Redox Changes of Cytochrome b$_{559}$ in the Presence of Plastoquinones*

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We have found that short chain plastquinones effectively stimulated photoreduction of the low potential form of cytochrome b$_{559}$ and were also active in dark oxidation of this cytochrome under anaerobic conditions in Triton X-100-solubilized photosystem II (PSII) particles. It is also shown that molecular oxygen competes considerably with the prenylquinones in cytochrome b$_{559}$ oxidation under aerobic conditions, indicating that both molecular oxygen and plastquinones could be electron acceptors from cytochrome b$_{559}$ in PSII preparations. α-Tocopherol quinone was not active in the stimulation of cytochrome photoreduction but efficiently oxidized it in the dark. Both the observed photoreduction and dark oxidation of the cytochrome were not sensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea. It was concluded that both quinone-binding sites responsible for the redox changes of cytochrome b$_{559}$ are different from either the QA or QB site in PSII and represent new quinone-binding sites in PSII.

Cytochrome b$_{559}$ (cyt b$_{559}$) is an integral component of the photosystem II (PSII) reaction center (RC) complex, whose function has been the subject of very extensive studies within recent years (1, 2). It was found that it has a crucial role in PSII assembly, and its electron transport properties are attributed to protection of PSII against photoinhibition (1, 3–5). It is found in thylakoid membranes and in PSII preparations in high potential (HP) and low potential (LP) forms differing in redox potentials ($E_m = 330–400$ and $20–80$ mV, respectively) (2), and both these forms are believed to be at least partially interconvertible (1, 4, 6). In native, fully functional PSII complexes, the LP form is probably a minor component whose levels increase under photoinhibitory conditions. Under strong light illumination (acceptor side inhibition), the LP form of cyt b$_{559}$ was found to accept electrons from the photoreduced phytoquinin, preventing PSII from photoinhibition (3, 5, 7). However, the natural electron acceptor from the cyt b$_{559}$ LP form is unknown. Among the potential candidates, plastoquinone and menaquinone were considered to be possible candidates by Kruk and Strzalka in their previous studies (16). Here, we report on the role of plastquinones in the redox changes of cyt b$_{559}$ under photoinhibitory conditions.

In the present study, we have performed measurements of the photoreduction and dark oxidation of the cyt b$_{559}$ LP form in Triton X-100-solubilized PSII particles under the influence of different plastquinone homologues in anaerobic and aerobic conditions. We have also included for these studies α-tocopherol quinone (α-TQ), a natural thylakoid prenylquinin of unknown function in plants (12) that is an effective quencher of PSII fluorescence (13) and also affects the cyclic electron flow around PSII (14).

MATERIALS AND METHODS

PSII particles were isolated according to the method of Berthold et al. (15). PQ-1-PQ-4 homologues were a kind gift of H. Koike, PQ-9 was a gift from Hoffmann-La Roche, PQ-decyl was from Sigma, and vitamin K$_3$ (menadione) was from Aldrich. α-TQ was prepared from α-tocopherol as described by Kruk (16). Menaquinone (K$_3$H$_{10}$, MD) was obtained by reduction of menadione with NaBH$_4$ in methanol, and its concentration was determined using the millimolar extinction coefficient $\varepsilon = 20.1$ at 245 nm in ethanol. Plastoquinone concentrations were calculated using $\varepsilon_{559} = 17.94$ m$m^2$/cm$^2$ (17) in absolute ethanol. All measurements of cyt b$_{559}$ redox changes on PSII particles were made in 50 mM Hepes buffer, pH 7.5, 10 mM KCl, and 5 mM MgCl$_2$ at a chlorophyll concentration of 50 µg/ml and in the presence of 0.2% (w/v) Triton X-100. Additionally, photoreduction of cyt b$_{559}$ was measured in the presence of 1 mM MnCl$_2$ as an electron donor to PSII. The cyt b$_{559}$ concentration was determined using $A_{560-770} = 20$ m$m^2$/cm$^2$ (18). Unless otherwise indicated, all kinetic measurements were performed under anaerobic conditions using 1% CO$_2$ in the gas phase.
RESULTS

Absorption Spectra of Different cyt $b_{559}$ Forms—To observe only redox changes of the LP form of cyt $b_{559}$ in PSII particles and to avoid possible absorption changes due to the HP form, we have performed our measurements in the presence of Triton X-100, which is known to convert the HP form into the LP form (19). This detergent was also found to increase the ascorbate-reducible fraction of the cyt $b_{559}$ as compared with the dithionite-reducible cyt $b_{559}$ in PSII RC (20), probably by disaggregation of the RC preparations, which in turn improves penetration of added redox compounds to the cytochrome. This detergent should also increase the solubility of the added hydrophobic prenylquinones. The difference absorption spectra in the $\alpha$-band region of cyt $b_{559}$ absorption show (Fig. 1) that our PSII preparation solubilized in 0.2% Triton X-100 does not contain any detectable cyt $b_{559}$ HP redox form, whereas the ascorbate reduces the cytochrome to practically the same level as that produced by MD, which is a stronger reductant than ascorbate. Dithionite reduced the cytochrome to the same level as that produced by MD and ascorbate (data not shown), indicating that Triton X-100-solubilized PSII particles contain only the low-potential, totally ascorbate-reducible cyt $b_{559}$ form. The presence of Triton X-100 also improves the optical properties of the sample.

Pho†oreduction of cyt $b_{559}$ in the Presence of Plastoquinones—Because the Triton X-100 treatment inactivates the oxygen-evolving complex, 1 mM MnCl$_2$ was added to all light-treated samples as an electron donor to PSII particles. Fig. 2 shows that under anaerobic conditions in the presence of even very low PQ-2 concentration (1.73 mol of PQ-2/mol of cyt $b_{559}$), the cytochrome is photoreduced at a fast rate and oxidized back to the original level after turning off the light. The second illumination of the same sample gives both faster photooxidation and photoreduction rates. Interestingly, the same sample in the presence of ambient oxygen shows no detectable redox changes of the cytochrome. This might indicate that the cytochrome is oxidized so quickly by molecular oxygen that there is no detectable steady-state level of the reduced cytochrome. The control sample, with no added quinone, shows almost no redox changes of the cytochrome. PQ-9 was also active in catalyzing the photoreduction and subsequent dark oxidation of the cytochrome.
However, much higher amounts of PQ-9 are required to obtain significant levels of the cytochrome photoreduction. Even though the photoreduction is considerably less sensitive to PQ-9 than to PQ-2 in our measurements, it is much more sensitive than in analogous experiments performed on PSII RCs (20), where the cytochrome could be photoreduced to not more than 62% of the total cytochrome present in the preparation and where this saturation was reached at over 300 mol of PQ-9/mol of cyt $b_{559}$. In our case, the reduction level of cyt $b_{559}$ is 65 and 40% for 110 PQ-9 and 55 PQ-9 samples (Fig. 2), respectively. It is also evident for samples with low PQ-9 content (55 PQ-9 and 28 PQ-9) that there is a residual fast photoreduction and dark oxidation absorbance change that is still present in the control sample (with no added quinone) but is not observed under aerobic conditions. These cytochrome redox changes could originate from the residual plastoquinone present in PSII preparations or a low efficient electron transport via cyt $b_{559}$ without the participation of the plastoquinone. In contrast to plastoquinones, α-TQ was not active in stimulation of the cytochrome photoreduction event at high concentrations (Fig. 2). None of the cytochrome redox changes shown in Fig. 2 was inhibited by DCMU, indicating that the Q$_i$ site is not involved in the electron transport to and from cyt $b_{559}$ under our conditions.

Fig. 3 shows that the cytochrome photoreduction rate is proportional to the PQ-2 content and is already observed at very low (substoichiometric) amounts of PQ-2 in relation to cyt $b_{559}$, indicating a very tight binding site of PQ-2 in the vicinity of the cytochrome. The cytochrome is totally reduced already at 30 mol PQ-2/mol of cyt $b_{559}$. In our case, the reduction level of cyt $b_{559}$ is 65 and 40% for 110 PQ-9 and 55 PQ-9 samples (Fig. 2), respectively. It is also evident for samples with low PQ-9 content (55 PQ-9 and 28 PQ-9) that there is a residual fast photoreduction and dark oxidation absorbance change that is still present in the control sample (with no added quinone) but is not observed under aerobic conditions. These cytochrome redox changes could originate from the residual plastoquinone present in PSII preparations or a low efficient electron transport via cyt $b_{559}$ without the participation of the plastoquinone. In contrast to plastoquinones, α-TQ was not active in stimulation of the cytochrome photoreduction event at high concentrations (Fig. 2). None of the cytochrome redox changes shown in Fig. 2 was inhibited by DCMU, indicating that the Q$_i$ site is not involved in the electron transport to and from cyt $b_{559}$ under our conditions.

Dark Autoxidation of cyt $b_{559}$—To analyze the possible oxidation routes of the photoreduced cyt $b_{559}$ (Fig. 2), we used chemical reduction of the cytochrome with a small excess of MD and followed the cytochrome oxidation under molecular oxygen (in air-saturated buffer) and exogenously added prenylquinones. Addition of MD under anaerobic conditions results in fast dark reduction of the cytochrome, which remains reduced for minutes (Fig. 6A). If MD is added under aerobic conditions, after transient reduction, the cytochrome is gradually oxidized by molecular oxygen (Fig. 6B). When the cytochrome is reduced under anaerobic conditions and H$_2$O$_2$ is added, which immediately releases molecular oxygen on contact with the catalase, which is a component of the oxygen trap, the cytochrome oxidation is observed (Fig. 6C). The second H$_2$O$_2$ addition, which should give a 200 μM oxygen concentration in the reaction mixture, causes a higher cytochrome oxidation rate than that under aerobic conditions, where the oxygen concentration should be about 250 μM. The reason for this could be that under the conditions illustrated in Fig. 6B there is an excess of MD left after the cytochrome reduction that slows down its autoxidation. In the case of the reaction illustrated in Fig. 6C, the first H$_2$O$_2$ addition probably at least partially removes the MD excess.
Dark Prenylquinone-mediated Oxidation of cyt b\textsubscript{559}—Fig. 7 illustrates PQ-2-induced dark cytochrome oxidation after its prereduction with MD. PQ-2 induces cytochrome oxidation at rates proportional to the quinone concentration, but under anaerobic conditions the extent of the cytochrome reduction decreases with increasing PQ-2 concentration (Table I). In the presence of oxygen, the cytochrome oxidation rate is considerably increased by the simultaneous cytochrome autoxidation. In this case, the cytochrome is completely oxidized. PQ-9 and α-TQ were also active in the oxidation of cyt b\textsubscript{559} (Fig. 8), but the oxidation rates in the presence of PQ-9 and α-TQ are considerably lower than those caused by PQ-2. In contrast to PQ-2, the oxidation extent is considerably higher for PQ-9 and α-TQ (Table I). The oxidation rates under aerobic conditions are evidently higher, and the rates correspond approximately to the oxidation rates under anaerobic conditions increased by the cytochrome autoxidation rate (Table I). Comparing the oxidation rates during dark periods in the photoreduced samples (Fig. 2) and the rates obtained from Figs. 7 and 8, it can be seen that the rates for the dark cytochrome oxidation obtained from experiments on cyt b\textsubscript{559} prereduced by MD would not be high enough to account for the observed cytochrome oxidation rates prereduced by light at the same quinone concentrations. The reason for this could be partially connected with the excess of MD that remains after the cytochrome reduction. This probably inhibits the oxidation of the cytochrome by the added quinones, which can be deduced from comparison of the ob-

![Fig. 5. Photoreduction of cyt b\textsubscript{559} in the presence of plastoquinones with the different length of the side chain and DBMIB at 1.73 mol of quinone/mol of cyt b\textsubscript{559} under anaerobic conditions. Other conditions were as described in the legends to Fig. 1 and 2.](image)

![Fig. 6. Dark cyt b\textsubscript{559} reduction under the addition of 3.6 μM MD and aerobic conditions (A), anaerobic conditions (B), or anaerobic conditions (C) after additions of H\textsubscript{2}O\textsubscript{2} (△) giving final H\textsubscript{2}O\textsubscript{2} concentrations of 0.4, 0.8, and 4 mM. Other conditions were as described in the legends to Figs. 1 and 2.](image)

**TABLE I**

| Sample | cyt b\textsubscript{559} LP oxidation rate | cyt b\textsubscript{559} LP oxidation extent |
|--------|------------------------------------------|----------------------------------------|
|        | nm/min | %  |                           | nm/min | %  |
| 1.73 PQ-2 (L-D) | 340–500 | 100 |
| 110 PQ-9 (L-D) | 120–200 | 100 |
| 220 PQ-2 | 120 | 30  |
| 110 PQ-2 | 620 | 40  |
| 28 PQ-2 | 200 | 50  |
| 28 PQ-2 + O\textsubscript{2} | 300 | 100 |
| 220 PQ-2 | 95  | 70  |
| 110 PQ-9 | 45  | 65  (n.s.) |
| 110 PQ-9 + O\textsubscript{2} | 100 | 100 |
| 55 PQ-9 | 30  | 60  (n.s.) |
| 220 α-TQ | 95  | 65  (n.s.) |
| 220 α-TQ + O\textsubscript{2} | 170 | 100 |
| control + O\textsubscript{2} | 67  | 100 |
| control + O\textsubscript{2} (H\textsubscript{2}O\textsubscript{2}) | 180 | 65  |
DISCUSSION

The observed effects described above of stimulation of cyt \( b_{559} \) photoreduction by different plastoquinones are similar to those observed with DBMIB, PQ-decyl, or PQ-9 on the isolated PSII reaction centers (9, 10, 20), but in our case the photoreduction proceeds with higher quinone sensitivity and to a higher cytochrome reduction extent. The efficient photoreduction of the cytochrome at very low PQ-2 concentrations (Fig. 3) suggests a very tight binding site close to the cytochrome heme. The question arises as to whether it is one of the known quinone-binding sites in PSII (QA or QB) or another high affinity quinone-binding site in PSII. The lack of DCMU sensitivity of the cytochrome redox changes and the fact that the QB site is detergent-sensitive (23) and easily damaged indicate that the base-line stability and signal to noise ratio were poor in that case (data not shown). Interestingly, the QA reconstitution experiments in PQ-9-depleted PSII core preparations (26) showed that this site shows a high structural specificity, and only PQ-9 or UQ-10 efficiently substituted for QA function, in contrast to PQ-2, DBMIB, or other short chain quinones. A similar specificity, only for PQ-9 or UQ-9, was observed in PSII core complexes where QA was exchanged by 1% Triton X-100-phenanthroline incubation (25). On the other hand, rather low structural quinone specificity is observed for cyt \( b_{559} \) quinone-catalyzed photoreduction (9). These data favor the view that the quinone-binding site active in cyt \( b_{559} \) photoreduction is different from the QA and QB sites. It is also evident that the QA site shows specificity for hydrophobic quinones (PQ-9, UQ-9 or UQ-10), whereas the cyt \( b_{559} \) quinone-binding site shows preference for polar plastoquinones or other artificial quinones, which is connected with the exposure of the heme of the cyt \( b_{559} \) LP form to the polar surroundings.

The question arises as to whether this cyt \( b_{559} \) LP quinone-binding site is occupied and functional in vivo in the native PSII. Some indication may come from analyses of the PQ-9 content of PSII membranes and PSII core complexes. Such analyses usually give about three or more PQ-9 molecules per RC in PSII membranes (27, 28); two of these molecules are strongly bound (non-extractable by hexane) (27), and about two are tightly bound PQ-9 molecules for PSII core complexes (28–30). In both preparations, one molecule is certainly the QA molecule, but the identity of the second PQ-9 in PSII core complexes is not certain (29, 30). Probably it is not a QA molecule, because this site is easily damaged by detergent treatment during the PSII core isolation procedure, and the QB

**Fig. 8.** Dark reduction of cyt \( b_{559} \) under the addition of 3.6 \( \mu \)M MD and the cytochrome oxidation after the addition of different amounts of PQ-9 or \( \alpha \)-TQ at the given quinone/cyt \( b_{559} \) molar ratios. The measurements were performed under anaerobic conditions unless otherwise indicated (+ \( O_2 \)). Other conditions were as described in the legends to Figs. 1 and 2.

**Fig. 9.** A model of the electron transport reactions within PSII showing the main and alternative electron transport pathways protecting PSII against photoinhibition. Bold arrows denote the electron transport route under non-inhibitory conditions in PSII; non-bold arrows indicate the electron transport reactions in isolated and quinone-reconstituted PSII RC complexes and occurring possibly in vivo under acceptor side inhibition (); dashed arrows indicate electron transport routes probably not occurring in isolated PSII RC complexes but taking place in vivo at low yield under non-inhibitory conditions and at higher yield under donor side photoinhibition (dashed line); \((-\rightarrow\) denotes interconversion between the cyt \( b_{559} \) HP and LP forms; and QC and Q are the quinone-binding sites to the cyt \( b_{559} \) LP form, responsible for its photoreduction and oxidation. Pheo, pheophytin; OEC, oxygen-evolving complex.
molecule is lost (23, 28–30). This also would explain poor or no DCMU sensitivity of the electron transport in PSII core complexes (29, 30). It was originally thought that the additional, tightly bound PQ-9 molecule to PSII membranes is Z (27), the primary electron donor to P680, but it turned out that it was tyrosine. This data may suggest that the second, tightly bound PQ-9 molecule in PSII core complexes is the PQ-9 molecule participating in cyt b$_{559}$ photoreduction via the semiquinone form and is different from the QA molecule.

The site of quinone interaction with the cyt b$_{559}$ causing its oxidation seems to be a low affinity quinone-binding site that is also DCMU-insensitive and is probably in contact and exchange with the PQ pool in thylakoid membranes. An argument supporting the concept of different photoreduction and dark oxidation sites of cyt b$_{559}$ LP is the inefficiency of $\alpha$-TQ in the stimulation of the cytochrome photoreduction; but at the same concentration $\alpha$-TQ efficiently oxidizes the reduced cytochrome.

The model of electron transport pathways in PSII showing the PQ-binding sites (QA and Q$_b$) together with the high (QC) and low (Q) affinity PQ-binding sites involved in cyt b$_{559}$ redox changes is shown in Fig. 9. The possible physiological role of the quinone-stimulated cyt b$_{559}$ photoreduction (via the QC site) and its reoxidation (via the Q site) could be the acceleration of the electron transport from plastoquinone to the PQ pool or to molecular oxygen via cyt b$_{559}$ LP upon acceptor side inhibition (Fig. 9) when the electron route via QA$\rightarrow$Q$_b$$\rightarrow$PQ pool is saturated or inhibited and electron donation to P680 from the oxygen-evolving complex is active. When PSII is fully functional, electron transport via QA$\rightarrow$Q$_b$ dominates, and the cyt b$_{559}$ exists mainly in the HP form, which is kept reduced by the plastoquinol of the PQ pool. Under donor side inhibition, P680 is reduced by the cyt b$_{559}$ HP form via the redox active Chl$Z$ molecule (1). Moreover, a $\beta$-carotene molecule probably mediates electron transfer between Chl$_Z$ and P680 (31, 32). The hypothetical molecular switch transforms the HP to the LP form, which is further oxidized by molecular oxygen or a neighboring PQ molecule (Q in Fig. 9), and the oxidized LP form is no longer able to donate electrons to P680. Then, Chl$Z$ is oxidized, giving Chl$Z^{-}$, which effectively quenches the excitation energy, preventing the photosynthetic apparatus against overexcitation (1). The electron route via QC upon acceptor side inhibition also requires the HP$\rightarrow$LP molecular switch to give a sufficient cyt b$_{559}$ LP level through which electrons could outflow from plastoquinol via QC and the cyt b$_{559}$ LP form to O$_2$ or PQ pool, preventing charge recombination in the RC, leading to P680 triplet state formation and consequently RC degradation (2, 21).