Interconversion of Low- and High-Potential Forms of Cytochrome b_{559} in Tris-Washed Photosystem II Membranes under Aerobic and Anaerobic Conditions

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ABSTRACT: In this study, the reversible conversion between the high- (HP) and low-potential (LP) forms of Cytb$_{559}$ has been analyzed in Tris-washed photosystem II (PSII) enriched membranes. These samples are deprived of the Mn cluster of the water-oxidizing complex (WOC) and the extrinsic regulatory proteins. The results obtained by application of optical and EPR spectroscopy reveal that (i) under aerobic conditions, the vast majority of Cytb$_{559}$ exhibits a low midpoint potential, (ii) after removal of O_2 in the dark, a fraction of Cytb$_{559}$ is converted to the high-potential form which reaches level of about 25% of the total Cytb$_{559}$, (iii) a similar dark transformation of LP → HP Cytb$_{559}$ occurs under reducing conditions (8 mM hydroquinone), (iv) under anaerobic conditions and in the presence of 8 mM hydroquinone, about 60% of the Cytb$_{559}$ attains the HP form, (v) the interconversion is reversible with the re-establishment of aerobic conditions, and (vi) aerobic and oxidizing conditions (2 mM ferricyanide or 0.5 mM potassium iridate) induce a decrease of the amount of the HP form, also showing that the conversion is reversible. This reversible interconversion between LP and HP Cytb$_{559}$ is not observed in PSII membrane fragments with an intact WOC. On the basis of these findings, the possibility is discussed that the O_2-dependent conversion of Cytb$_{559}$ in PSII complexes lacking a functionally competent WOC is related to a protective role of Cytb$_{559}$ in photoinhibition and/or that it is involved in the regulation of the assembly of a competent water-oxidizing complex in PSII.

Cytochrome b_{559} (Cytb$_{559}$)[1] is an integral part of the PSII reaction center. It consists of two membrane-spanning polypeptides, α and β (*1*, *2*), both of which are required for the stable assembly of PSII. Mutations in the Cytb$_{559}$ genes often result in the absence of functional PSII complexes (*3*). Solubilized PSII preparations with the minimum polypeptide composition which are able to perform efficient light-induced primary charge separation although they are deprived of the non-heme iron center (*4*) and quinone acceptors Q_A and Q_B still retain Cytb$_{559}$ (*5*, *6*). Rather harsh treatments are required to remove Cytb$_{559}$ from PSII (*7*, *8*). The tight connection of Cytb$_{559}$ with the D1/D2 heterodimer, which houses all cofactors for the radical pair formation, $Q_A^- Y_Z^{ox}$, together with its relevance for PSII assembly are striking features which might be indicative of an essential role of Cytb$_{559}$. Despite numerous investigations, this problem has not been clarified. Surprisingly, even the much more simple question about the stoichiometric number of Cytb$_{559}$ per PSII is still a matter of controversy and discussion.

Isolated thylakoids have been reported to contain two hemes per PSII (*9*, *10*). Conflicting results with either one or two Cytb$_{559}$ exist for PSII membrane fragments (for a recent compilation of data, see refs *11* and *12*). Recent detailed analysis led to the conclusion that PSII core complexes from spinach with high oxygen evolution activity contain two Cytb$_{559}$ per PSII (*13*), whereas only one Cytb$_{559}$ was found in the PSII core complex from *Synechocystis* PCC6803 (*14*). In D1/D2/Cytb$_{559}$ preparations, probably only one Cytb$_{559}$ exists (*6*, *15*). Possible explanations for this dilemma of conflicting Cytb$_{559}$ stoichiometries are a loss of Cytb$_{559}$ during the purification of PSII preparations and/or the existence of differences in the composition of PSII complexes among different organisms (*11*, *12*, *16*, *17*).

The main reason for the difficulties in clarifying the role of Cytb$_{559}$ probably originates from the unique properties of this heme protein. The apoprotein of isolated Cytb$_{559}$ consists of two subunits (α and β) each containing only one His residue as the potential ligand for the heme iron (*18*). The two subunits of 9 and 4 kDa are encoded by the *psbE* and *psbF* genes, respectively (*11*). They are assumed to form an αβ heterodimer (*2*, *19*). Recent data, however, led to the alternative suggestion that the native protein exists in the form of two different homodimers [α_2 and β_2 (*20*)]. This idea gained support with the recent successful assembly of

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[1] Abbreviations: Chl, chlorophyll; Cytb$_{559}$, cytochrome b_{559}; DPC, 1,5-diphenylcarbazide; E_m, midpoint redox potential; EPR, electron paramagnetic resonance; VLP, LP, IP, and HP, very low-, low-, intermediate-, and high-potential forms of Cytb$_{559}$, respectively; ferricyanide, $K_3[Fe(CN)_6]$; iridate, $K_2[IrCl_6]$; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; PpBQ, phenyl-*p*-benzoquinone; PSII, photosystem II; Q_A and Q_B, primary and secondary quinone acceptors in PSII; Tris, tris(hydroxymethyl)aminomethane; Y_Z, secondary electron donor in PSII; WOC, water-oxidizing complex.

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a synthetic β_2 $Cytb_{559}$ which closely resembles the native low-potential enzyme in its spectral and redox properties (*21*).

Despite the lack of straightforward evidence, it seems likely that the unique coordination of the heme group in a dimeric protein matrix is responsible for the existence of $Cytb_{559}$ form(s) with unusually high midpoint redox potential (E_m) compared with other *b* type cytochromes and the remarkble variability of its E_m value toward different treatments. On the basis of redox titrations, four different $Cytb_{559}$ forms can be distinguished: the high-potential (HP) form with an E_m of 360−435 mV at pH 7.0−8.0 (*22*), the intermediate-potential (IP) form with an E_m of 150−270 at pH 6.0 (*10*), the low-potential (LP) form with an E_m of 20−120 mV (*22*), and the very low-potential (VLP) form with an E_m of −45 mV at pH 7.2 (*23*). However, the exact molecular factors which control the redox properties of $Cytb_{559}$ still have to be clarified.

The HP form of $Cytb_{559}$ dominates in PSII membrane fragments with an intact water-oxidizing complex, and it has been suggested that granal PSII complexes contain only HP $Cytb_{559}$ which is stabilized by Ca^{2+} ligation (*24*). Several treatments which lead to disintegration of the membrane or of the WOC cause a conversion of $Cytb_{559}$ into forms with lower E_m values (*11*, *12*). A partial reversal to high E_m values can be achieved by incubation of purified $Cytb_{559}$ into digalactosylglycerol liposomes (*25*). The HP form in thylakoid membranes can be restored by illumination in the presence of aqueous Mn^{2+} or other different electron donors such as NH_2OH, DPC, and semicarbazide (*26*). During assembly of the Mn cluster by photoactivation, the HP form of $Cytb_{559}$ is also enriched (*27*) and the latter process was found to be faster than the restoration of a functionally competent WOC (*26*). Despite this correlation, it is clear from previous studies that HP $Cytb_{559}$ is not a prerequisite for a functionally competent WOC (*28*). A partial restoration of HP $Cytb_{559}$ was reported for salt-washed PSII membranes after rebinding of the extrinsic 23 kDa protein (*29*, *30*).

On the basis of the peculiar properties, several possibilities have been considered for the functional role of $Cytb_{559}$. It has been proposed that $Cytb_{559}$ may be involved in the function of the WOC (*31*−*33*). The most widely discussed function is the participation of $Cytb_{559}$ in protection against photoinhibition under light stress. The HP form is suggested to protect against donor side-induced photoinhibition by electron transfer to $P680^+$ in the absence of a functional Mn cluster (*34*), while the LP form might prevent acceptor side-induced photoinhibition by oxidation of $Pheo^-$ (*35*); reactions supporting these functions have been observed during photoactivation of PSII in a mutant grown in the dark in *Chlamydomonas reinhardtii* (*36*). On the basis of these conclusions, a model was proposed where $Cytb_{559}$ serves as a molecular switch between different redox forms to regulate between donor and acceptor side photoinhibition (*37*). Furthermore, the possibility exists that $Cytb_{559}$ is involved in endogenous superoxide dismutase activity (*38*). Recently, it was suggested that the redox state of $Cytb_{559}$ regulates the formation of Chl_z^+, which acts as a powerful quencher of excited singlet states of Chl (*39*).

In this study, we concentrate on the conversion between HP and LP forms of $Cytb_{559}$ in Tris-washed PSII membrane fragments which are lacking the tetranuclear Mn cluster of the WOC and the extrinsic regulatory proteins of 17, 23, and 33 kDa. The results obtained clearly show that a reversible LP ↔ HP conversion of $Cytb_{559}$ takes place in the dark. The equilibrium population is shifted toward the HP form under anaerobic conditions and/or by the addition of reductants (hydroquinone). This phenomenon is not observed in PSII membrane fragments with a functionally competent WOC.

MATERIALS AND METHODS

PSII membrane fragments were isolated from market spinach (*40*, *41*). To remove the Mn cluster and the three extrinsic oxygen-evolving proteins, the PSII membranes were suspended in 1.0 M Tris (pH 9.1) and incubated in light on ice for 2 h. After a second wash in the same buffer, the pellets were washed once in 10 mM Mes (pH 6.5), 0.4 M sucrose, 4 mM $MgCl_2$, and 10 mM NaCl and stored at −80 °C at a Chl concentration of 2−3 mg/mL until they were used. The same buffer was used in all experiments.

The different thermodynamic forms of $Cytb_{559}$ were identified at 559 nm (*42*) from reduced minus oxidized difference spectra between 520 and 580 nm on a Shimadzu UV-3000 spectrophotometer. The wavelength accuracy was ±0.4 nm, the wavelength reproducibility ±0.2 nm, the scan speed 50 nm/min, and the spectral resolution 5 nm/cm. As a reference spectrum, we used the absorbance spectrum, obtained in the presence of 2 mM potassium ferricyanide. The reduction of $Cytb_{559}$ was achieved by adding 4 or 8 mM hydroquinone ($E_m \approx 280$ mV). Following reduction with hydroquinone, we added, to the same cuvette, 5 mM sodium ascorbate ($E_m \approx 60$ mV), reducing IP and LP forms, and subsequently 10 mM sodium dithionite to reduce $Cytb_{559}$ quantitatively. After each addition, the absorbance spectrum was recorded. The total amount of $Cytb_{559}$ (100%) was obtained from the dithionite-reduced minus ferricyanide-oxidized spectrum. The amount of HP $Cytb_{559}$ was estimated from the hydroquinone-reduced minus ferricyanide-oxidized spectrum. The IP and LP forms were identified from the difference between the ascorbate-reduced and the HP forms. The VLP form of $Cytb_{559}$ was estimated as the difference between total and ascorbate-reduced forms. The Chl concentration in the measurements was 75 μg/mL.

The redox midpoint potential of the medium containing Tris-washed PSII membranes after the various additions was measured with a micro calomel combination redox electrode, manufactured by Broadley James Corp. The E_m was found to be 270 and 230 mV after hydroquinone addition under our aerobic and anaerobic conditions, respectively.

X-band EPR spectra of $Cytb_{559}$ in the $g_z = 3$ turning point region (*10*, *12*) were recorded at 15 K with a Bruker ESP380E spectrometer equipped with an Oxford Instruments cryostat and temperature controller. EPR conditions were as follows: microwave frequency of 9.47 GHz, microwave power of 5.85 mW, and modulation amplitude of 2 mT. The sample concentration was about 2 mg of Chl/mL. The spectra were evaluated and normalized for varying Chl concentrations using the ESP300 and the Bruker Win-EPR software.

The oxygen evolution was assessed in the presence of 2 mM ferricyanide and 0.2 mM PpBQ as electron acceptors at a Chl concentration of 10 μg/mL at 20 °C in a buffer, containing 20 mM Mes (pH 6.5) and 10 mM NaCl using a

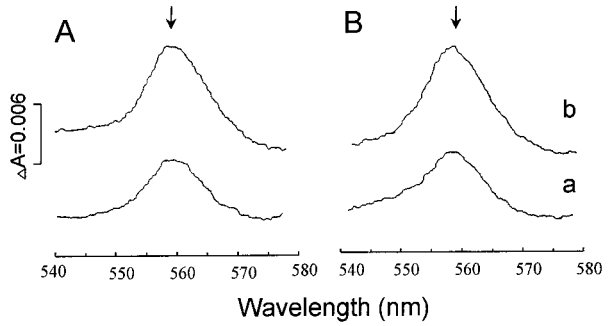

FIGURE 1: Representative redox difference spectra of $Cytb_{559}$ in oxygen-evolving PSII membranes under (A) aerobic and (B) anaerobic conditions. Difference spectra are shown as follows: (a) hydroquinone (8 mM) minus ferricyanide (2 mM) (representing the HP form of $Cytb_{559}$) and (b) dithionite (10 mM) minus ferricyanide (2 mM) (representing the total $Cytb_{559}$). The anaerobic conditions were established by the glucose oxidase/catalase/glucose system (see Materials and Methods).

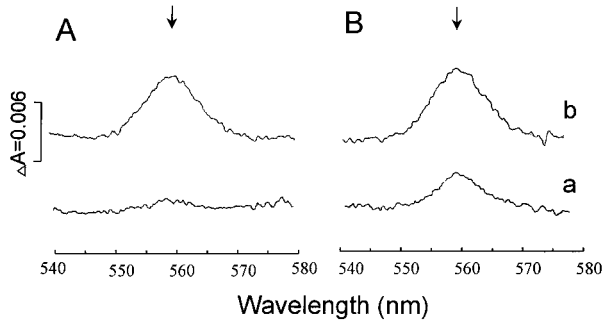

FIGURE 2: Representative redox difference spectra of $Cytb_{559}$ in Tris-treated PSII membranes under (A) aerobic and (B) anaerobic conditions (created as described in the legend of Figure 1). Difference spectra are shown as follows: (a) hydroquinone (8 mM) minus ferricyanide (2 mM) (representing the HP form of $Cytb_{559}$) and (b) dithionite (10 mM) minus ferricyanide (2 mM) (representing the total $Cytb_{559}$).

Clark type electrode. The absence of the peripheral proteins was examined by electrophoresis according to the method of ref *43*, followed by staining of gels according to the method of ref *44*.

Anaerobic conditions were created by N_2 flushing or by the addition of glucose oxidase (75 units/mL), catalase (1500 units/mL), and glucose (50 mM). In the EPR measurements, the concentrations of these reactants were doubled.

RESULTS

The starting material for oxygen-evolving PSII membrane fragments (activity of >400 μmol of O_2/mg of Chl *h*) contained 50−65% of the HP form of $Cytb_{559}$ under aerobic conditions. This value is typical for this kind of PSII preparation. Under anaerobic conditions, the amount of the HP form slightly decreased by about 10% (Figure 1). A markedly different feature, however, is observed after Tris washing of the PSII membrane fragments. These samples do not evolve oxygen, contain less than one manganese per PSII, and are deprived of the three extrinsic subunits of 33, 23, and 16 kDa (not shown). Figure 2 shows difference spectra of the hydroquinone minus ferricyanide and dithionite minus ferricyanide samples measured under both aerobic and anaerobic conditions. The amounts of the HP, IP, and VLP forms of $Cytb_{559}$ gathered from the spectral data are compiled in Table 1. An inspection of these results reveals

Table 1: Different Thermodynamic Forms of $Cytb_{559}$ (Percentage of the Total $Cytb_{559}$) in Tris-Treated PSII Membranes under Various Conditions[a]

$Cytb_{559}$	aerobic	anaerobic[e]	anaerobic and 2 mM $K_3Fe(CN)_6$	anaerobic and 0.5 mM K_2IrCl_6
HP[b]	21.8 (14−27)	54.3 (48−65)	48.4 (46−49)	31.3 (27−33)
IP and LP[c]	25.6 (24−27)	11.0 (10−12)	37.3 (35−39)	6.2 (5−7)
VLP[d]	52.6 (48−54)	34.7 (24−46)	14.3 (10−15)	62.0 (60−66)

[a] The data represent the average of eight independent measurements in different preparations (numbers in parentheses indicate the variations between experiments). [b] HP $Cytb_{559}$ was estimated from the hydroquinone (8 mM) minus ferricyanide (2 mM) difference optical spectrum. [c] IP and LP forms of $Cytb_{559}$ were estimated from (ascorbate minus ferrycyanide) minus (hydroquinone minus ferricyanide) difference spectra. [d] The VLP form of $Cytb_{559}$ was estimated from (dithionite minus ferricyanide) minus (ascorbate minus ferricyanide) difference spectra. [e] Anaerobic conditions were established by the addition of glucose oxidase (75 units/mL), catalase (1500 units/mL), and glucose (50 mM).

that after Tris washing most of the $Cytb_{559}$ was converted into the LP form and the samples contained only about 20% (14−27%, Table 1) of the HP form under aerobic conditions. A very interesting feature emerges when O_2 is removed from the suspension. In this case, the amount of the HP form increased significantly to about 54% of the total $Cytb_{559}$ (see Table 1). Concomitantly, the fraction of the IP and LP forms decreased significantly by almost 15%. The amount of the VLP form also decreased under anaerobic conditions by about 18%. Thus, the removal of O_2 resulted in the conversion of $Cytb_{559}$ into the HP form in a large fraction of PSII. This conversion occurred in the dark which shows that PSII photochemistry was not involved. Another interesting observation in these experiments is that the amount of hydroquinone-reducible $Cytb_{559}$ was dependent on the concentration of hydroquinone. As much as 8 mM hydroquinone was required to reduce all HP $Cytb_{559}$ (not shown).

Furthermore, comparison of Figures 1 and 2 reveals that there is about 30% less $Cytb_{559}$ in the Tris-washed membranes (compare spectra b in Figures 1 and 2). Such a decrease (20−25%) was observed earlier in PSII complexes from *Synechocystis* (*16*), and it was suggested that Tris washing seemingly labilizes some $Cytb_{559}$ (*16*).

The optical measurements were always performed in the presence of reductants (hydroquinone, ascorbate, or dithionite). It has recently been reported that PSII redox chemistry might induce a potential shift of $Cytb_{559}$ (*26*). Therefore, another method has to be used which permits a separation of effects caused by addition of hydroquinone from those that are due to removal of O_2. EPR spectroscopy appears to be the most promising tool because it allows measurements in the absence of hydroquinone. Furthermore, this method has an additional advantage in that low-temperature EPR spectra from oxidized $Cytb_{559}$ (*10*, *12*) exhibit g_z peaks which are characterized by slightly different g values for the HP ($g \approx 3.05$) and LP forms ($g \approx 2.97$).

Figure 3 shows the EPR spectra of Tris-washed PSII membrane fragments, recorded under aerobic and anaerobic conditions in the presence and absence of hydroquinone. The spectrum of dark-adapted Tris-washed PSII membrane fragments under aerobic conditions exhibits a large peak from $Cytb_{559}$ at $g \approx 2.96$ which mainly represents the oxidized state of the dominating LP form. Under anaerobic conditions, the amount of oxidized $Cytb_{559}$ is diminished (Figure 3A,

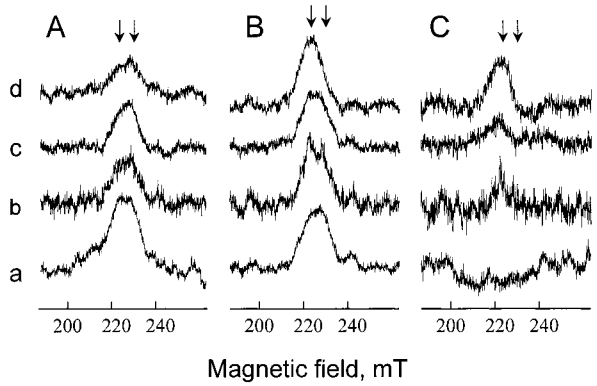

FIGURE 3: EPR spectra of the oxidized $Cytb_{559}$. (A) Spectra recorded in the dark, (B) spectra recorded after illumination for 10 min at 77 K, and (C) difference spectra between A and B (times 2): (a) aerobic, (b) anaerobic, (c) aerobic and 8 mM hydroquinone, and (d) anaerobic and 8 mM hydroquinone. The spectra were normalized for Chl concentration and tube factors. The arrows denote $g = 3.03$ (left) and $g = 2.96$ (right) positions where the spectra represent high- and low-potential $Cytb_{559}$, respectively.

Table 2: Amount of Oxidized $Cytb_{559}$ (Perecentage of the Total Measured after Illumination at 77 K) in Tris-Treated PSII Membranes before and after Illumination at 77 K As Determined by Single Integration of the g_z Peak of the EPR Spectra[a]

conditions	dark	after illumination at 77 K	light minus dark
aerobic[b] (a)	100	100	0
anaerobic[b] (b)	75	100	25
aerobic and hydroquinone[b] (c)	66	100	34
anaerobic and hydroquinone[b] (d)	37	100	63

[a] The error was estimated to be ±5% after three different measurements. [b] For a−d, the EPR spectra are shown in Figure 2.

spectrum b). A similar effect is observed after addition of 8 mM hydroquinone to the aerobic sample (Figure 3A, spectrum c). The most pronounced decrease of about 60% is achieved after addition of 8 mM hydroquinone to the anaerobic sample (Figure 3, spectrum d). The latter result is in good agreement with the LP → HP shift derived from the optical measurements (comparative data in Tables 1 and 2).

Reduced $Cytb_{559}$ can be photooxidized at low temperatures (for an explanation of the photochemistry involved, see ref *45*). Therefore, the spectra recorded after illumination for 10 min at 77 K reflect the total amount of oxidizable $Cytb_{559}$ in the samples. As a consequence, the illuminated minus dark difference spectra represent a measure of the amount of photooxidized HP $Cytb_{559}$. In the aerobic samples, illumination under 77 K conditions did not result in any signal increase (Figure 3B, spectrum a). This finding is indicative of a very small (if any) content of reduced $Cytb_{559}$ which is below the detection limit in the EPR difference spectra between the illuminated and dark-adapted samples. It has to be emphasized that traces a were recorded in aerobic samples in the absence of hydroquinone. Therefore, it can be concluded that in Tris-washed PSII membrane fragments almost all of the $Cytb_{559}$ attains the LP form in the presence of O_2 and absence of reductants. It can thus to be concluded that the small fraction of HP $Cytb_{559}$ detected by optical spectroscopy (14−27%; see Table 1) was caused by a partial LP → HP conversion due to the presence of hydroquinone in the optical measurement samples. This conclusion is in qualitative agreement with the EPR data (Figure 3A, curve c).

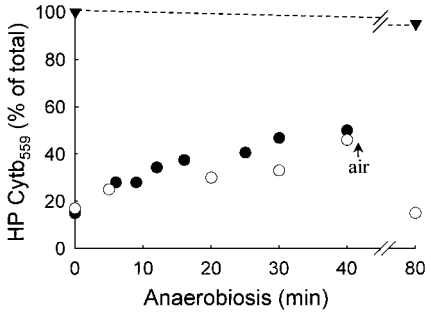

FIGURE 4: Time dependence of the reversible conversion of the LP form of $Cytb_{559}$ to the HP form after the removal of O_2 by glucose oxidase (75 units/mL), catalase (1500 units/mL), and glucose (50 mM) (●) or by N_2 (○). After 40 min, air was introduced in the system where O_2 was removed by N_2 flushing. The total amount of $Cytb_{559}$ (▼) was determined under aerobic conditions from dithionite minus ferricyanide difference spectra. The amount of HP $Cytb_{559}$ was determined from the difference spectrum of hydroquinone (8 mM) minus ferricyanide (2 mM) and is plotted as a percentage of the total $Cytb_{559}$.

After removal of O_2 from the suspension, a clearly resolved difference is observed between the spectra of illuminated and dark samples (Figure 3C, spectrum b) which can be ascribed to a dark conversion of 25−30% of $Cytb_{559}$ into the reduced HP form because the latter is not EPR detectable (Figure 3A, spectrum b). This finding shows that the simple removal of oxygen from the suspension gives rise to partial conversion of 25% of $Cytb_{559}$ into the HP form. To analyze the effect of hydroquinone, experiments were performed with aerobic and anaerobic samples. In the former case, addition of hydroquinone reduced 34% of $Cytb_{559}$, whereas in the latter case, up to about 60% of the $Cytb_{559}$ was transformed (Figure 3, spectra c and d, and Table 2). Independent support for the conclusion of a LP → HP $Cytb_{559}$ transformation induced either by removal of oxygen alone or by addition of hydroquinone comes from the finding that the peak of the light minus dark EPR difference spectra is at $g \approx 3.03$ (see Figure 3C) which is typical for the oxidized form of HP $Cytb_{559}$.

Taken together, the optical and EPR experiments led to a consistent picture. $Cytb_{559}$ is almost entirely in the LP form in dark-adapted aerobic Tris-washed PSII membranes. Removal of O_2 leads to conversion of about 25% of the $Cytb_{559}$ to the HP form, and the addition of 8 mM hydroquinone causes a further increase of about 35% in the HP form.

The following question then arises: how fast do these transitions occur, and are they reversible? To investigate the kinetics of reversible LP ↔ HP $Cytb_{559}$ conversion, the following experiments were performed. The initial amount of the HP form of Tris-washed PSII membrane fragments was measured under aerobic conditions in the presence of 4 mM hydroquinone. It was found (in this experiment) that approximately 15% of the $Cytb_{559}$ attained the HP form (see Figure 4, time zero). Then anaerobic conditions were created in the same cuvette by the addition of glucose oxidase, catalase, and glucose and spectra recorded at different times after the removal of O_2. The results depicted in Figure 4 show that the level of the HP form of $Cytb_{559}$ slowly increased and reached about 50% of the total $Cytb_{559}$ after incubation for 40 min in the dark. To exclude the possibility that this effect is specific for the catalase/glucose/glucose oxidase system, comparative experiments were carried out

where anaerobic conditions were created by flushing the sample with N_2 in the absence of hydroquinone (Figure 4, ○). Also in this case, the amount of the HP form increased, and after 40 min, a level of about 50% of the total $Cytb_{559}$ was reached (the total content of $Cytb_{559}$ did not change in this experiment as indicated by the inverted triangles in Figure 4). The reversibility of the LP ↔ HP transformation was studied in an experiment where at first the HP form of $Cytb_{559}$ was enriched by the removal of O_2 (through N_2 flushing) and subsequently the suspension was saturated with air. The results depicted in Figure 4 (○) show that after incubation for 40 min with air the initial ratio of LP to HP $Cytb_{559}$ was re-established. On the basis of these findings, the LP ↔ HP form conversion is inferred to be slow but fully reversible when switching between aerobic and anaerobic conditions took place.

Apart from removing O_2 from the suspension, a partial conversion of the LP form into the HP form can also be induced by the addition of a reducing agent. This idea is supported by the observation that a doubling of the hydroquinone concentration from 4 to 8 mM increased the amount of HP $Cytb_{559}$ by 8−10 and 15−20% under aerobic and anaerobic conditions, respectively (data not shown). Accordingly, the question about a possible opposite effect of oxidants arises. The results that were obtained are summarized in Table 1. They reveal that after incubation of anaerobic samples with 2 mM ferricyanide or 0.5 mM iridate the fraction of HP $Cytb_{559}$ was lower (48 and 31%, respectively) than in the absence of the oxidants (54%). This shift was accompanied by a corresponding increase of the LP and VLP forms. It has to be emphasized that the ambient redox potential of the suspension after addition of 8 mM hydroquinone was the same in both cases (≈250 mV); i.e., the observed changes originate neither from a change of the available amount of reduced hydroquinone in the measurements (note that the optical measurements shown in Table 1 were performed in the presence of 8 mM hydroquinone) nor from a higher redox potential in the medium. The latter suggestion is supported by the fact that in suspensions containing either ferricyanide or iridate the addition of ascorbate and dithionite shifted the ambient redox potential down to 225 and −250 mV, respectively. The amounts of the IP plus LP and VLP forms strongly differ in both cases (see Table 1, columns 3 and 4). The fact that oxidizing conditions lead to conversion of HP into the LP form is in agreement with redox titration measurements of $Cytb_{559}$ performed in ref *24*. The content of HP $Cytb_{559}$ in PSII membranes was different at the same E_m, depending crucially on the direction in which the titration was performed. When $Cytb_{559}$ was maintained in the reduced state, it was present in the HP form, but after oxidation, some conversion into the LP forms occurred. In addition, it was shown (*46*) that rapid oxidation of $Cytb_{559}$ and its change from the HP form to the LP form take place during photoinhibition.

DISCUSSION

The results of this study reveal two striking and new properties of $Cytb_{559}$: (i) in the dark $Cytb_{559}$ undergoes reversible LP ↔ HP form transitions which are induced either by a switch between aerobic and anaerobic conditions or by addition of reductants and oxidants, respectively, and (ii) these features are not observed in PSII membranes with a functionally competent WOC but only arise after Tris washing of the sample, thus leading to WOC loss. These findings raise questions about the molecular mechanism and the functional role of these transitions.

With respect to the mechanism, it is important to note the LP → HP $Cytb_{559}$ conversion takes place in the dark. Therefore, this process seems to be different from that described in ref *26*, where the level of the HP form in Tris-washed PSII membrane fragments was increased by illumination (continuous or flash light) in the presence of exogenous electron donors such as Mn^{2+}. In addition, the presence of only 0.72 Mn per PSII in our Tris-washed particles makes it unlikely that Mn can donate electrons to PSII to any substantial extent.

It has also been suggested that the E_m of $Cytb_{559}$ is linked to the redox state of Q_A (*12*) and conversion of LP $Cytb_{559}$ into the HP form might be linked to redox reactions on the acceptor side of PSII involving the generation of Q_A^- under illumination. Q_A is more easily reduced under anaerobic conditions. However, a key role of Q_A^- seems to be unlikely as an explanation of our results. The E_m of Q_A was recently shown to depend on treatments which influence the donor side of PSII, on redox mediators, on freezing and thawing of PSII membranes, etc. Redox titrations of Q_A lead to values in the range from −300 to 120 mV (see the discussion in ref *47* and references therein). We measured the content of the HP form of $Cytb_{559}$ under anaerobic conditions in the presence of 8 mM hydroquinone. In this case, the redox potential of the medium was about 230 mV. Thus, Q_A cannot be chemically reduced, and we can consequently exclude that the redox state of Q_A was changed in the dark. Nevertheless, we observed a marked difference in the content of the HP form of $Cytb_{559}$ under aerobic and anaerobic conditions (see Table 1).

Thus, it appears to be reasonable to conclude that the conversion of LP into HP $Cytb_{559}$ does not necessarily depend on the presence of exogenous electron donors, on electron transport through PSII, or on the generation of Q_A^-. Also, the ligation of Ca^{2+} at the lumenal surface of PSII (*24*) can be excluded because Ca^{2+} was omitted in our experiments. Therefore, another mechanism has to be considered for the conversion of LP into HP $Cytb_{559}$. It has been proposed that a molecular switch between different redox forms of $Cytb_{559}$ is determined by interactions with protons (*48*−*50*). On the basis of the correlation between pH and the relative levels of the HP and LP forms of $Cytb_{559}$, the interconversion was inferred to be linked to protonation and deprotonation reactions (*42*). This idea is supported by measurements in the presence of protonophores (*51*−*53*). With respect to a possible influence of protons, it is interesting to note that according to ref *54* the HP state is most likely characterized by a hydrogen bond between an NH of each histidine and a peptide bond carbonyl of each helix of the dimeric protein matrix of the heme group. In the LP form, one of these bonds is probably absent. Therefore, it appears to be attractive to speculate that $Cytb_{559}$ in Tris-washed samples becomes susceptible to an interaction with oxygen which gives rise to the break of one hydrogen bond and transformation into the LP form. Accordingly, removal of oxygen restores this hydrogen bond and as a consequence the HP form. In a similar way, hydroquinone, supplying electrons and protons, can facilitate the protonation of carboxyl group(s) and the

restoration of hydrogen bonds. The absence of O_2 creates favorable conditions for such a reaction. If the hydroquinone concentration was doubled (from 4 to 8 mM), the amount of the HP form increased under aerobic and anaerobic conditions by 8−10 and 15−20%, respectively (data not shown).

For considerations about the possible physiological role of this O_2-dependent interconversion of the $Cytb_{559}$, it is important to keep in mind that this effect was not observed in PSII membrane fragments with a functionally competent WOC. Furthermore, PSII complexes lacking the WOC are especially prone to photoinhibition at the PSII donor side (*55*−*57*). Presently, we see two possibilities for the fraction of the LP ↔ HP conversion: (i) it has a protective function against photoinhibition or (ii) it participates in the regulation of the content of PSII with a fully competent WOC.

In the first case, as a result of photoinhibition, the HP form converts into the LP form (*46*) which can accept electrons from the acceptor side (*35*, *37*, *58*) and protons from the local environment. Under aerobic conditions, O_2 is able to intercept electrons from $Pheo^-$ or reoxidize $Cytb_{559}$. In PSII complexes containing a damaged WOC, the microenvironment of $Cytb_{559}$ could attain an anaerobic state, thus facilitating protonation and switching into the HP form. This process leads to reduction of HP $Cytb_{559}$ which becomes now available as an electron donor to $P680^+$ (*59*). As a consequence, the population probability of the harmful radicals $P680^+$ and Y_Z^{ox} will be diminished and in this way partial protection from irreversible photoinhibition at the level of P680 is established (*34*). In this respect, it is worth mentioning that the rate constant for the photooxidation of $Cytb_{559}$ in Mn-depleted PSII is much faster (50 s^{-1}) than in O_2-evolving PSII [0.9 s^{-1} (*13*, *50*, *59*)].

In the second case, the interconversion of the $Cytb_{559}$ forms could be important for the recovery of PSII during stress conditions (temperature, salt, and light) via regulation of the content of PSII with an intact WOC. The LP form is localized in inactive PSII centers which are mainly in stroma lamellae, while the HP form dominates in active centers in the granal part of the thylakoid membrane (*60*). Under stress conditions, the population of inactive PSII complexes with the LP form is increasing and concentrates in the stroma region. Since these PSII complexes do not evolve oxygen, the level of O_2 around $Cytb_{559}$ might decrease and conditions close to anaerobiosis could arise, thus leading to transformation of the LP form into the HP form. It should be noted that this transformation is also stimulated by the reduction of the LP $Cytb_{559}$ from the acceptor side (*37*). The conversion of the LP form to the HP form might then induce the activation of WOC. In subsequent steps, the linear electron transport through PSII recovers and PSII centers begin to accumulate in the grana region. The idea that the LP → HP $Cytb_{559}$ conversion is involved in PSII recovery is in agreement with recent photoactivation experiments (*26*) where the Mn cluster was shown to be reconstituted only after the restoration of the HP form. The details of this mechanism remain to be unravelled in future studies.

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REFERENCES

1. Hermann, R. G., Alt, J., Schiller, B., Widger, W. R., and Cramer, W. A. (1984) *FEBS Lett. 176*, 239−244.
2. Widger,. W. R., Cramer, W. A., Hermodson, M., and Herrmann, R. G. (1985) *FEBS Lett. 191*, 186−190.
3. Pakrasi, H. B., Williams, J. G. K., and Arntzen, C. J. (1988) *EMBO J. 7*, 325−332.
4. Kurreck, J., Garbers, A., Parak, F., and Renger, G. (1997) *FEBS Lett. 403*, 283−286.
5. Nanba, O., and Satoh, K. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 109−112.
6. Gounaris, K., Chapman, D. J., and Barber, J. (1989) *Biochim. Biophys. Acta 973*, 269−301.
7. Garewal, H. S., and Wasserman, A. R. (1974) *Biochemistry 13*, 4063−4071, 4072−4079.
8. Widger, W. R., Cramer, W. A., Hermodson, M., Meyer, D., and Gullifor, M. (1984) *J. Biol. Chem. 259*, 3870−3876.
9. Whitmarsh, J., and Ort, D. R. (1984) *Arch. Biochem. Biophys. 231*, 378−389.
10. Thompson, L. K., Miller, A. F., Buser, C. A., De Paula, J. C., and Brudvig, G. W. (1989) *Biochemistry 28*, 8048−8056.
11. Whitmarsh, J., and Pakrasi, H. B. (1996) in *Oxygenic Photosynthesis: The Light Reactions* (Ort, D. R., and Yocum, C. F., Eds.) Advances in Photosynthesis, Vol. 4, pp 249−264, Kluwer Academic Publishers, Dordrecht, The Netherlands.
12. Stewart, D. H., and Brudvig, G. W. (1998) *Biochim. Biophys. Acta 1367*, 63−87.
13. Irrgang, K.-D., Lekauskas, A., Franke, P., Reifarth, F., Smolian, H., Karge, M., and Renger, G. (1998) in *Photosynthesis: Mechanisms and Effects* (Garab, G., Ed.) Vol. 2, pp 977−980, Kluwer Academic Publishers, Dordrecht, The Netherlands.
14. Tang, X. S., and Diner, B. A. (1994) *Biochemistry 33*, 4594−4603.
15. Vacha, F., Joseph, D. M., Durrant, J. R., Telfer, A., Klug, D. R., Porter, G., and Barber, J. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 2929−2933.
16. MacDonald, G. M., Boerner, R. J., Everly, R. M., Cramer, W. A., Debus, R. J., and Barry, B. A. (1994) *Biochemistry 33*, 4393−4400.
17. Bricker, T. M., Morvant, J., Masri, N., Sutton, H. M., and Frankel, L. K. (1998) *Biochim. Biophys. Acta 1409*, 50−57.
18. Cramer, W. A., Theg, S. M., and Widger, W. R. (1986) *Photosynth. Res. 19*, 393−403.
19. Babcock, G. T., Widger, W. R., Cramer, W. A., Oertling, W. A., and Metz, J. G. (1985) *Biochemistry 24*, 3638−3645.
20. McNamara, V. P., Sutterwala, F. S., Pakrasi, H. B., and Whitmarsh, J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 14173−14178.
21. Francke, C., Loyal, R., Ohad, I., and Haehnel, W. (1999) *FEBS Lett. 442*, 75−78.
22. Cramer, W. A., and Whitmarsh, J. (1977) *Annu. Rev. Plant Physiol. 28*, 133−172.
23. Shuvalov, V. A. (1994) *J. Bioenerg. Biomembr. 26*, 619−626.
24. McNamara, V. P., and Gounaris, K. (1995) *Biochim. Biophys. Acta 1231*, 289−296.
25. Matsuda, H., and Butler, W. L. (1983) *Biochim. Biophys. Acta 725*, 320−324.
26. Mizusawa, N., Miyao, M., and Yamashita, T. (1997) *Biochim. Biophys. Acta 1318*, 145−158.
27. Mizusawa, N., Ebina, M., and Yamashita, T. (1995) *Photosynth. Res. 45*, 71−77.
28. Völker, M., Renger, G., and Rutherford, A. W. (1986) *Biochim. Biophys. Acta 851*, 424−430.
29. Larsson, C., Jansson, C., Ljungberg, H. E., Åkerlund, B., and Andersson, B. (1984) in *Advances in photosynthesis research* (Sybesma, C., Ed.) Vol. 1, pp 363−366, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands.

30. Briantains, J. M., Vernotte, C., Miyao, M., Murata, N., and Picaud, M. (1985) *Biophim. Biophys. Acta 808*, 348−351.
31. Shuvalov, V. A., Schreiber, U., and Heber, U. (1994) *FEBS Lett. 337*, 226−230.
32. Fiege, R., Schreiber, U., Renger, G., Lubitz, W., and Shuvalov, V. A. (1995) *FEBS Lett. 377*, 325−329.
33. Hulsebosch, R. J., Hoff, A. J., and Shuvalov, V. A. (1996) *Biochim. Biophys. Acta 1277*, 103−106.
34. Thompson, L. K., and Brudvig, G. W. (1988) *Biochemistry 27*, 6653−6658.
35. Nedbal, L., Samson, G., and Whitmarsh, J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 7929−7933.
36. Magnusson, A., Rova, M., Mamedov, F., Fredriksson, P.-O., and Styring, S. (1999) *Biochim. Biophys. Acta 1411*, 180−191.
37. Barber, J., and De Las Rivas, J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10942−10946.
38. Ananyev, G., Renger, G., Wacker, U., and Klimov, V. (1994) *Photosynth. Res. 41*, 327−338.
39. Schweitzer, R. H., and Brudvig, G. W. (1997) *Biochemistry 36*, 11351−11359.
40. Berthold, D. A., Babcock, G. T., and Yocum, C. A. (1981) *FEBS Lett. 134*, 231−234.
41. Völker, M., Ono, T., Inoue, Y., and Renger, G. (1985) *Biochim. Biophys. Acta 806*, 25−34.
42. De Las Rivas, J., Klein, J., and Barber, J. (1995) *Photosynth. Res. 46*, 193−202.
43. Laemmli, U. K. (1970) *Nature 227*, 680−685.
44. Oakley, B. R., Kirsch, D. R., and Morris, R. N. (1980) *Anal. Biochem. 105*, 361−363.
45. Miller, A. F., and Brudvig, G. W. (1991) *Biochim. Biophys. Acta 1056*, 1−18.
46. Styring, S., Virgin, I., Ehrenberg, A., and Andersson, B. (1990) *Biochim. Biophys. Acta 1015*, 269−278.
47. Krieger, A., Rutherford, A. W., and Johnson, G. N. (1995) *Biochim. Biophys. Acta 1229*, 193−201.
48. Losada, M., Hervas, M., De Las Rosa, M. A., and De La Rosa, F. F. (1983) *Bioelectrochem. Bioenerg. 11*, 193−230.
49. Ortega, J. M., Hervas, M., and Losada, M. (1990) *Plant Sci. 68*, 71−75.
50. Buser, C. A., Diner, B. A., and Brudvig, G. W. (1992) *Biochemistry 31*, 11449−11459.
51. Ortega, J. M., Hervas, M., and Losada, M. (1988) *Eur. J. Biochem. 171*, 449−455.
52. Arnon, D. I., and Tang, G. M. S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9524−9528.
53. Barabas, K., Kravkova, T., and Garab, G. (1993) *Photosynth. Res. 36*, 59−64.
54. Berthomieu, C., Boussac, A., Mäntele, W., Breton, J., and Nabedryk, E. (1992) *Biochemistry 31*, 11460−11471.
55. Eckert, H.-J., Geiken, B., Bernarding, J., Napiwotzki, A., Eichler, H.-J., and Renger, G. (1991) *Photosynth. Res. 27*, 97−108.
56. Chen, G. X., Kazimir, J., and Cheniae, G. M. (1992) *Biochemistry 31*, 11072−11083.
57. Jegerschöld, C., Virgin, I., and Styring, S. (1990) *Biochemistry 29*, 6179−6186.
58. Poulson, M., Samson, G., and Whitmarsh, J. (1995) *Biochemistry 34*, 10932−10938.
59. Buser, C. A., Thompson, L. K., Diner, B. A., and Brudvig, G. W. (1990) *Biochemistry 29*, 8977−8985.
60. Chylla, R. A., Garab, G., and Whitmarsh, J. (1987) *Biochim. Biophys. Acta 894*, 562−671.

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