn^{2+} and Ca^{2+} (photoreactivation treatment), 50-80% of the LP form of Cyt *b*559 was restored to the HP form, concomitantly with recovery of the O_2-evolving activity. Mn^{2+} and illumination, which are required for the photoreactivation of O_2 evolution, were also necessary for the restoration of the HP form. However, the restoration of the HP form was found to precede the recovery of O_2-evolving activity. The times required for the half-maximum restoration were less than 2.5 min for the HP form and 6 min for the O_2-evolving activity. This observation suggests that the restoration of the HP form is a primary process of the photoreactivation of O_2 evolution which occurs prior to the reconstitution of the Mn cluster. It was also found that inhibitors of electron transport from PSII to PSI, namely, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone (DBMIB) completely suppressed the recovery of O_2-evolving activity while they did not at all affect the restoration of the HP form. These results suggest that the restoration of the HP form of Cyt *b*559 and the reconstitution of the Mn cluster proceed via different reaction

mechanisms. Uncouplers such as nigericin and gramicidin-S inhibited both the restoration of the HP form and the recovery of O_2-evolving activity. This result might imply the possibility that the pH gradient across the thylakoid membrane would participate in the restoration of the HP form.

INTRODUCTION

It has been known that the presence of the HP form of Cyt *b*559 closely correlates with the presence of the Mn cluster in the PSII complex *in vivo* (Cramer et al., 1986). Almost all Cyt *b*559 exists in the LP and/or VLP form in materials in which PSII lacks in the Mn cluster and is inactive in O_2 evolution, as in the LF1 mutant cells of *Scenedesmus* (Metz et al., 1980), in wheat seedlings grown under intermittent illumination (Ono et al., 1984), and in dark-grown pine cotyledons (Shinohara et al., 1992). Conversely, the HP form of Cyt *b*559 can be restored when O_2 evolution is activated by constitution of the Mn cluster in the PSII complex *in vivo* (Shinohara et al., 1992).

In isolated thylakoids which contain the Mn cluster and are active in O_2 evolution, more than half of Cyt *b*559 is present in the HP form (Thompson et al., 1989). The HP form can be converted to the LP form when the Mn cluster is depleted (Erixon et al., 1972; Mizusawa et al., 1992). However, the change in redox potential of Cyt *b*559 upon reconstitution of the Mn cluster (photoreactivation of O_2 evolution) has not been studied *in vitro* yet.

I investigated whether the restoration of the HP form of Cyt *b*559 also occurred upon the reconstitution of the Mn cluster *in vitro*, using isolated thylakoids which retained the native membrane structure as *in vivo*.

MATERIALS AND METHODS

Preparation of Tris-Treated and DCPIP-Treated Spinach Thylakoids.

Thylakoids were isolated from young spinach leaves by differential centrifugation as described by Yamashita and Tomita (1975). To deplete the Mn cluster from the PSII complex completely, the thylakoids were suspended in 0.8 M Tris-HCl (pH 9.1)/5 mM EDTA at 0.1 mg Chl/ml and incubated in darkness at $0^{o}C$ for 2 h (Tris treatment). After the incubation, the Tris-treated thylakoids were collected by centrifugation at 1000 x *g* for 10 min and the pelleted thylakoids were suspended at 0.4 mg Chl/ml in 0.4 M sucrose/50 mM Hepes-NaOH (pH 7.8)/20 mM NaCl (designated hereafter as SHN) that contained 20 μM DCPIP and 2 mM sodium ascorbate, and incubated in darkness at $0^{o}C$ for 30 min (DCPIP treatment). The DCPIP-treated thylakoids were collected by centrifugation at 1000 x *g* for 10 min, washed once with SHN by suspension and recentrifugation, and finally suspended in SHN at about 3 mg Chl/ml. The optimum concentration of DCPIP for the photoreactivation was 300 μM (data not shown), but in the present study, the concentration of DCPIP was reduced to 20 μM to minimize the adsorption of DCPIP to thylakoids, since DCPIP has absorption maximum at 590 nm and interferes with the determination of cytochromes. Chlorophyll concentration was determined by the method of Arnon (1949).

Photoreactivation of O_2 Evolution.

Thylakoids which had been treated with Tris and subsequently with DCPIP were photoreactivated essentially according to Yamashita and Tomita (1975). The thylakoids were suspended at 50 μg Chl/ml in a solution that contained 0.2 M sucrose, 50 mM Hepes-NaOH (pH 7.8), 50 μM $Mn(CH_3COO)_2$, 1 mM

$Ca(CH_3COO)_2$, 20 μM DCPIP, 2 mM sodium ascorbate and 20 mM NaCl. Two or 30 ml of the suspension was put in a small test tube (internal diameter, 10 mm) or in a 300-ml beaker and illuminated with white fluorescent light at 400 lux (0.9 W/m^2) at 20°C for 20 min. After illumination, the thylakoids were collected by centrifugation and washed once with SHN that contained 1 mM EDTA by resuspension and recentrifugation. The O_2-evolving activity of the thylakoids was measured at 50 μg Chl/ml and 20°C in the presence of 0.05% BSA, 2 mM potassium ferricyanide and 2 mM NH_4Cl using a Clark-type oxygen electrode in a 1-ml cell under illumination with white incandescent light at 60,000 lux (240 W/m^2). When indicated, the photoreactivation mixture was directly subjected to the assay of O_2-evolving activity.

Determination of Cyt b*559.*

Thylakoids which had been suspended in SHN at 100 μg Chl/ml were incubated in darkness for 10 min and then subjected to the analysis of cytochromes. Levels of Cyt *b*559 in different potential forms were determined from reduced-minus-oxidized difference spectra recorded at room temperature with a computer-linked spectrophotometer (UV-2200; Shimadzu, Kyoto, Japan). Each spectrum was recorded five times and averaged to improve the signal-to-noise ratio.

In addition to Cyt *b*559, since thylakoids contained Cyt *f* (λ max = 554 nm) which is reducible by hydroquinone and Cyt *b*563 (λ max = 563 nm) which is reducible by sodium dithionite, levels of Cyt *b*559 in the HP and LP forms were obtained as follows. (1) To reduce the HP form of Cyt *b*559 and Cyt *f*, the sample was supplemented with 2 mM hydroquinone. The reference sample was supplemented with 0.5 mM potassium ferricyanide to oxidize all cytochromes. Then, the difference spectrum was recorded. The resultant

difference spectrum, (HQ)-(FeCN) spectrum, represented the sum of the reduced forms of Cyt *f* and of Cyt *b*559 HP form [Fig. 4 (I), B]. (2) Next, the sample which contained hydroquinone was incubated at 45°C for 10 min. This heat treatment converted the HP form of Cyt *b*559 to the LP form but did not affect the midpoint potential of Cyt *f*. Therefore, only Cyt *f* remained in the reduced form after the heat treatment, and the resultant difference spectrum [Fig. 4 (I), C] represented the level of Cyt *f*. (3) Subsequently, 5 mM sodium ascorbate was added to the heated sample to reduce the LP form of Cyt *b*559. The resultant difference spectrum represented the sum of the reduced forms of Cyt *f* and Cyt *b*559 (HP + LP) forms [Fig. 4 (I), A]. (4) Finally, a few mg of sodium dithionite were added to reduce all cytochromes in the sample. The resultant difference spectrum represented the sum of the reduced forms of Cyt *f*, Cyt *b*559 (HP + LP + VLP) forms and Cyt *b*563 [Fig. 4 (I), D]. The difference spectra of each cytochrome were obtained by subtraction of the reduced-minus-oxidized spectra as follows.

(a) Cyt *b*559 (HP + LP) forms: (Spectrum A)-(Spectrum C).

(b) HP form of Cyt *b*559: (Spectrum B)-(Spectrum C).

(c) LP form of Cyt *b*559: (Spectrum A)-(Spectrum B).

(e) VLP form of Cyt *b*559 and Cyt *b*563: (Spectrum D)-(Spectrum A).

The subtracted fractions of difference spectra for each cytochrome are shown in II, III and IV of Fig. 4. The amounts of cytochromes were determined using the following differential extinction coefficients (Cramer and Whitmarsh, 1977) : Cyt *b*559, $\varepsilon^{\Delta}_{(559-570)}$=15 $mM^{-1} \cdot cm^{-1}$; Cyt *f*, $\varepsilon^{\Delta}_{(554-540)}$= 20 $mM^{-1} \cdot cm^{-1}$; and Cyt *b*563, $\varepsilon^{\Delta}_{(563-570)}$=14 $mM^{-1} \cdot cm^{-1}$.

RESULTS AND DISCUSSION

Fig. 4 shows reduced-minus-oxidized difference spectra of cytochromes in the untreated (I and II), the Tris-treated and DCPIP-treated (III) and the photoreactivated (IV) thylakoids. From the difference spectra, the contents of Cyt *b*559 in the (HP + LP) forms and of Cyt *f* in the untreated thylakoids were determined to be 1 and 0.5/400 Chl unit (mol/mol), respectively [Fig. 4 (II), a and d]. The content of Cyt *b*559 in the VLP form could not be determined directly because not only this cytochrome but also Cyt *b*563 were reduced by dithionite, giving rise to the spectra which overlapped with each other. However, the content of Cyt *b*563 could be estimated from the content of Cyt *f* to be 1/400 Chl unit (mol/mol), on the basis of the heme ratio of Cyt *b*563/Cyt *f* of two in the Cyt *b*563/*f* complex (Hurt and Hauska, 1982). Then the size of the spectrum of Cyt *b*563 could be calculated by estimating the concentration to be twice of Cyt *f* and by using the $\Delta\varepsilon$ for Cyt *b*563. The content of Cyt *b*559 in the VLP form was determined to be 1/400 Chl unit (mol/mol) by subtracting the calculated spectrum of Cyt *b*563 from the (Dith) - (ASC) spectrum [Fig. 4 (II), e]. Thus the total content of Cyt *b*559 was 2/400 Chl unit (mol/mol).

In fresh and untreated thylakoids with O_2-evolving activity of 150-250 μmol O_2 mg $Chl^{-1} \cdot h^{-1}$, 0.5 to 0.7 molecule of Cyt *b*559 was present in the HP form and 0.5 to 0.3 was in the LP form, on the basis of 400 Chl [Fig. 4 (II), b and c]. In the Tris-treated and DCPIP-treated thylakoids, the O_2-evolving activity was markedly depressed and 82% of the HP form of Cyt *b*559 was converted to the LP form [Fig. 4 (III), c]. Treatment with Tris releases Mn, and the 33-, 23- and 18-kDa proteins from the PSII complex (Yamamoto et al., 1981; Kuwabara and Murata, 1982). In the case of thylakoids, these released

Fig. 4. Reduced-minus-oxidized difference spectra of cytochromes in the untreated, the Tris-treated and DCPIP-treated, and the photoreactivated thylakoids. (I) and (II): Untreated thylakoids. (III): Tris-treated and DCPIP-treated thylakoids. (IV): Photoreactivated thylakoids. The Tris-treated and DCPIP-treated thylakoids were photoreactivated in the presence of 50 μM Mn^{2+}, 1 mM Ca^{2+}, 20 μM DCPIP, and 2 mM sodium ascorbate for 20 min. After photoreactivation, thylakoids were washed with SHN that contained 1 mM EDTA before measurement of different spectra. Redox difference spectra shown are as follows: A, ASC - FeCN; B, HQ - FeCN; C, the same as B but after heat treatment; D, Dith - FeCN; a, (Spectrum A - Spectrum C) which represents Cyt *b*559 (HP + LP); b, (Spectrum B - Spectrum C) which represents Cyt *b*559 HP; c, (Spectrum A - Spectrum B) which represents Cyt *b*559 LP; d, Spectrum C which represents Cyt *f* ; e, (Spectrum D - Spectrum A) which represents (Cyt *b*559 VLP + Cyt *b*563), respectively. The O_2-evolving activities of untreated, Tris-treated and DCPIP-treated, and photoreactivated thylakoids were 190, 10, and 148 μmol · mg Chl^{-1} · h^{-1}, respectively. For details, see "MATERIALS AND METHODS".

I
II
III
IV
D
A
B
C
e
a
b
d
c
e
a
c
d
b
e
a
b
d
c
0.0192
0.0160
0.0128
0.0096
0.0064
0.0032
0.0000
ΔA
540
560
580
Wavelength (nm)

proteins remain in the lumen, and only the 33-kDa protein reassembles back to the PSII complex during the subsequent treatment with reduced DCPIP (Ebina and Yamashita, 1992). Therefore, it is suggested that the reassembly of the 33-kDa protein by itself does not cause the restoration of the HP form of Cyt *b*559 or the recovery of the O_2-evolving activity [Fig. 4 (III), b]. When the Tris-treated and DCPIP-treated thylakoids were photoreactivated by illuminating them in the presence of Mn^{2+} and Ca^{2+}, about 0.5/400 Chl unit (mol/mol) of the LP form of Cyt *b*559 was brought back to the HP form [Fig. 4 (IV), b] while 78% of the O_2-evolving activity was recovered. During the photoreactivation treatment, the Mn cluster is reconstituted and the 23- and 18-kDa proteins reassemble to the PSII complex (Ebina and Yamashita, 1992).

Addition of chloramphenicol (0.1 mg/ml), an inhibitor of chloroplast protein synthesis, did not affect the recoveries of HP form and O_2-evolving activity by the photoreactivation (data not shown).

Mn^{2+}, Ca^{2+} and illumination with weak light are the factors required for the photoreactivation of O_2 evolution in Tris-treated thylakoids (Yamashita and Tomita, 1974). The effects of these factors on the restoration of the HP form of Cyt *b*559 are shown in Table 1. It was demonstrated that illumination in the presence of Mn^{2+} alone can lead to the reconstitution of Mn cluster and Ca^{2+} is required for the expression of O_2-evolving activity of the reconstituted cluster (Tamura and Cheniae, 1989). Also in the case of the restoration of the HP form of Cyt *b*559, weak light and Mn^{2+} were both indispensable (Table 1). However, Ca^{2+} exhibited different effects. When Ca^{2+} was omitted from the photoreactivation mixture, the restoration of the HP form was reduced to 54% while the recovery of O_2-evolving activity decreased to 28%. The inhibition by the omission of Ca^{2+} was not enhanced even when thylakoids had been treated with Tris in the presence of 5 mM EGTA

to decrease the level of Ca^{2+} in thylakoids (data not shown).

The recovery levels of the O_2-evolving activity and the HP form of Cyt *b*559 increased in parallel with the increase of Mn^{2+} concentration in the photoreactivation mixture and reached a maximum at around 20 μM Mn^{2+} (Fig. 5).

The time courses of the recoveries of the HP form of Cyt *b*559 and the O_2-evolving activity are shown in Fig. 6. After the photoreactivation in the presence of 1 mM Ca^{2+}, 50 μM Mn^{2+}, 2 mM ascorbate and 20 μM DCPIP, the O_2-evolving activity was measured with or without further addition of 10 mM Ca^{2+} as a substitute for the 23-kDa protein (Murata and Miyao, 1985), but the addition of 10 mM Ca^{2+} did not enhance the O_2-evolving activity under any conditions (data not shown). The recovery of the HP form occurred at a high rate and reached the maximum level in only 5 min ($t_{1/2} \leqq 2.5$ min), while the maximum recovery of O_2-evolving activity required 20 min ($t_{1/2} > 7$ min). It has been reported (Ebina and Yamashita, 1992) that, in the photoreactivation of Tris-treated thylakoids, rebinding of Mn to the PSII complex occurred first ($t_{1/2} = 1$ min), the extrinsic 23- and 18-kDa proteins then reassembled ($t_{1/2} = 3$-4 min), and finally the O_2-evolving activity was restored ($t_{1/2} = 6$ min). Thus, it appears that the restoration of the HP form of Cyt *b*559 in this study occurred a little more rapidly than the reassembly of the proteins. This result raises the question of whether the reassembly of these proteins is indispensable for the restoration of the HP form of Cyt *b*559.

From these results, it seems that the restoration of the HP form of Cyt *b*559 could be one of the primary processes involved in the photoreactivation of O_2 evolution, as is the photooxidation of Mn^{2+} possibly by Y_Z^+ (Miller and Brudvig, 1990).

Table 1
Effects of factors essential for the photoreactivation of O_2 evolution on the restoration of HP form of Cyt *b*559

Conditions	O_2-evolving activity (μmol·mg $Chl^{-1}\cdot h^{-1}$)	Cytochrome content (mol · 400 mol Chl^{-1})		
		*b*559 HP	*b*559 LP	*f*
Non-photoreactivated	26	0.17 (18.7%)	0.75 (81.3%)	0.51
Photoreactivated				
(Complete)	184	0.81 (80.9)	0.19 (19.1)	0.48
($-Mn^{2+}$)	52	0.11 (12.3)	0.77 (87.7)	0.54
($-Ca^{2+}$)	70	0.51 (52.2)	0.47 (47.8)	0.48
(-light)	40	0.24 (27.6)	0.62 (72.4)	0.57

Tris-treated and DCPIP-treated thylakoids were photoreactivated for 20 min and the O_2 evolution and the content of cytochromes were measured after the photoreactivated thylakoids had been washed with SHN that contained 1 mM EDTA. The conditions for photoreactivation were the same as those described in the legend to Fig. 4. 100% corresponds to the sum of Cyt *b*559 (HP + LP) forms.

Fig. 5. Effects of Mn^{2+} concentration on the recoveries of O_2-evolving activity and the HP form of Cyt *b*559 by the photoreactivation of Tris-treated and DCPIP-treated thylakoids. The conditions for photoreactivation were the same as described in the legend to Fig. 4. The O_2-evolving activity and the content of HP form of Cyt *b*559 were measured after washing of the photoreactivated thylakoids. The content of the HP form of Cyt *b*559 of 100% corresponds to the sum of Cyt *b*559 in the HP and LP forms.

Fig. 6. Time courses of the recoveries of the O_2-evolving activity and the HP form of Cyt *b*559 during the photoreactivation of Tris-treated and DCPIP-treated thylakoids. The conditions for photoreactivation were the same as described in the legend to Fig. 4. The photoreactivation reaction was terminated at designated times by adding 1 mM EDTA to the photoreactivation mixture. Then, the thylakoids were washed twice with SHN that contained 1 mM EDTA. The O_2-evolving activity and the difference spectra were measured after washing. A value of the HP form of Cyt *b*559 of 100% corresponds to the sum of Cyt *b*559 in the HP and LP forms.

During the photoreactivation of Tris-treated thylakoids, exogenously added Mn^{2+} must be transported into the lumen across the thylakoid membrane and subsequently reassembled into the Mn cluster. It has been reported that the photoreactivation of Tris-treated thylakoids is inhibited by DCMU (Yamashita and Tomita, 1976) and by various uncouplers (Yamashita, 1982). It can be assumed that DCMU suppresses the photooxidative process of the transported Mn^{2+} and that uncouplers inhibit the transport of Mn^{2+} by disrupting the pH gradient or the high energy state of the thylakoid membrane. As shown in Table 2, neither DCMU, an inhibitor of electron transport from Q_A to Q_B, nor DBMIB, an inhibitor of electron transport from plastoquinol to the Cyt *b*563/*f* complex, inhibited the restoration of the HP form, while the recovery of the O_2-evolving activity was severely suppressed. This observation implies that the requirements for electron transport reactions are different between two processes. On the other hand, uncouplers such as nigericin and gramicidin-S, each at 100 μM, severely inhibited both processes. The half-inhibition concentration of nigericin (I_{50}) was 30 μM (data not shown). This result might imply that the pH gradient and/or the high energy state of the thylakoid membrane would contribute to some extent to the recoveries of the HP form of Cyt *b*559 and the O_2-evolving activity. However, the concentrations of uncouplers required for the inhibition in this study were higher than that required to uncouple photophosphorylation. Thus, the possibility could not be ignored that the secondary effects of uncouplers might have been responsible for the inhibition of the photoreactivation and the restoration of the HP form. Even so, these findings clearly indicate that the inhibitors of electron transport (DCMU and DBMIB) and the uncouplers (nigericin and gramicidin-S) act at different processes which occur during the photoreactivation process. The restoration of the HP form of Cyt *b*559 might

involve DCMU-insensitive partial electron transport driven by PSII or PSI alone.

It was previously reported that the O_2-evolving capacity can remain unaffected under conditions where the HP form of Cyt *b*559 is converted to the LP form (Briantais et al., 1985; Ghanotakis et al., 1986; Völker et al., 1986). In addition, it was reported (Tamura and Cheniae, 1987) that, in NH_2OH-treated PSII membranes, more than half of Cyt *b*559 remained in the HP form although the Mn cluster and the 23- and 18-kDa proteins in PSII had been completely depleted. Thus, it is likely that the HP form of Cyt *b*559 does not directly participate in the O_2-evolving process by active Mn cluster. On the other hand, the present study suggests the possibility that the HP form of Cyt *b*559 might participate in the photoreactivation of O_2 evolution. It can be presumed that the restoration of the HP form is a primary process which is necessary for the reconstitution of the Mn cluster into the PSII complex.

It was proposed previously that Cyt *b*559 exists in the HP form when the heme is located inside a hydrophobic niche that is shielded from water molecules by protein segments, while the HP form is converted to the LP form when the heme is exposed to the outer aqueous phase (Krishtalik et al., 1993). The interconversion between the HP and LP forms by treatment with Tris and the subsequent photoreactivation observed in this study might be ascribable to "lumenal side effects", since depletion and reconstitution of the Mn cluster and the extrinsic proteins are events occurring at the lumenal surface of the PSII complex. Such lumenal side effects might be transmitted to the heme of Cyt *b*559 which is located at the stromal side of the PSII complex (see Figs. 2,3). However, in studies using thylakoids, it is not easy to clarify factor(s) responsible for the restoration of the HP form of Cyt *b*559, since the protein environment at the lumenal surface of the PSII complex, such as

binding of the extrinsic proteins, cannot be controlled. Further studies using purified PSII preparations are required for understanding of the mechanism of the restoration of the HP form of Cyt *b*559.

Table 2

Effects of inhibitors and uncouplers on the recoveries of O_2-evolving activity and the HP form of Cyt *b*559 by photoreactivation

Conditions	O_2-evolving activity (μmol·mg Chl^{-1}·h^{-1})		Cyt *b*559 HP (%)
	before washing	after washing	
A) Non-photoreactivated	12	–	12.2
Photoreactivated			
(Complete)	132	110	54.5
(+1 μM DCMU)	10	16	52.3
(+10 μM DBMIB)	26	44	54.5
B) Non-photoreactivated	13	–	7.8
Photoreactivated			
(Complete)	141	148	68.6
(+0.1 mM Nigericin)	22	16	12.0
(+0.1 mM Gramicidin-S)	16	24	7.6

Tris-treated and DCPIP-treated thylakoids were preincubated with designated inhibitors or uncouplers for 2 min at 20°C, supplemented with Mn^{2+} and Ca^{2+} and then photoreactivated for 20 min. Nigericin was added together with 10 mM KCl. The other conditions for photoreactivation were the same as those described in the legend to Fig. 4. The O_2-evolving activity was measured before and after washing. The content of the HP form of Cyt *b*559 was determined after washing. 100% corresponds to the sum of Cyt *b*559 (HP + LP) forms.

CHAPTER II

THE MECHANISM OF RESTORATION OF THE HIGH-POTENTIAL FORM OF CYTOCHROME *b*559 DURING PHOTOACTIVATION OF O_2 EVOLUTION: THE STUDY WITH ISOLATED PHOTOSYSTEM II MEMBRANES

SUMMARY

The conversion of Cyt *b*559 from the LP to the HP form under conditions for reconstitution of the Mn cluster (photoactivation of O_2 evolution) was investigated using spinach PSII membranes that had been depleted of the Mn cluster by treatment with Tris.

As in the case of isolated thylakoids, the HP form of Cyt *b*559 was converted to the LP form upon inactivation of O_2 evolution by treatment with Tris, and the LP form was converted to the HP form upon the subsequent photoactivation of O_2 evolution, although the extent of interconversion between the HP and the LP forms was smaller in PSII membranes than in thylakoids. Ca^{2+} was not essential for the restoration of the HP form but was necessary for retention of the HP form after illumination. When the photoactivation was achieved with flashing light, the restoration of the HP form and the recovery of O_2-evolving activity exhibited quite different dependences on flash number. The restoration of the HP form occurred after 2 flashes, while the reactivation of O_2 evolution required more than 6 flashes. The concentrations of Mn^{2+} required for the restoration of the HP form and for the reactivation of O_2 evolution were also different. The concentrations of Mn^{2+} that gave the half-maximum restoration were 5 and 17 μM for the HP form and the O_2 evolution, respectively. It was also found that the HP form could be restored when the PSII membranes were illuminated in the presence of artificial electron donors instead of Mn^{2+}, such as NH_2OH, 1,5-diphenylcarbazide and semicarbazide. These observations suggest that the restoration of the HP form of Cyt *b*559 is a process that is independent of the reconstitution of the Mn cluster and that the electron donation by Mn^{2+} to PSII is responsible for the restoration of the HP form.

The restoration of the HP form by illumination in the presence of Mn^{2+} was not affected by DCMU, an inhibitor of electron transport from Q_A to Q_B in PSII. This result suggests that electron transport from Q_A to Q_B is not required for the restoration of the HP form. By contrast, the restoration of the HP form was completely suppressed by artificial electron acceptors which bind to the Q_B site and reoxidize Q_A^-, namely, DBMIB and 2,6-dichloro-1,4-benzoquinone. These results suggest that Q_A^- formed by photochemical reaction in PSII is involved in the process of the restoration of the HP form. It is known that the half-lifetime of Q_A^- is greatly extended by exogenous electron donors. Therefore, it is likely that the electron donors support the restoration of the HP form by stabilizing Q_A^-.

INTRODUCTION

As demonstrated in CHAPTER I, the LP form of Cyt *b*559 was restored to the HP form during photoreactivation of O_2 evolution in isolated thylakoids which had been depleted of the Mn cluster by treatment with Tris. The restoration of the HP form seemed not to be ascribable to the reconstitution of the Mn cluster, since the restoration of the HP form occurred more rapidly than the recovery of O_2-evolving activity (CHAPTER I). It has been proposed that the interactions of Mn^{2+} (Cramer et al., 1986), Ca^{2+} (McNamara and Gounaris, 1995) and/or the extrinsic 23- and 18-kDa proteins (Briantais et al., 1985) with the reaction center complex of PSII might be the factors responsible for the restoration of the HP form. However, it is difficult to determine the effects of these factors in studies using thylakoids, since the site(s) with which the factor(s) interacts is located in the inner surface of the closed vesicle of the thylakoid membrane.

In PSII-enriched membranes which are prepared by mild treatment of intact thylakoids with a non-ionic detergent, Triton X-100, the active site for O_2 evolution is exposed to the outer aqueous phase. This preparation contains the PSII complexes but practically lacks in the PSI complex, the Cyt *b*563/*f* complex and the ATP synthase complex (Berthold et al., 1981; Kuwabara and Murata, 1982; Murata et al., 1984). These characteristics of PSII membranes had made great progress in biochemical and biophysical studies of the PSII complex concerning the extrinsic proteins and the cofactors of O_2 evolution that are located at the lumenal surface of the complex (Murata and Miyao, 1985; Brudvig et al., 1989; Vermass and Ikeuchi, 1991; Debus et al., 1992). In addition, absorption spectra of redox components in the PSII complex can be easily determined without any interference by the spectra

of components of the PSI and Cyt *b*563/*f* complexes.

When the PSII membranes are treated with concentrated Tris, the Mn cluster, one molecule of calcium ion and the three extrinsic proteins are almost completely depleted from the PSII complex, with concomitant inactivation of O_2 evolution (Yamamoto et al., 1981; Kuwabara et al., 1982; Tamura et al., 1985; Tamura et al., 1987). Procedures for reconstitution of these proteins and cofactors to the PSII complex had already been established (Murata and Miyao, 1985; Tamura and Cheniae, 1987; Ono and Inoue, 1988), and this enables us to determine the effects of these factors on the restoration of the HP form of Cyt *b*559. Procedures for reconstitution of the Mn cluster (photoactivation of O_2 evolution) in PSII membranes have also been established recently (Tamura and Cheniae, 1987; Miyao and Inoue, 1991).

In this Chapter, the photoactivation system of PSII membranes was employed to investigate the mechanism of the conversion of the Cyt *b*559 from the LP to the HP form in detail. It was found that, under the photoactivation conditions, not the reconstitution of the Mn cluster but the electron donation by Mn^{2+} to PSII is responsible for the restoration of the HP form of Cyt *b*559. In addition, It was found that illumination in the presence of artificial electron donors other than Mn^{2+} also supports the restoration of the HP form. A possible mechanism for the restoration of the HP form is discussed.

MATERIALS AND METHODS

Preparation of Thylakoids, PSII Membranes and Extrinsic Proteins.

Thylakoids were prepared from spinach leaves, treated with Tris and, subsequently, with reduced DCPIP, before they were subjected to the photoactivation treatment (CHAPTER I). PSII membranes were prepared from spinach leaves as described by Kuwabara and Hashimoto (1990) with the exception that a ratio of Triton X-100 to Chl of 15 (w/w) was used. The PSII membranes were suspended in 30% (v/v) ethylene glycol/20 mM NaCl/0.4 M sucrose/50 mM Mes-NaOH (pH 6.5) and stored at -80 ^{o}C. Before use, the PSII membranes were thawed on ice and washed three times with 20 mM NaCl/0.4 M sucrose/50 mM Mes-NaOH (pH 6.5; low-salt medium) by centrifugation and resuspension. To deplete the Mn cluster and the three extrinsic proteins from the PSII complex, the PSII membranes were suspended in 0.8 M Tris-HCl (pH 9.1)/5 mM EDTA/2 mM sodium ascorbate at 0.5 mg Chl/ml and incubated in darkness for 2 h. The Tris-treated PSII membranes were collected by centrifugation at 35,000 x *g* for 20 min and washed twice with the low-salt medium by resuspension (0.3 mg Chl/ml) and centrifugation. They were finally suspended in the same medium supplemented with 30% (v/v) ethylene glycol and stored at -80 ^{o}C. Before use, the Tris-treated PSII membranes were thawed and washed three times with the low-salt medium by centrifugation and resuspension. Handling of the Tris-treated PSII membranes was performed under dim light.

The extrinsic proteins of 33, 23 and 18 kDa were prepared from the Tris extract of PSII membranes. The supernatant after centrifugation that followed the treatment with Tris, prepared as described above, was dialyzed against 10 mM Mes-NaOH (pH 6.5) for 8 h and concentrated with a Centriprep 10 system

(Amicon, USA). No contamination by other proteins was detected by SDS-polyacrylamide gel electrophoresis. All procedures were performed at 0-4 ^{o}C. Chlorophyll concentration was determined as described by Arnon (1949).

Photoactivation of O_2 Evolution.

Photoactivation of Tris-treated thylakoids was performed as described in CHAPTER I. Photoactivation of Tris-treated PSII membranes was performed at 25°C by the method of Miyao and Inoue (1991) with some modifications, as follows. Tris-treated PSII membranes, which had been kept in darkness at 0 ^{o}C for more than 4 h, were suspended in 1 M NaCl/0.4 M sucrose/50 mM Mes-NaOH (pH 6.5; high-salt medium). Final chlorophyll concentrations were 125 and 250 μg/ml for photoactivation with continuous and flashing light, respectively. The suspension (6 and 1 ml for photoactivation with continuous and flashing light, respectively) was placed in a flat glass dish of 30 mm in diameter. Final thicknesses of the suspension were 8.6 and 1.4 mm for photoactivation with continuous and flashing light, respectively. After incubation in darkness at 25 ^{o}C for 5 min, the suspension was supplemented with 0.1 mM $Mn(CH_3COO)_2$, 50 mM $CaCl_2$ and, when indicated, 2 μM DCPIP, and further incubated in darkness at 25 ^{o}C for 5 min. Then, the suspension was illuminated from above with continuous light from a white fluorescent lamp at 33 $\mu E \cdot m^{-2} \cdot s^{-1}$ or it was illuminated with flashing light from a 10-μs xenon flash lamp (PS-240; Sugawara, Japan), with gentle stirring. Saturation of the light intensity of the flash was confirmed by use of a neutral-density filter (75%). After illumination, 50 μl of the photoactivation mixture was diluted 20-fold with the low-salt medium that contained the 33-, 23- and 18-kDa proteins at levels sufficient to maximize the O_2-evolving

activity. The O_2-evolving activity was measured at 20 ^{o}C with 0.5 mM phenyl-1,4-benzoquinone as an artificial electron acceptor using a Clark-type oxygen electrode. Another portion of the photoactivation mixture was subjected to the analysis of Cyt *b*559.

Analysis of Cyt b*559.*

Levels of Cyt *b*559 in the different potential forms were determined from reduced-minus-oxidized difference spectra recorded with a spectrophotometer (UV-2200; Shimadzu, Japan), essentially as described in CHAPTER I. When Tris-treated PSII membranes had been photoactivated with continuous light, the photoactivation mixture was directly subjected to analysis. When photo-activation was achieved with flashing light, the photoactivation mixture was diluted 5-fold with the high-salt medium that contained 0.1 mM $Mn(CH_3COO)_2$ and 50 mM $CaCl_2$ prior to the analysis. These concentrations of Mn^{2+} and Ca^{2+} and 2 μM DCPIP did not interfere with the analysis of Cyt *b*559. For the reduction of Cyt *b*559, the sample was supplemented sequentially with 2 mM hydroquinone (Eo'= +290 mV), 5 mM sodium ascorbate (Eo'= +60 mV) and a few mg of sodium dithionite. After each addition, the absorbance spectrum was recorded at the scanning rate of 100 nm/min with the slit width of measuring beam of 2 nm. The amounts of Cyt *b*559 reduced by addition of hydroquinone (HP form), sodium ascorbate (LP form) and sodium dithionite (VLP form) were estimated from the difference spectra, with the absorbance spectrum recorded in the presence of 0.5 mM potassium ferricyanide taken as the reference spectrum. The half width of the α-band of reduced Cyt *b*559 in the difference spectrum was 12 $\pm$ 0.5 nm and it was unchanged upon treatment with Tris and subsequent photoactivation treatment. A difference extinction coefficient $[\varepsilon^{\Delta}{}_{(559-570\ nm)}]$ of 15 $mM^{-1}\cdot cm^{-1}$ (Cramer and Whitmarsh, 1977) was used.

The deviation of results was less than 5%.

RESULTS

Changes in Levels of the Different Potential Forms of Cyt b*559 upon Treatment with Tris and Subsequent Photoactivation Treatment.*

The PSII membranes used in this study contained about two molecules of Cyt *b*559 per 220 Chl molecules, a PSII unit. The levels of the different potential forms of Cyt *b*559 in these PSII membranes were 1.0–1.2, 0.2–0.1 and 0.8–0.6/220 Chl unit (mol/mol) for the HP, LP and VLP forms, respectively. Treatment with Tris of the PSII membranes decreased the level of the HP form only by 0.3–0.4/220 Chl unit (mol/mol) with a concomitant increase in the level of the LP form. The level of the VLP form remained unchanged. The treatment with Tris had completely removed Mn and the three extrinsic proteins from the PSII membranes, as confirmed by atomic absorption spectroscopy and SDS-polyacrylamide gel electrophoresis (data not shown). These observations are consistent with results of previous studies (Tamura and Cheniae, 1987; Miller and Brudvig, 1990; McNamara and Gounaris, 1995) that the depletion of the Mn cluster and the extrinsic proteins does not necessarily cause the change in redox potential of Cyt *b*559.

Next, the experimental conditions that would further lower the level of the HP form of Cyt *b*559 in the Tris-treated PSII membranes were investigated. It was found that incubation of the membranes with 1 M NaCl in darkness lowered the level of the HP form by 0.1–0.4/220 Chl unit (mol/mol) to give a final level of 0.3–0.6/220 Chl unit (mol/mol). Besides NaCl, NaF, NaBr and sodium formate at 1 M were each effective in lowering the level of the HP form, while 1 M Na_2SO_4 was ineffective. Thus, some factor other than the ionic strength appeared to affect the level of the HP form. The use of 1 M NaCl had another advantage in the present study since 1 M NaCl has been

shown effectively to enhance the photoactivation of O_2 evolution in PSII membranes (Miyao and Inoue, 1991). Therefore, to investigate the effects of the photoactivation on the redox potential of Cyt *b*559, photoactivation treatment was performed in the presence of 1 M NaCl.

Table 3 shows the changes in the amount of the HP form of Cyt *b*559 in Tris-treated PSII membranes by photoactivation treatment with continuous light. When the membranes were illuminated in the absence of Mn^{2+} and Ca^{2+}, the level of the HP form decreased by about 0.2/220 Chl unit (mol/mol). This decrease probably resulted from photoinhibition of PSII that had been depleted of the Mn cluster (Tamura and Cheniae, 1987; Iwasaki et al., 1995). When the membranes were illuminated in the presence of Mn^{2+}, the level of the HP form increased by 0.13/220 Chl unit (mol/mol). The additional presence of Ca^{2+} during illumination enhanced the increase in the level of the HP form and about 0.2/220 Chl unit (mol/mol) of the HP form was restored. DCPIP did not further affect the increase in the level of the HP form. On the other hand, illumination in the presence of Ca^{2+} alone did not increase the level of the HP form, even though Ca^{2+} suppressed the decrease in the level of the HP form during illumination. When the level of the HP form increased, the level of the LP form decreased concomitantly, while the level of the VLP form remained unchanged (data not shown). These results suggest that the LP form of Cyt *b*559 is converted to the HP form by illumination in the presence of Mn^{2+}. The O_2-evolving activity also increased when the membranes were illuminated in the presence of Mn^{2+} (Table 3). The recovery of activity, however, was unrelated with the extent of the restoration of the HP form.

The photoactivation treatment and the subsequent analysis of Cyt *b*559 in this study were performed in the absence of the three extrinsic proteins. Thus, it is suggested that the restoration of the HP form of Cyt *b*559 does

Table 3

Changes in the amount of the HP form of Cyt *b*559 and in the O_2-evolving activity of Tris-treated PSII membranes upon photoactivation treatment

Additives	Cyt *b*559 HP/220 Chl (mol/mol)			O_2 evolution after illumination [μmol·mg Chl^{-1}·h^{-1}]	
	Before illu.	After illu.	Δ	− Ca^{2+}	+ Ca^{2+}
None	0.60	0.38	−0.22	8	−
Mn^{2+}	0.50	0.63	+0.13	8	47
Ca^{2+}	0.50	0.46	−0.04	8	−
Mn^{2+}, Ca^{2+}	0.50	0.70	+0.20	92	−
Mn^{2+}, Ca^{2+}, DCPIP	0.54	0.73	+0.19	172	−

Tris-treated PSII membranes were suspended in 1 M NaCl/0.4 M sucrose/ 50 mM Mes-NaOH (pH 6.5; high-salt medium) and incubated in darkness for 5 min. The suspension was supplemented with the indicated additive(s), incubated in darkness for 5 min, and then illuminated with continuous light for 10 min. Final concentrations of additives were 0.1 mM, 50 mM and 2 μM for Mn^{2+}, Ca^{2+} and DCPIP, respectively. The amount of the HP form of Cyt *b*559 was determined from the (hydroquinone)-(ferricyanide) difference absorbance spectrum. The total amount of Cyt *b*559 was 1.94/220 Chl unit (mol/mol). The O_2-evolving activity was measured in the presence of the three extrinsic proteins. When Ca^{2+} had been absent during illumination, 10 mM $CaCl_2$ was added to the assay mixture. The O_2-evolving activity of PSII membranes before Tris treatment was 405 μmol·mg Chl^{-1}·h^{-1}.

not require these extrinsic proteins. The extent of the restoration of the HP form varied among preparations, ranging from 0.15 to 0.35/220 Chl unit (mol/mol) when the membranes were illuminated in the presence of Mn^{2+} and Ca^{2+}.

Fig. 7 shows the effects of Ca^{2+} on the HP form of Cyt *b*559 that had been restored by the photoactivation treatment. In this experiment, the Tris-treated PSII membranes were illuminated in the presence of Mn^{2+} alone and then they were incubated in darkness in the presence or in the absence of Ca^{2+}. It is obvious that the level of the HP form that had been restored by illumination remained unchanged in the presence of Ca^{2+}, while in the absence of Ca^{2+} the level decreased within 10 min to the level before illumination (Fig. 7A). Mg^{2+} did not have such stabilizing effects. By contrast, the O_2-evolving activity that had been restored by illumination remained unchanged during incubation in darkness both in the presence and in the absence of Ca^{2+} (Fig. 7B). These observations suggest that Ca^{2+} stabilizes the HP form of Cyt *b*559 that had been restored by photoactivation treatment and that this stabilizing effect does not result from the stabilization by Ca^{2+} of the reconstituted Mn cluster. Ca^{2+} also appeared to stabilize the HP form of Cyt *b*559 that had originally been present in the Tris-treated PSII membranes since the presence of Ca^{2+} suppressed the decrease in the level of the HP form during illumination in the absence of Mn^{2+} (Table 3). Thus, the stabilizing effects of Ca^{2+} appear to be independent of the presence of the Mn cluster.

Fig. 8 shows the restoration of the HP form of Cyt *b*559 by photoactivation treatment with flashing light. Illumination with flashes in the presence of Mn^{2+} and Ca^{2+} restored both the HP form and the O_2-evolving activity, but the two phenomena exhibited quite different dependence on the flash number. The level of the HP form was unchanged by illumination with a

single flash but it greatly increased after 2 flashes and reached a maximum with 3 flashes (Figs. 8A,8B). By contrast, the recovery of O_2-evolving activity was detected only after illumination with 6 flashes and reached a maximum after 100 flashes (Fig. 8B). These observations indicate that the restoration of the HP form of Cyt *b*559 is completed before the Mn cluster is reconstituted. It is also suggested that the HP form might be restored by two photoreactions in PSII.

Fig. 8B also shows the changes in the level of Cyt *b*559 that remained in the reduced form after illumination. In general, the HP form can remain in the reduced form even under aerobic conditions, while the lower-potential forms are, if reduced, readily reoxidized by ambient oxygen molecules. Therefore, in the experiment of Fig. 8B in which the absorption spectrum of the photoactivation mixture was recorded about 2 minutes after illumination, only the reduced HP form could be detected. The results indicate that the level of the reduced HP form was unchanged with 1 and 2 flashes but it gradually increased after 3 flashes. After illumination with 50 flashes, all the HP form was in the reduced form. Two interpretations can be considered for the gradual increase in the level of the reduced HP form: (1) the quantum efficiency of the reduction of the HP form was low and 50 flashes were required to reduce all the HP form, or (2) the HP form was, once reduced, reoxidized by some reason and had to be reduced repeatedly for the accumulation of the reduced form.

To examine these possibilities, the change in the level of the reduced Cyt *b*559 during illumination with flashes was followed by direct measurement of the absorbance change at 559 nm of the photoactivation mixture (Fig. 9). The absorbance change at 570 nm (isosbestic point for the redox spectra of Cyt *b*559) was also measured to follow the change in the base line of the

Fig. 7. Effects of Ca^{2+} on the stability of the HP form of Cyt *b*559 and of the O_2-evolving activity which had been restored by photoactivation treatment. Tris-treated PSII membranes, suspended in the high-salt medium, were illuminated with continuous light in the presence of 0.1 mM Mn^{2+} for 10 min. After illumination, the suspension was incubated in darkness with no addition (○), with 50 mM $CaCl_2$ (●) or with 50 mM $MgCl_2$ (▲) for the designated times. (A) The amounts of HP form of Cyt *b*559. The total amount of Cyt *b*559 was 1.96/220 Chl unit (mol/mol). (B) O_2-evolving activity measured in the presence of 10 mM Ca^{2+}. The O_2-evolving activity of PSII membranes before treatment with Tris was 480 $\mu mol \cdot mg\ Chl^{-1} \cdot h^{-1}$. Broken lines represent the levels before illumination.

Fig. 8. Restoration of the HP form of Cyt *b*559 and reactivation of O_2 evolution by photoactivation treatment with flashing light. Tris-treated PSII membranes, suspended in the high-salt medium, were illuminated with the designated numbers of flashes at intervals of 1 s in the presence of 0.1 mM Mn^{2+} and 50 mM Ca^{2+}. (A) Changes in the (hydroquinone)-(ferricyanide) difference spectrum of Cyt *b*559. a: Before illumination; b, c and d: after illumination with 1, 2 and 3 flashes, respectively. (B) Changes in the amount of the HP form of Cyt *b*559 (○), in the amount of Cyt *b*559 that remained in the reduced form after illumination (●), and in the O_2-evolving activity (△). The total amount of the Cyt *b*559 was 1.8/220 Chl unit (mol/mol). The O_2-evolving activity of PSII membranes before Tris treatment was 450 $\mu mol \cdot mg\ Chl^{-1} \cdot h^{-1}$.

ΔA=0.001
d
c
b
a
A
540
560
580
Wavelength (nm)

Cyt b559/220 Chl (mol/mol)
0.6
0.4
0.2
0
B
0 1 2 3 5 10 20 50 100
Flash number
40
20
0
O2 evolution [μmol·(mg Chl)-1·h-1]

Fig. 9. Changes in the level of Cyt *b*559 in the reduced form during and after photoactivation treatment with flashing light. Tris-treated PSII membranes were suspended in the high-salt medium that contained 0.1 mM Mn^{2+} and 50 mM Ca^{2+} at 100 μg Chl/ml and placed in a plastic cuvette (light-path length, 10 mm; light-path width, 4 mm). The cuvette was set in a spectrophotometer (UV-2200, Shimadzu) and the suspension was illuminated from the side of the cuvette with flashing light from a 10-μs xenon flash lamp, which had been passed through a red filter (R-62, Toshiba), at 1-s intervals. The photomultiplier was protected from red light with a blue filter (4-96, Corning). The absorbance of the suspension was recorded at 559 or 570 nm with the data-sampling interval of 0.1 s and the slit width of 5 nm. (A) Illumination with 10 flashes. (B) Illumination with 40 flashes. (a and b) Absorbance changes at 559 and 570 nm, respectively. Upward and downward arrows indicate the beginning and the end of illumination with a train of flashes, respectively. The absorbance change at 559 nm of 0.001 corresponds to 0.13 molecule of the reduced Cyt *b*559 per 220 Chl molecules. The averages of five different experiments are presented.

absorption band of Cyt *b*559. As seen in Fig. 9, the level of Cyt *b*559 in the reduced form gradually increased during illumination with increasing number of flashes. After illumination with 10 flashes, however, the level of the reduced Cyt *b*559 gradually decreased in darkness for about 30 s. On the other hand, after illumination with 40 flashes, the level of the reduced Cyt *b*559 did not decrease but remained almost constant in darkness. In this experiment, a fraction of Cyt *b*559 which was once reduced but reoxidized within 1 s could not be detected because of the limitation of time resolution of the instrument. Provided that only the reduced HP form was detected in this experiment, it is suggested that the reduced HP form was first unstable and susceptible to reoxidation but that it became more stable toward reoxidation by further illumination.

Fig. 10 shows the dependence on the flash interval of the restoration of the HP form. The dependence gave a bell-shaped curve, with the extent of the restoration was maximum at the interval of 1 s. If only one photoreaction were sufficient for the restoration, the extent of the restoration should increase with increasing intervals between flashes and then remain at a maximum plateau level at longer intervals. Therefore, it appeared that one photoreaction was insufficient for the restoration of the HP form. From this result and the dependence on the flash number in Fig. 8, it is suggested that two photoreactions are required for the restoration of the HP form.

The dependence on the flash interval also indicates that two photoreactions must occur successively for the restoration of the HP form. Illumination with flashes at intervals longer than 5 s was totally ineffective in the restoration (Fig. 10). This observation suggests that the restoration of the HP form proceeds via an unstable intermediary state which is generated after the first photoreaction but decays in darkness within several seconds.

Fig. 10. Effects of the flash interval on the restoration of the HP form of Cyt *b*559 by photoactivation treatment with 5 flashes. Tris-treated PSII membranes, suspended in the high-salt medium, were illuminated with 5 flashes at the designated flash intervals in the presence of 0.1 mM Mn^{2+} and 50 mM Ca^{2+}. The total amount of Cyt *b*559 was 2.1/220 Chl unit (mol/mol). A broken line represents the level of the HP form before illumination. Three different experiments gave a similar dependence with a maximum restoration at 1-s intervals.

The photoactivation of O_2 evolution yields a similar bell-shaped curve for the dependence on the flash interval (Radmer and Cheniae, 1977; Tamura and Cheniae, 1987). However, the dependence of the restoration of the HP form in Fig. 10 was distinct from that for photoactivation since photoactivation treatment in this experiment was performed with 5 flashes, which had practically no reactivating effect on O_2 evolution (see Fig. 8B).

Fig. 11 shows the dependence on Mn^{2+} concentration of the restoration of the HP form and of the recovery of O_2-evolving activity upon the photoactivation treatment with 30 flashes. The HP form was effectively restored at Mn^{2+} concentrations below 10 μM, while the recovery of activity required much higher concentrations of Mn^{2+}. The concentrations that gave the half-maximal restoration were 5 and 17 μM for the HP form and the O_2-evolving activity, respectively. These differences in the requirement for Mn^{2+} again suggest that the restoration of the HP form of Cyt *b*559 occurs independently of the reconstitution of the Mn cluster.

Factors Involved in Restoration of the HP Form of Cyt b*559.*

As described above, the LP form of Cyt *b*559 could be converted to the HP form by illumination with 2 flashes in the presence of Mn^{2+}. Two different roles for Mn^{2+} in this process can be considered: (1) Mn^{2+} could be essential, and the process could involve photooxidation of Mn^{2+} and binding of the resultant Mn^{3+} to the PSII reaction center complex, as in the case of the reconstitution of the Mn cluster (Radmer and Cheniae, 1977; Tamura and Cheniae, 1987) or (2) Mn^{2+} could merely act as artificial electron donors and electron-transport reactions through PSII are responsible for the restoration of the HP form. It was examined whether artificial electron donors other than Mn^{2+} could replace Mn^{2+}. As shown in Table 4, three of five artificial

Fig. 11. Effects of the concentration of Mn^{2+} during photoactivation treatment on the restoration of the HP form of Cyt *b*559 and of the reactivation of O_2 evolution. Tris-treated PSII membranes, suspended in the high-salt medium, were illuminated with 30 flashes at intervals of 1 s in the presence of the designated concentrations of Mn^{2+} and 50 mM Ca^{2+}. The increases in levels of the HP form of Cyt *b*559 (●) and O_2-evolving activity (△) upon illumination are presented. The amount of the HP form and the O_2-evolving activity before illumination were 0.47/220 Chl unit (mol/mol) and 16 μmol·mg $Chl^{-1}\cdot h^{-1}$, respectively.

donors tested were effective in restoring the HP form of Cyt *b*559 during illumination. NH_2OH (10–1000 μM), semicarbazide (0.5–1 mM) and DPC (50–100 μM) allowed restoration of the HP form more effectively than Mn^{2+}. Benzidine (10–100 μM) was ineffective, but it prevented any decrease in the level of the HP form during illumination. I^- did not restore the HP form but, in fact, tended to enhance the decrease in the level of the HP form. Identical results were obtained even after removal of artificial donors from those illuminated PSII membranes by washing twice with the low-salt medium prior to analysis of Cyt *b*559 (data not shown).

The electron donors that were effective in the restoration of the HP form are all efficient electron donors to PSII (Bennoun and Joliot, 1969; Izawa et al., 1969; Vernon and Shaw, 1969; Yamashita and Butler, 1969). Benzidine, which did not support the restoration, is also an efficient electron donor comparable to Mn^{2+} since benzidine and Mn^{2+} reduce Y_Z^+ with similar rate constants (Hoganson et al., 1989). Thus, the rate of electron donation seems not to be correlated with the effectiveness in the restoration. The midpoint potentials of the artificial donors were also uncorrelated (Table 4). I^- is a poor electron donor. Illumination in the presence of I^- causes the specific iodination of Y_Z or the non-specific iodination of proteins of PSII in samples depleted of the Mn cluster, with resultant inactivation of electron transport in PSII (Takahashi and Asada, 1985; Ikeuchi et al., 1988). These deleterious effects of I^- might be responsible for the decrease in the level of the HP form under illumination in the presence of I^-.

From the above observations, it is suggested that Mn^{2+} is not essential but electron donation by exogenous donors to PSII is responsible for the restoration of the HP form of Cyt *b*559. Next, the events were investigated that occur at the acceptor side of PSII during the course of the restoration

of the HP form.

Table 5 shows the effects of DCMU and benzoquinone-type electron acceptors on the restoration of the HP form of Cyt *b*559 upon illumination in the presence of Mn^{2+} and Ca^{2+}. DCMU did not at all affect the restoration. This result suggests that electron transfer from Q_A to Q_B is not required for the restoration. By contrast, benzoquinone-type electron acceptors, namely, DCBQ and DBMIB, completely suppressed the restoration. This result contrasts with the effects of DCPIP which did not suppress the restoration (see Table 3). DCBQ and DBMIB bind to the Q_B site and directly oxidize Q_A^- (Tanaka-Kitatani et al., 1990; Satoh et al., 1992) while DCPIP accepts electrons from Q_B and/or plastoquinone pool. Therefore, it seems likely that DCBQ and DBMIB had a suppressive effect when they were bound to the Q_B site. This possibility is supported by the observations that, when the Q_B site was first occupied by DCMU, further addition of DCBQ or DBMIB did not have a suppressive effect, while the addition of these compounds in the opposite order had a suppressive effect (Table 5). The most probable action of DCBQ and DBMIB in the Q_B site is to oxidize Q_A^-. Therefore, it seems likely that Q_A^- generated under illumination is necessary for the restoration of the HP form. It is also possible that DCBQ and DBMIB in the Q_B site affect some other redox component(s) in PSII which is involved in the restoration of the HP form.

Even in darkness the increase in the level of the HP form was observed when DBMIB and DCMU were added sequentially (Table 5). This restoration in darkness depended on the conditions of the dark adaptation of the Tris-treated PSII membranes prior to the experiment. When the membranes had been adapted to darkness for 5 h on ice, the extent of the restoration in darkness was about 0.3/220 Chl unit (mol/mol). However, when the membranes had been dark-adapted at 25 ^{o}C, the extent of the restoration decreased by about 50%.

Table 4

Changes in the amount of the HP form of Cyt *b*559 upon illumination in the presence of artificial electron donors

Electron donor added	Eo' (mV)	Cyt *b*559 HP/220 Chl (mol/mol)		
		Before illu.	After illu.	Δ
None		0.58	0.45	−0.13
100 μM Mn^{2+}	+1,090	0.48	0.62	+0.14
1 mM NH_2OH	–	0.42	0.69	+0.27
1 mM semicarbazide	> +430	0.43	0.60	+0.17
100 μM DPC	+215 – +430	0.42	0.63	+0.21
100 μM benzidine	+610	0.42	0.42	0
10 mM NaI	+560	0.50	0.27	−0.23

Tris-treated PSII membranes were suspended in the high-salt medium and incubated in darkness for 5 min. The suspension was supplemented with the indicated artificial electron donor, incubated in darkness for 5 min, and illuminated with continuous light for 10 min. The total amount of Cyt *b*559 was 2.0/220 Chl unit (mol/mol). Eo' represents midpoint potentials taken from Clark (1960). For Mn^{2+}, the Eo' value of the Mn^{2+}/Mn^{3+} couple is presented (Bachofen, 1966). For semicarbazide and DPC, the Eo' values were estimated to be higher than +430 mV and to be between +215 and +430 mV, respectively, since semicarbazide does not reduce ferricyanide (Eo'= +430 mV) and DPC reduces ferricyanide but not DCPIP (Eo'= +215 mV).

Table 5

Effects of DCMU and artificial electron acceptors on the restoration of the HP form of Cyt *b*559 by photoactivation treatment

Additives	Cyt *b*559 HP/220 Chl (mol/mol)		
	Before illu.	After illu.	Δ
None	0.47	0.69	+0.22
DCMU	0.50	0.71	+0.21
DCBQ	0.50	0.50	0
DBMIB	0.50	0.50	0
DCMU -> DCBQ	0.50	0.66	+0.16
DCMU -> DBMIB	0.50	0.74	+0.24
DCBQ -> DCMU	0.50	0.50	0
DBMIB -> DCMU	0.84	0.79	−0.05

Tris-treated PSII membranes were suspended in the high-salt medium, supplemented with 0.1 mM Mn^{2+} and 50 mM Ca^{2+} and incubated in darkness for 5 min. Then the suspension was further supplemented with the indicated additive(s), incubated in darkness for 2 min, and illuminated with 5 flashes at flash intervals of 1 s. The final concentration of each additive was 10 μM. When two reagents were added sequentially, the second reagent was added one min after addition of the first one (indicated by an arrow). The total amount of Cyt *b*559 was 2.1/220 Chl unit (mol/mol).

Thus, some redox power that remained in PSII appeared to be responsible for this restoration in darkness, via some as yet unexplained mechanism.

It was proposed in CHAPTER I that, in Tris-treated thylakoids, the pH gradient across the thylakoid membrane might participate in the restoration of the HP form of Cyt *b*559 upon the photoreactivation treatment, since uncouplers, namely, nigericin and gramicidin-S at 100 μM, almost completely inhibited the restoration. The PSII membranes used in the present study were not closed membrane vesicles but were membrane fragments (Dunahay et al., 1984) and, thus, no pH gradient could be formed across the membrane. The effects of nigericin on the restoration of the HP form in Tris-treated thylakoids and Tris-treated PSII membranes were reinvestigated (Fig. 12). At 10 μM, nigericin inhibited the restoration of the HP form by about 20% in the thylakoids but no inhibition at all was noted in the PSII membranes. At concentrations above 10 μM, nigericin had inhibitory effects in both cases, though the extent of inhibition in the PSII membranes was about 50% of that in the thylakoids at all tested concentrations of nigericin. In general, nigericin at 10 μM is sufficient to diminish the pH gradient across the thylakoid membrane. Therefore, it seems likely that, while the inhibition by nigericin at 10 μM observed in the thylakoids might be ascribable to disruption of the pH gradient, the inhibition by nigericin at concentrations above 10 μM results from some secondary effect of nigericin. It appears that a pH gradient across the membrane is not a prerequisite for the restoration of the HP form of Cyt *b*559, even though such a gradient acts to enhance the restoration in thylakoids.

Fig. 12. Effects of nigericin on restoration of the HP form of Cyt *b*559 by photoactivation treatment. Tris-treated PSII membranes (○) were suspended in the high-salt medium that contained 0.1 mM Mn^{2+} and 50 mM Ca^{2+}. Thylakoids that had been treated with Tris and then with DCPIP (●) were suspended in a medium that contained 50 μM Mn^{2+}, 1 mM Ca^{2+}, 20 μM DCPIP and 2 mM ascorbate (CHAPTER I). Then the suspension was supplemented with designated concentrations of nigericin and 10 mM KCl, incubated in darkness for 2 min, and illuminated with continuous light. The duration of illumination was 10 and 20 min for the PSII membranes and the thylakoids, respectively. The amount of the HP form of Cyt *b*559 in the thylakoids was determined after the thylakoids had been washed twice with a medium without Mn^{2+}, Ca^{2+} and DCPIP. 100% corresponds to the amount of the HP form of Cyt *b*559 restored by photoactivation treatment in the absence of nigericin, namely, 0.22/220 Chl unit (mol/mol) in the PSII membranes and 0.56/400 Chl unit (mol/mol) in the thylakoids. The total amounts of Cyt *b*559 were 2.1/220 Chl unit (mol/mol) and about 2/400 Chl unit (mol/mol) in the PSII membranes and the thylakoids, respectively.

DISCUSSION

Interconversion of the LP and HP Forms of Cyt b*559.*

Using isolated thylakoids, it was demonstrated in CHAPTER I that the HP form of Cyt *b*559 can be converted to the LP form by treatment with Tris and that the LP form can be recovered to the HP form by photoreactivation of O_2 evolution. The present study showed that the same interconversion of the LP and HP forms can also occur in isolated PSII membranes. However, the extent of interconversion was smaller in PSII membranes than in thylakoids.

Under the conditions employed in this study, only about 30-40% of the HP form in PSII membranes was converted to the LP form when the Mn cluster and the three extrinsic proteins had been completely depleted by treatment with Tris. In the case of intact thylakoids, by contrast, about 80% of the HP form was readily converted to the LP form upon treatment with Tris (Mizusawa et al., 1995). To lower further the level of the HP form in the Tris-treated PSII membranes, the membranes were incubated with 1 M NaCl in darkness.

It was reported previously that Ca^{2+} bound to the donor side of PSII, possibly to the Mn cluster, is released under illumination in the presence of concentrated NaCl, with resultant conversion of the HP form of Cyt *b*559 to the lower-potential forms (McNamara and Gounaris, 1995). However, it is unlikely that the effects of 1 M NaCl observed in this study resulted from the release of Ca^{2+}, since the releasable Ca^{2+} at the donor side would have already been lost upon depletion of the Mn cluster by treatment with Tris (Tamura and Cheniae, 1985). The effects of concentrated NaCl are considered to be ascribable to the chaotropic effects of Cl^-. It was proposed previously that Cyt *b*559 exists in the HP form when the heme is located inside a hydrophobic niche that is shielded from water molecules by protein segments, while the HP

form is converted to the LP form when the heme is exposed to the outer aqueous phase (Krishtalik et al., 1993). Since Cl^- is a chaotropic anion that destroys the water structure at the surface of proteins and membranes, it is likely that concentrated Cl^- alters the environment around the heme to allow water molecules access to the heme.

The extent of restoration of the HP form by photo(re)activation was also smaller in PSII membranes than in thylakoids: 30–50% and 50–80% of the total LP form were converted to the HP form in the PSII membranes and in the thylakoids, respectively. If the interconversion of the HP and LP forms were to result from changes in the hydrophobicity around the heme, it seems likely that protein segments that shield the heme of Cyt *b*559 can undergo conformational change more easily in thylakoids than in PSII membranes upon treatment with Tris and subsequent photo(re)activation.

Under photoactivation conditions, illumination in the presence of Mn^{2+} was sufficient for the restoration of the HP form (Table 3). The extrinsic proteins and Ca^{2+} were not required for this process. However, it was found that Ca^{2+} was necessary for stabilization of the HP form of Cyt *b*559 and that this stabilizing effect was independent of the presence of the Mn cluster (Table 3, Fig. 7). A similar stabilizing effect of Ca^{2+} on the HP form was also proposed previously in the case of PSII membranes that contained the Mn cluster but lacked the extrinsic 23-kDa protein (McNamara and Gounaris, 1995).

Intact PSII membranes contain two molecules of Ca^{2+} per PSII (Ono and Inoue, 1988). One of the two Ca^{2+} is released from PSII upon depletion of the Mn cluster (Tamura and Cheniae, 1985) and this Ca^{2+} is considered to interact with the Mn cluster (Ono et al., 1991). The other Ca^{2+} is tightly bound to the light-harvesting Chl complex and is not releasable (Han and Katoh, 1993).

In the case of PSII membranes that contain the Mn cluster, it is possible that Ca^{2+} binds to its specific binding site close to the Mn cluster and stabilizes the HP form of Cyt *b*559 as proposed previously (McNamara and Gounaris, 1995). By contrast, in the case of PSII membranes depleted of the Mn cluster, it is unclear if the specific binding site for Ca^{2+} remains intact. Provided that Ca^{2+} can bind to its specific binding site both in the presence and in the absence of the Mn cluster, the effects of bound Ca^{2+} on the HP form would be conformational since the Ca^{2+}-binding site is located at the lumenal surface of the PSII reaction center complex, at a distance from the heme of Cyt *b*559.

Mechanism of the Restoration of the HP Form of Cyt b*559.*

It was reported previously (Okayama and Butler, 1972) that the HP form of Cyt *b*559 was restored when isolated thylakoids that had been extracted with hexane were reconstituted with β-carotene and plastoquinone molecules. The restoration of the HP form was also observed when purified Cyt *b*559 was incorporated into liposomes that consisted of digalactosyldiacylglycerol (Butler and Matsuda, 1983). The restoration of the HP form in these cases would be ascribable to the effects of exogenously added hydrophobic molecules which could directly alter the hydrophobicity around the heme of Cyt *b*559. In the case of the restoration of the HP form upon photoactivation, illumination of PSII in the presence of Mn^{2+} was sufficient for the restoration.

The results of the present study show clearly that the restoration of the HP form and the reconstitution of the Mn cluster are different processes. The restoration of the HP form occurred with high quantum efficiency and was completed before the Mn cluster was reconstituted (Fig. 8). In addition, the concentration of Mn^{2+} required for the restoration was much lower than

that for the reconstitution of the Mn cluster (Fig. 11). The only similarity between these two processes is that both require two successive photoreactions in PSII (Figs. 8,10) (Radmer and Cheniae, 1977; Tamura and Cheniae, 1987).

Artificial electron donors other than Mn^{2+} also supported the restoration of the HP form under illumination (Table 4). This finding clearly indicates that Mn^{2+} is not essential while electron donation from exogenous donors to PSII is required for the restoration of the HP form.

It was also found that some redox component(s) that interacts with benzoquinone-type electron acceptors in the Q_B site participates in the restoration of the HP form (Table 5). The most probable candidate for this component is Q_A^- generated during illumination. According to Johnson et al. (1995), upon depletion of the Mn cluster, Q_A is converted from an active form with a low potential (Eo'= -80 mV) to an inactive form with a high potential (Eo'= +55 mV), which is unable to transfer electrons to Q_B. When Q_A is in the inactive form, Q_A^- generated by a photoreaction in PSII decays back to Q_A as a consequence of a charge recombination reaction with Y_Z^+, with a half time of 120 ms, unless Y_Z^+ is not re-reduced by exogenous donors (Dekker et al., 1984). If Q_A^- were to participate in the restoration of the HP form of Cyt *b*559, it might be possible that the role of exogenous electron donors in the restoration is to stabilize Q_A^- by reducing Y_Z^+ and suppressing the charge recombination reactions. In fact, it has been reported that the presence of exogenous donors extends the lifetime of Q_A^- to more than a few seconds (Dekker et al., 1984; Nixon and Diner, 1992).

If some redox component(s) other than Q_A^- could participate in the restoration, the non-heme iron at the acceptor side of PSII would be a likely candidate. In general, the non-heme iron is in the Fe(II) state both in the light and in the dark, but it is oxidized under illumination in the presence

of benzoquinone-type electron acceptors via the reduction-induced oxidation mechanism that involves oxidation of Q_A^- by a benzoquinone molecule bound to the Q_B site (Diner and Petrouleas, 1987). This oxidation of the non-heme iron does not occur when the Q_B site has been occupied by DCMU prior to addition of benzoquinone-type electron acceptors (Diner and Petroureas, 1987). This effect of the sequential addition of DCMU and benzoquinone-type electron acceptors on the oxidation of the non-heme iron is quite similar to that on the restoration of the HP form (Table 5). Since addition of benzoquinone-type electron acceptors completely suppressed the restoration (Table 5), it seems likely that the non-heme iron had to stay in the Fe(II) state during the course of the restoration of the HP form.

The most probable action of the putative redox component(s), Q_A^- and/or the non-heme iron, in the restoration of the HP form would be to reduce the LP form of Cyt *b*559. It has been demonstrated recently that, in the case of horse heart Cyt *c*, the native protein in the reduced form is more stable toward unfolding than in the oxidized form, and that the reduction of the heme shifts the equilibrium from the denatured and low-potential form toward the native and high-potential form (Pascher et al., 1996). In the case of Cyt *b*559, it is known that the HP form in the reduced form is more resistant to treatments which lower the redox potential of Cyt *b*559 than in the oxidized form (Ortega et al., 1990; McNamara and Gounaris, 1995). In addition, it was reported that the LP form of Cyt *b*559 can be reduced by a photoreaction in PSII under some certain conditions (Ortega et al., 1995; Poulson et al., 1995). Therefore, it is not unlikely that the LP form of Cyt *b*559 is reduced during the course of the restoration of the HP form. If the LP form were to be reduced for the restoration, the reduced LP form appeared to be reoxidized within 1 s since, in the experiments in Fig. 9, significant reduction of

Cyt *b*559 was not detected after illumination with 3 flashes with which the restoration of the HP form was completed. It was observed that chemical reduction of the LP form of Cyt *b*559 by incubating PSII membranes with sodium ascorbate in darkness did not restore the HP form (data not shown). This suggests that the LP form of Cyt *b*559 has to be reduced by a photochemical reaction(s) in PSII, but not by chemical reductants, for the restoration of the HP form.

It was observed that the HP form of Cyt *b*559 restored by illumination with a few flashes remained in the oxidized form after illumination, but that the level of its reduced form increased by further illumination with flashes (Fig. 8B). It was also found that the reduced HP form was first susceptible to reoxidation but became more stable toward reoxidation by further illumination with flashes (Fig. 9). Under the conditions in this study, not only the HP form of Cyt *b*559 (Eo'= +370 mV) but also the intermediate-potential form(s) of the Eo' value higher than +200 mV could be detected as the HP form that was reducible with 2 mM hydroquinone (see "MATERIALS AND METHODS"). Therefore, it might be possible that the redox potential of the hydroquinone-reducible HP form restored with a few flashes was lower than that of the real HP form but increased gradually during further illumination so that the reduced form became stabilized. In fact, our preliminary experiments have shown that the hydroquinone-reducible HP form restored by illumination with 2 flashes was not reduced by 2 mM potassium ferrocyanide, while about 50% of the restored HP form was reduced by ferrocyanide after illumination with 40 flashes (data not shown). Thus, it is likely that the conversion of the LP form of Cyt *b*559 to the real HP form proceeds via the intermediate-potential form(s), the reduced form(s) of which is unstable and gradually reoxidized by ambient oxygen molecules. I presume that the LP form could be first

converted to such an intermediate-potential form by two photoreactions in PSII, and the further conversion to the real HP form would require additional photoreactions in PSII. These photoreactions might lead to reduction of Cyt *b*559 and shift the equilibrium toward higher-potential forms.

The effect of the flash interval on the restoration of the hydroquinone-reducible HP form (Fig. 10) suggests that an unstable intermediary state would be formed between two photoreactions. The origin of this intermediary state is obscure. It also remains to be solved if the LP form had to be reduced twice for the conversion to the putative intermediate-potential form. Further studies are required to clarify the precise mechanism of the restoration of the HP form of Cyt *b*559.

GENERAL DISCUSSION

The Mechanism of the Reconstitution of the HP Form of Cyt b*559.*

It had long been considered that the HP form of Cyt *b*559 might be involved in the photosynthetic O_2 evolution both *in vivo* and *in vitro* (for a review, Cramer et al., 1986). This hypothesis had been proposed from the apparent correlation between the presence of the HP form of Cyt *b*559 and the capacity to evolve oxygen (See "GENERAL INTRODUCTION"). However, most of previous studies merely described the phenomena, and did not investigate the factors responsible for the change in redox potential of Cyt *b*559. In this thesis, the treatment with Tris and the photo(re)activation treatment were employed to inactivate and reactivate O_2 evolution and the system to analyze the interconversion between the HP and LP forms of Cyt *b*559 was established.

Treatment with Tris of isolated thylakoids and PSII membranes converted the HP form of Cyt *b*559 to the LP form, with concomitant inactivation of O_2 evolution [Fig. 4 (II,III), Table 3]. The LP form in Tris-treated materials could be recovered to the HP form by illumination in the presence of Mn^{2+} [Fig. 4 (III,IV), Fig. 5, Table 3]. Almost similar characteristics in the restoration of the HP form of Cyt *b*559 were observed both in thylakoids and PSII membranes except the requirement for Mn^{2+} and the effects of uncouplers. In thylakoids, the concentrations of Mn^{2+} required for the restoration of the HP form of Cyt *b*559 and for the recovery of O_2-evolving activity were almost identical and the concentration that gave the half-maximum restoration was 3-4 μM in both cases (Fig. 5). By contrast, in PSII membranes, two processes exhibited different requirements for Mn^{2+}: the concentrations of Mn^{2+} that gave the half-maximum restoration were 5 and 17 μM for the HP form and the O_2 evolution, respectively (Fig. 11). I

considered that this difference could be ascribable to a difference in illumination conditions for the photo(re)activation. In experiments with thylakoids, they were illuminated with weak light for 20 min, during which the recoveries of the HP form and the O_2-evolving activity reached the maximum (Fig. 5). In experiments with PSII membranes, they were illuminated with 30 flashes, with which the recovery of the O_2-evolving activity was about 30% of the maximum (Fig. 8). Thus, it is likely that the concentration of Mn^{2+} that gave the half-maximum recovery of the O_2-evolving activity would be underestimated in the case of thylakoids.

It was suggested in CHAPTER I that the pH gradient across the membrane might be involved in the restoration of the HP form of Cyt *b*559 in the case of thylakoids. This hypothesis was deduced from the suppressive effects of uncouplers on the restoration of the HP form (Table 2). However, nigericin at 10 μM, which is sufficient to diminish the pH gradient across the thylakoid membrane, inhibited only 20% of the restoration of the HP form in the thylakoids (Fig. 12). The suppression by nigericin was observed in PSII membranes only at the concentration of nigericin above 10 μM (Fig. 12). Thus, the suppression of the restoration of the HP form by nigericin in PSII membranes can be ascribed to the secondary effects of nigericin. It appears that a pH gradient across the membrane is not a prerequisite for the restoration of the HP form of Cyt *b*559, even though such a gradient acts to enhance the restoration in thylakoids.

One of the important conclusions of this study is that the restoration of the HP form of Cyt *b*559 is a process distinct from the reconstitution of the Mn cluster. In the photoactivation treatment of Tris-treated thylakoids, illumination in the presence of Mn^{2+} was found to be indispensable for the restoration of the HP form, as in the case of the reactivation of O_2 evolution

(Table 1). However, the time courses of the restoration of the HP form and of the O_2-evolving activity were quite different. The restoration of the HP form occurred more rapidly than the restoration of O_2-evolving activity (Fig. 6). The effects of DCMU were also different. DCMU inhibited the restoration of O_2-evolving activity but did not affect that of the HP form. These observations support the conclusion that these two processes proceed via different reaction mechanisms.

This was also the case in isolated PSII membranes. When the photo-(re)activation treatment was achieved with flashes, the restoration of the HP form occurred with only 2 flashes while that of O_2-evolving activity required more than 6 flashes (Fig. 8). It was also found that artificial electron donors of PSII (NH_2OH, DPC and semicarbazide) other than Mn^{2+} also supported the restoration of the HP form (Table 4). Thus, it is suggested that not the reconstitution of the Mn cluster but the electron donation by Mn^{2+} to PSII are responsible for the restoration of the HP form of Cyt *b*559. From the effects of DCMU and artificial electron acceptors, DBMIB and DCBQ (Table 5), it is suggested that Q_A^- generated under illumination is involved in the restoration of the HP form of Cyt *b*559, and that some redox reaction(s) at the acceptor side of PSII, probably reduction of the LP form of Cyt *b*559 by Q_A^-, occurs during the course of the restoration of the HP form (Fig. 13).

Q_A^- generated by a photoreaction in PSII rapidly decays back to Q_A as a consequence of a charge recombination reaction with Y_Z^+, with a half time of 120 ms, unless Y_Z^+ is not re-reduced by exogenous electron donors (Dekker et al., 1984). The presence of exogenous electron donors extends the lifetime of Q_A^- to more than a few seconds (Dekker et al., 1984; Nixon and Diner, 1992). Therefore, it might be possible that the role of exogenous electron donors in the restoration is to stabilize Q_A^- by reducing Y_Z^+ and suppressing the

Fig. 13. Electron transport pathway in PSII depleted of the Mn cluster proposed to restore the HP form of Cyt *b*559. In this model, Q_A^- generated by a photochemical reaction in PSII reduces the LP form of Cyt *b*559 (indicated by a broken line with an arrow). The reduction of the LP form leads to the restoration of the HP form. Arrows indicate the direction of transport of electrons.

Fe: the non-heme iron; Pheo: the primary electron acceptor pheophytin; Q_A and Q_B: quinone-type electron acceptors.

charge recombination reactions.

The Physiological Significance of the Restoration of the HP Form of Cyt b*559.*

When PSII is newly assembled *in vivo*, protein components of PSII, including Cyt *b*559, are newly synthesized, inserted into the thylakoid membrane and assembled into PSII that lacks the Mn cluster. Then, the Mn cluster is constituted by photochemical reactions in the nascent PSII (Fig. 14; van Wijk et al., 1994). It is known that Cyt *b*559 is present in the LP form before the Mn cluster is constituted (Metz et al., 1980; Shinohara et al., 1992) but is converted to the HP form during constitution of the Mn cluster under illumination (Shinohara et al., 1992). This study has revealed that electron donors to PSII are necessary for the conversion of Cyt *b*559 from the LP to the HP form (Table 4). *In vivo*, Mn^{2+} present in the thylakoid lumen at a micromolar level (Ono and Inoue, 1983) would presumably be a sole substance for the electron donor to PSII. Thus, it is likely that, *in vivo*, the LP form of Cyt *b*559 is first converted to the HP form by electron donation by Mn^{2+}, and subsequently, the Mn cluster is constituted by photooxidation of Mn^{2+}. It is considered that the conversion of Cyt *b*559 from the LP to the HP form is a primary process of the photoactivation of the nascent PSII *in vivo*.

It has been proposed that, among two different types of PSII, namely, PSIIα and PSIIβ, PSIIβ is a transient and intermediary form during the formation of the functional PSII, PSIIα (Guenther and Melis, 1990; Melis, 1991). PSIIβ is unable to transfer electrons from Q_A to Q_B since Q_A in PSIIβ is present in the inactive and high-potential form (Thielen and van Gorkom, 1981). However, it contains an active Mn cluster since it can evolve oxygen in the presence of benzoquinone-type electron acceptors that can draw electrons from Q_A directly (Henrysson and Sundby, 1990; Nedbal et al., 1991).

If PSIIβ were the intermediate for the functional PSII, it is suggested that the Mn cluster can be constituted even when Q_A is inactive. Q_A in the inactive form can accept only one electron during illumination unless Q_A^- is re-oxidized by some component in PSII or by autooxidation. However, the constitution of the Mn cluster requires at least two or more successive charge-separation reactions since the quantum yield of the reconstitution is not high (See Fig. 8B; Radmer and Cheniae, 1977; Miyao-Tokutomi and Inoue, 1992). As judged from the midpoint potential of Cyt *b*559, it is quite possible that the HP (and also intermediate-potential) form of Cyt *b*559 might act as the oxidant for Q_A^-. In fact, the reduction of the HP form was observed during the photo(re)activation process (Figs. 8, 9). I presume that, during the photo(re)activation of O_2 evolution, the HP form of Cyt *b*559 is first restored and then acts as an electron acceptor to support the reconstitution of the Mn cluster.

Fig. 14 The schematic model of assembly of the PSII complex in the thylakoid membrane. A) Protein components of the PSII complex including Cyt *b*559 are newly synthesized and inserted into the thylakoid membrane. B) The inserted protein components are assembled into PSII that lacks the Mn cluster. Components involved in the photochemical and electron transport reactions in PSII, namely, P680, pheophytin, Q_A, Q_B and non-heme iron are ligated to the reaction center complex and the heme is ligated to the α and β subunits of Cyt *b*559. Cyt *b*559 in this nascent PSII complex is in the LP form. C) The LP form of Cyt *b*559 is converted to the HP form under illumination, utilizing Mn^{2+} present in the thylakoid lumen as an electron donor. D) The Mn cluster is constituted to the PSII complex via photooxidation of Mn^{2+} in PSII, the three extrinsic proteins are also assembled and then the O_2-evolving activity is expressed. RC: reaction center of PSII; 33, 23, 18: the extrinsic proteins of 33, 23, 18 kDa.

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Photochemical reactivation of photosynthetic O2 gas by nitrite

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