Spin conversion of cytochrome $b_{559}$ in photosystem II induced by exogenous high potential quinone

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Abstract

The spin-state of cytochrome $b_{559}$ (cyt $b_{559}$) was studied in photosystem II (PSII) membrane fragments by low-temperature EPR spectroscopy. Treatment of the membranes with 2,3-dichloro-5,6-dicyano-p-benzoquinone (DDQ) converts the native low-spin (LS) form of cyt $b_{559}$ into the high-spin (HS) form characterized by the $g = 6.19$ and $g = 5.95$ split signal. The amount of HS cyt $b_{559}$ was pH dependent with the amplitude increasing toward more acidic pH values (pH 5.5-8.5). The HS state was not photochemically active upon continuous illumination at 77 K and 200 K under our conditions and was characterized by a low reduction potential ($\leq 0$ V). It was also demonstrated that DDQ treatment damages the oxygen evolving complex, leading to inhibition of oxygen evolution, decrease of the $S_2$-state EPR multi-line signal and release of Mn$^{2+}$. In parallel, studies of model systems containing iron(III) protoporphyrin IX chloride (Fe$^{III}$Por), which is a good model compound for the cyt $b_{559}$ prosthetic group, were performed by using optical and EPR spectroscopy. The interaction of Fe$^{III}$Por with imidazole (Im) in weakly polar solvent results in formation of bis-imidazole coordinated heme iron (Fe$^{III}$PorIm$_2$) which mimics the bis-histidine axial ligation of cyt $b_{559}$. The reaction of DDQ with the LS Fe$^{III}$PorIm$_2$ complex leads to its conversion into the HS state ($g_{\perp} = 5.95$, $g_{\parallel} = 2.00$). It was shown that the spin conversion is due to donor-acceptor interaction of coordinated imidazole with this high-potential quinone causing the displacement of imidazole from the axial position. A similar mechanism of DDQ-induced spin change is assumed to be responsible for cyt $b_{559}$ in PSII centers.
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Introduction

Cytochrome \( b_{559} \) is an intrinsic component of photosystem II (PSII) of all oxygen evolving photosynthetic organisms. It is closely associated with the reaction center \( D_1 \) and \( D_2 \) proteins forming the minimum unit (\( D_1D_2\text{ cyt } b_{559} \) complex) capable to perform light-induced primary charge separation [1-3]. According to current ideas, cyt \( b_{559} \) is not involved in the primary electron transfer reactions leading to the oxidation of water but rather its functional role is associated with protecting PSII against photoinhibition [3-6] or with photoactivation [7]. The structural organization of cyt \( b_{559} \) includes two polypeptide subunits called \( \alpha \) (9 kDa) and \( \beta \) (4 kDa). Recent determination of the PSII crystal structure from two cyanobacterial species [8, 9] showed the presence of only one cyt \( b_{559} \) per reaction center (RC), although the question on cyt \( b_{559} \) stoichiometry in complete PSII complexes from higher plants is still debatable. Two histidine residues in the \( \alpha \beta \) heterodimer coordinate heme \( b \) which is oriented perpendicular to the membrane plane, closer to the stromal part of the membrane [8, 9]. It is expected that, like in other hemoproteins [10], the \( \text{bis}\)-histidine ligation of the heme iron keeps it in a low-spin (LS) state (\( S = 1/2 \)). Indeed, low-temperature EPR spectroscopy confirmed the LS state for isolated cyt \( b_{559} \) [11, 12] and for cyt \( b_{559} \) in a variety of PSII preparations (reviewed in [3]). The EPR signals occasionally observed in chloroplasts and membrane fragments in the Fe high-spin (HS) region (\( g \sim 6-7 \)) were mostly attributed to modified cyt \( b_6 \) ( cyt \( b_{563} \)) [13-16], known to adopt the HS state upon degradation [17-19]. However, in a number of recent papers both low- and high-spin (\( S = 5/2 \)) signals were observed for cyt \( b_{559} \) in photochemically active photosynthetic preparations, such as chloroplasts [20, 21], PSII membrane fragments [22] and \( D_1D_2\text{ cyt } b_{559} \) complexes [23]. Contrary to previous work [24], the observed HS state was ascribed to the native form of cyt \( b_{559} \). According to Fiege and Shuvalov [22] the major part of high-potential (HP) cyt \( b_{559} \) (~95 \%) in intact chloroplasts is present in the HS form. The intense sharp components with \( g = 5.6 \) and \( g = 6.1 \) were believed to be a result of distortion of the axial symmetry at the sixth coordination position, while the photoactive shoulder at \( g = 6.8 \) was ascribed to OH-ligation of the heme Fe(III) at this site [22]. In \( D_1D_2\text{ cyt } b_{559} \) complexes the HS signal with \( g = 5.9 \) and \( g = 6.4 \) (shoulder) was associated with an extra low-potential (LP) form
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of cyt $b_{559}$ formed at high pH [23]. The samples in many of the above-mentioned investigations were treated with high-potential quinones, especially DDQ with the purpose to oxidize the cyt $b_{559}$ heme moiety [13, 14, 16, 20-22]. However, it is rather likely that chemical treatment with DDQ also changes the local environment of the cyt $b_{559}$ heme group, so that a low- to high-spin conversion may take place. The present study aims to clarify the role of these high-potential quinones in formation of the HS state of cyt $b_{559}$. Therefore the EPR signals induced by DDQ treatment of PSII membrane fragments were studied in detail. In parallel, an artificial system containing iron(III) protoporphyrin IX chloride, which is a good model compound for the cyt $b_{559}$ prosthetic group [11, 12], was examined. The results obtained show that there are strong indications that the appearance of the HS form of cyt $b_{559}$ in PSII membranes is due to complex formation between DDQ and the histidine imidazoles ligated to the heme iron.

Materials and methods

BBY-type PSII membrane fragments were isolated from spinach according to refs. [25, 26]. The pellets at a Chl concentration ~10 mg/ml were stored at -80 °C in 10 mM MES-NaOH (pH 6.5), 3 mM MgCl$_2$, 15 mM NaCl and 0.4 M sucrose. The measurements at different pH values (pH range 5.5-9.0) were performed by washing and resuspension of the pellet in a corresponding buffer at the appropriate pH (100 mM MES or 100 mM HEPES). Quinone treatment of PSII membranes was carried out under aerobic conditions by addition of DDQ from a 200 mM stock solution in dimethyl sulfoxide (DMSO) to final quinone concentrations of 1-10 mM with a maximum DMSO content of ~5 % (v/v). The control experiment showed that at these concentrations DMSO alone did not have any influence on the EPR spectra or on the O$_2$-evolving activity of PSII membranes. The samples were incubated on ice in darkness for 20 min. To remove oxygen the suspensions were carefully bubbled with argon followed by freezing in liquid nitrogen for further measurements. The oxygen-evolving activity of PSII membranes was measured with a Clark-type electrode (Hansatech, Pentney, U.K.) at 20 °C. The average oxygen evolution rate for untreated PS membrane fragments was 430 ± 20 µmol O$_2$ per mg of Chl per hour at
pH 6.5 with DCBQ (250 µM) as an electron acceptor. At alkaline pH where
DCBQ is chemically unstable, pPBQ (250 µM) was used as an exogenous
acceptor. Iron(III) protoporphyrin IX chloride (Eastman Kodak), DDQ and
imidazole (both from Aldrich) were used without further purification.
Spectral grade solvents (methylene chloride, DMSO) were dried on 4 Å
molecular sieves. Low temperature cw X-band EPR spectra were recorded
with a Bruker ESP500E Elexys or with a Varian E9 spectrometer. The
temperature was regulated with an Oxford-900 liquid helium cryostat and
ITC 4 temperature controller (Oxford Instruments Ltd, Oxon, UK). The EPR
conditions are given in the figure legends. Low temperature illumination was
carried out for 15 min outside the EPR cavity in a bath containing liquid
nitrogen (77 K) or a dry CO$_2$-ethanol mixture (200 K). DPPH and myoglobin
(aqueous solution, pH 9.2) were used as standards for $g$-value
determinations. Visible absorption spectra were recorded at room
temperature with a Perkin-Elmer Lambda 900 UV/VIS/NIR spectrometer.

Results

**DDQ treated PSII membrane fragments**

In untreated dark-adapted PSII membrane fragments cyt $b_{559}$ is present in the
LS state characterized by a highly anisotropic EPR signal [3, 27]. The exact
intensity and shape of the most easily observed $g_z$-peak depends on the
ambient redox potential during the isolation procedure which determines the
amount of oxidized cyt $b_{559}$. The $g_z$-peak in PSII membranes usually shows
the superposition of the HP form ($g_z = 3.08-3.03$ [12, 28]), intermediate-
potential (IP) form ($g_z = 3.04-3.01$ [28]) and LP form ($g_z = 2.95-2.92$ [3, 12])
of cyt $b_{559}$. In dark-adapted preparations the contribution from the HP (HP +
IP) form to the $g_z$ signal is low (Fig. 1A, spectrum a). Illumination of the
sample at 77 K yields a $g_z = 3.07$ signal which may represent the mixture of
‘non-relaxed’ photo-oxidized HP cyt $b_{559}$ ($g_z = 3.08$ [3, 12, 14, 28]) and IP
($g_z = 3.04$ [28]) (Fig. 1A, spectrum b). The signal obtained represented the
total amount of oxidized cyt $b_{559}$ and was used for estimation of the
proportion between different redox forms of cyt $b_{559}$. Integration of the $g_z$-
peak for photo-oxidized and initial sample gives a ratio of 2.1:1, which
-corresponds to the 65:35 % stoichiometry between HP and LP forms. This is
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well correlated to the values known for cyt b_{559} from PSII membrane fragments [28, 29]. For a given preparation the intensity of the LS signal was approximately constant within a pH range of 7.0–9.0 and slightly increased at lower pH (pH 5.5 - 6.5) as a consequence of much slower dark reduction of cyt b_{559} at acidic pH [3].

Figure 1. (A) EPR spectra of PSII membrane fragments (pH 6.5; [Chl] = 6.7 mg/ml). (a) Dark-adapted untreated membranes, (b) Untreated membranes illuminated at 77 K; (c) DDQ-treated membranes; [DDQ] = 2 mM. (B) EPR spectra of PSII membrane fragments ([Chl] = 3.9 mg/ml). Untreated membranes at pH 6.5 (a) and pH 5.5 (c); DDQ-treated membranes at pH 6.5 (b) and at pH 5.5 (d); [DDQ] = 2 mM. EPR conditions: microwave frequency 9.41 GHz; microwave power 5 mW; modulation amplitude 20 G; temperature 9 K.

At increased pH (pH 8.0-9.0) the contribution of the HP form to the g_{z}-peak was usually lower which may be connected with alkaline-induced conversion of the HP form into IP [29]. For dark-adapted photosystem II membranes in the HS region (g = 5.5–7.0) a number of small peaks are
present (with its most pronounced feature at \( g = 6.10 \)) which amplitudes are increased upon acidification of the solution (Fig. 1B spectra a and c). Treatment of PSII membrane fragments with DDQ in the dark results in appearance of the intense HS signal characterized by \( g = 6.19 \) and \( g = 5.95 \) (± 0.02) splitting (Fig. 1A spectrum c and Fig.1B spectra b and d). The shape of the DDQ-induced HS signal is very reproducible and is different from the weak, structureless HS signals observed in untreated membranes. Upon going to lower pH the HS signal becomes larger without changing its shape (Fig. 1B spectra b and d). Similar split cytochrome signals have been observed before [13, 14, 16, 24], while in other studies a shoulder at \( g = 6.8-6.4 \) was additionally present [20, 21, 23]. The appearance of the \( g = 6 \) signal took place upon DDQ treatment at pH < 7 independent of whether HP LS cyt \( b_{559} \) was present in the oxidized or reduced state. DDQ addition to the samples with non-oxidized HP cyt \( b_{559} \) increased the amount of the \( g = 3.07 \) signal showing the ability of DDQ to oxidize the HP form of cyt \( b_{559} \) (Fig. 1A). When the HP heme was initially present in the oxidized form, upon DDQ treatment the amplitude of the \( g_z \)-peak was ~30% diminished without changes in overall shape (Fig. 1B). The amplitude of the LS \( g_z \)-peak of a DDQ-treated sample is smaller compared to the \( g_z \)-peak of control untreated sample with fully photo-oxidized cyt \( b_{559} \) (Fig. 1A spectra b and c). It will be shown below that upon complete conversion of iron(III) protoporphyrin IX, a model compound for the cyt \( b_{559} \) prosthetic group, from the LS to the HS state, the increase in the amplitude of the \( g = 6 \) peak is about 20 times larger than the decrease of the \( g = 3 \) peak. Therefore the appearance of the strong HS cyt \( b_{559} \) signal is not expected to be accompanied by a drastic reduction in the LS \( g_z \)-peak. Taking into account that for cyt \( b_{559} \) the HS signal is split and therefore broader than for the model compound, the ratio between HS/LS amplitude changes is expected to be smaller. For the spectra shown in Fig. 1 the HS \( g = 6 \) signal rises 5-8 fold at the expense of the LS \( g = 3 \) signal. Difference spectra (illuminated minus DDQ-treated, not shown) indicate the contribution from both HP and LP forms to the \( g_z \)-peak. Thus, the HS state of cyt \( b_{559} \) is formed from its LS state independent of the redox characteristics of the LS heme. The amplitude of the \( g = 6 \) peak is strongly pH-dependent (Fig. 2), such that upon DDQ treatment the HS signals could only be obtained at acidic pH (pH 5.5-6.5). Another requirement for the appearance of the HS signal is a prolonged (10-20 min) dark incubation of
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the membranes with DDQ at 0 °C. Increasing the DDQ concentration to about 5 mM is also favorable for the formation of the HS signal.

Figure 2. The effect of pH on the intensity of HS signals for untreated and DDQ-treated PSII membrane fragments. The intensities of the HS signals were estimated by double integration over the g = 5.5-8.0 region of the spectra.

In the presence of DDQ the six-line EPR signal of Mn$^{2+}$ is often observed (Fig. 1B spectra b and d, marked with asterisks). Upon increasing the DDQ concentration (> 5 mM) the signal becomes larger, indicating that DDQ treatment leads to destruction of the Mn$_4$ cluster in PSII. The release of Mn$^{2+}$ is one of the reasons for the loss of oxygen-evolving activity observed in the presence of DDQ (Fig. 3).
The oxygen-evolution activity of PSII membrane fragments pretreated with different concentrations of DDQ. The rate of O$_2$ evolution measured for untreated sample at respective pH was taken for 100%. pPBQ (250 µM) was used as an electron acceptor.

When membranes were incubated with DDQ in the dark at 0°C for 10-20 min (conditions similar to those for EPR measurements) the rate of O$_2$-evolution was decreased as compared to a control sample (Fig. 3). pPBQ was added as an electron acceptor. The O$_2$-evolving activity was gradually diminished upon increasing the DDQ concentration with stronger inhibition at the more acidic pH. At DDQ:Chl molar ratios similar to those used for EPR studies ([DDQ]:[Chl] ≈ 0.5) about 30-50% of the initial activity was lost. Illumination of DDQ-treated sample at 77 K and further at 200 K (without intermediate annealing) did not change the amplitude of the $g = 6.19$ and $g = 5.95$ peaks (data not shown). Subsequent incubation of the sample at 0°C for 15-30 min also did not result in any changes in the
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intensity of the $g \approx 6$ signals. Similar to the untreated sample, illumination at 200 K produced the S$_2$-state EPR signal from the Mn$_4$ cluster. In the case when a strong Mn$^{2+}$ signal was present after the DDQ treatment, the amplitude of the light-induced multi-line signal was considerably diminished compared to untreated membranes. The redox properties of the HS state of cyt b$_{559}$ compared to those of the LS state were tested by addition of hydroquinone, sodium ascorbate and sodium dithionite to DDQ-treated samples. No changes in amplitude of the HS peaks were observed upon hydroquinone (4 mM) addition, but it caused the disappearance of about 80 % of the $g_z \approx 3$ peak. Only in the presence of sodium dithionite (4 mM) was partial reduction of the HS signal (up to ~50 % from the initial intensity) achieved (data not shown).

Coordination of imidazole by hemin in model systems
Iron(III) protoporphyrin IX (hemin) chloride [Fe$^{III}$Por]$^+$ Cl$^-$ was used as a model compound of the cyt b$_{559}$ prosthetic group [11, 12]. The reaction of Fe(III) porphyrins with different axial ligands is often monitored by optical spectroscopy, which permits studies of the kinetics of complexation and determination of equilibrium constants. The optical spectrum of hemin in CH$_2$Cl$_2$ (Fig. 4A) shows three absorption peaks in the visible region at 511 nm ($\beta$-band), 542 nm and 640 nm. In the Soret region a peak is present at 386 nm. In non-coordinating solvents like chloroform, hemin exists as a five-coordinated complex with a Cl$^-$ anion as a weak axial ligand and its absorption spectrum is characteristic of high-spin ($S = 5/2$) ferric complexes [30]. Fig. 4A shows the spectra obtained during spectro-photometric titration of hemin in CH$_2$Cl$_2$ by imidazole. Upon addition of imidazole, the 386 and 640 nm bands disappear, while new bands appear at 411 and 534 nm. The presence of isosbestic points at 401, 471, 522 and 591 nm shows that the solution contains only two absorbing species. The changes in the absorbance at several wavelengths (411, 386, 640 nm) as a function of imidazole concentration (Fig. 4B) were used to determine the number of bound ligands ($n$), as well as a binding constant ($\beta_2$). For the reaction according to the equation

$$[\text{Fe}^{III}\text{Por}]^+\text{Cl}^- + n \text{Im} \leftrightarrow [\text{Fe}^{III}\text{Por}\text{Im}_n]^+ + \text{Cl}^-$$
Figure 4. (A) Spectrophotometric titration of iron(III) protoporphyrin IX (hemin) with imidazole in CH$_2$Cl$_2$ solution. Inset: The enlarged $\alpha$-band region. [Fe$^{III}$Por] = 0.01 mM; [Im], mM: 0 (1); 0.10 (2); 0.21 (3); 0.31 (4); 0.41 (5); 0.99 (6). (B) Plot of absorbance at several wavelengths versus imidazole concentration upon spectrophotometric titration. The dashed lines represent the best fit to the experimental data to determine the equilibrium constant for hemin-imidazole interaction.
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the experimental data were fitted with variable $n$ and $\beta_2$ and yielded $n = 2.1$ and equilibrium stability constant $\beta_2 = [\text{Fe}^{III}\text{PorIm}_2^+]/[\text{Fe}^{III}\text{Por}]^*[\text{Im}]^2 = 1.8 \times 10^7 \text{ M}^{-2}$ ($\log(\beta_2) = 7.26$). Due to poor solubility of hemin in pure CH$_2$Cl$_2$, some amount of DMSO (10% v/v) had to be added for further EPR studies. For this reason the complexation between hemin and imidazole was studied by optical spectroscopy in a similar way in pure DMSO solutions and yielded $n = 1.9$ and $\beta_2 = 6.5 \times 10^4 \text{ M}^{-2}$ ($\log(\beta_2) = 4.81$) which is in good agreement with the published value for this solvent ($\beta_2 = 7.0 \times 10^4 \text{ M}^{-2}$ [31]). Although $\beta_2$ decreases with increasing solvent polarity, in both solvents the sixth-coordinate hemine complex is formed with two imidazole molecules occupying both axial positions. Such bis-coordinate (with ‘strong’ ligands) iron(III) porphyrins are usually LS, in contrast to HS five-coordinate adducts (see Discussion). The next model to be studied was the imidazole-DDQ system in CH$_2$Cl$_2$. The absorption spectrum of DDQ in CH$_2$Cl$_2$ (Fig. 5A) was characterized by a strong $\pi-\pi^*$ band at 288 nm and a weaker $n-\pi^*$ transition at ~395 nm [32]. The addition of imidazole to a DDQ solution caused the appearance of a new broad absorbance in the visible region with a maximum at 465 nm. The increase of the band intensity was rather slow, so that even after reaction for one hour, saturation in the absorbance changes was not observed. For a given reaction time, the intensity of the band at 465 nm increased with increasing imidazole concentration (for Im:DDQ molar ratio in the range 1:1 to 150:1). The room temperature EPR spectrum of the DDQ-imidazole solution was characterized by a stable 2.1 Gauss broad radical signal at $g = 2.006 (\pm 0.003)$, not shown. This signal is attributed to the DDQ anion-radical, which was not present in DDQ-only solutions. Thus, similar to other quinones [32, 33], DDQ forms a stable electron donor-acceptor (or charge-transfer) complex with imidazole, an N-heterocyclic aromatic donor (see Discussion). Based on the above findings, it could be inferred that DDQ interferes with the hemin-imidazole complex. Indeed, Fig. 5B shows that upon addition of DDQ to a solution containing hemin which was completely bound to imidazole, the 411 nm maximum corresponding to $[\text{Fe}^{III}\text{PorIm}_2^+]^-$ complex was diminished with a concomitant increase of the 386 nm band of free $[\text{Fe}^{III}\text{Por}]^-$. The increase in absorbance in the 500-700 nm range represents the absorbance of the DDQ-imidazole charge-transfer complex (compare Fig. 5A). Thus, the optical measurements clearly show that DDQ could induce the dissociation of hemin-imidazole complexes due to DDQ-
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imidazole interactions. This in turn might change the Fe(III) spin-state analogous to what was observed in PSII. This was checked further with EPR spectroscopy.

![Figure 5](image)

**Figure 5.** (A) Optical absorbance spectra of DDQ in CH$_2$Cl$_2$ solution before (1) and after addition of imidazole (2-5). Spectra (2-5) were recorded at different times after the addition: 5 min (2); 10 min (3); 20 min (4); 40 min (5). [DDQ] = 0.13 mM; [Im] = 1.87 mM. (B) Optical absorbance spectra in CH$_2$Cl$_2$ solution: hemin without additions (1); upon addition of imidazole (2); upon subsequent addition of DDQ to the hemin-imidazole solution (3). Spectrum (3) was recorded 1 hour after the DDQ addition to the hemin-imidazole mixture. [Fe$^{III}$Por] 0.01 mM; [Im] = 0.20 mM. [DDQ] = 0.13 mM.

The EPR results directly confirmed the spin-state of hemin inferred from the optical studies. The EPR spectrum of ‘free’ hemin (measured at 4 K) in the mixed solvent CH$_2$Cl$_2$-DMSO (9:1, v/v) is shown in Fig. 6A and is typical for the HS state of iron porphyrins. The principle values of the g-tensor are: $g_\perp = 5.95 \pm 0.02$ and $g_\parallel = 2.00 \pm 0.02$; for the cross-point $g = 5.82 \pm 0.02$. 

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Figure 6. (A) EPR spectrum of iron(III) protoporphyrin IX ([Fe$^{III}$Por] = 0.5 mM) in CH$_2$Cl$_2$-DMSO (9/1, v/v) solution (solid line, with enlarged g = 2 region shown in the inset) and after the addition of imidazole (dashed line, [Im] = 20 mM). (B) The enlarged spectrum of iron(III) protoporphyrin IX in the presence of imidazole. (C) The same solution as in (B) after the subsequent addition of DDQ ([DDQ] = 20 mM) followed by 30 min incubation at room temperature. EPR conditions: microwave frequency 9.23 GHz; microwave power 6 mW; modulation amplitude 20 G; temperature 4 K.
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Reaction of hemin with imidazole yielded the hexa-coordinated LS state ($S = 1/2$). The resulting EPR spectrum measured with the same amplification as the HS spectrum has a low intensity (Fig. 6A) and is characterized by the following anisotropic $g$-values $g_z = 2.92$, $g_y = 2.24$, $g_x = 1.53$ (± 0.02 for all $g$-values) (Fig. 6B). This is in agreement with previously reported $g$-values for *bis*-imidazole coordinated iron(III) protoporphyrin IX [11, 34, 35]. Thus, LS to HS conversion in the model system resulted basically in the appearance of a sharp $g = 5.95$ line whose intensity is about 20 times higher compared to that for the $g_z = 3$ peak of the LS state. This observation is important to correlate the amplitudes of HS and LS cyt $b_{559}$ signals observed in PSII membranes (see data above). Thus, the high- to low-spin change indeed occurred upon formation of *bis*-imidazole complexes of hemin in solution. This is similar to previously observed spin changes of iron porphyrins (including hemin) in the presence of imidazole [31, 34-37]. Upon addition of DDQ at room temperature to the LS hemin-imidazole complex Fe$^{III}$PorIm$_2^+$, the LS signals disappear with concomitant increase of the $g = 5.82$ line belonging to the high-spin state of hemin (Fig. 6C). For the solutions with DDQ:Im ≥ 1 the HS signal is developed within ~10 min after DDQ addition. In contrast, the HS signal is not obtained at low DDQ:Im ratios, even after several hours of incubation at room temperature (not shown). The line shape of the $g = 5.82$ signal in the presence of DDQ is similar to that obtained for hemin in CH$_2$Cl$_2$ solution. In the EPR spectrum in Fig. 6C, the $g_{||} = 2.00$ signal belonging to the HS state of hemin (compare Fig. 6A, inset) is obscured by the strong signal from the DDQ anion-radical ($g = 2.006$). The mechanism of DDQ-induced spin change is associated with the removal (or displacement) of one of the two imidazoles from the axial positions of hemin as was discussed earlier. Thus, the EPR model studies strongly indicate that DDQ can induce changes in the spin-state of iron(III) protoporphyrin IX, a prosthetic group similar to that of natural cyt $b_{559}$.

Discussion

In the present study, iron(III) protoporphyrin IX was used as a model compound of the cyt $b_{559}$ prosthetic group. The optical absorption and EPR spectra (Fig. 4A and 6A) showed that in a solution of CH$_2$Cl$_2$, iron(III)
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Protoporphyrin IX forms a stable LS complex with two imidazole molecules as axial ligands. This may serve as a good model for the \textit{bis}-histidine coordination of the heme group in cyt \textit{b}_{559}. To understand how the addition of high-potential quinone DDQ converts this complex into the HS-state, quinone and imidazole in CH\textsubscript{2}Cl\textsubscript{2} solution were studied in detail (Fig. 5A). Quinones are well known to serve as electron acceptors in donor-acceptor (DAC) or charge-transfer complexes. High-potential quinones including DDQ (E\textsubscript{1/2} = + 0.75 V), chloranil (E\textsubscript{1/2} = + 0.25 V), DCBQ (E\textsubscript{1/2} = + 0.06 V) and others (all standard one-electron reduction potentials for Q/Q\textsuperscript{-} redox couples are given for MeCN solutions, versus standard hydrogen electrode [38]), were shown to form DACs with a number of electron donors such as aromatic hydrocarbons, amines, phenols, amino acids, nitrones and N-heterocycles [32, 33 and Ref. therein]. The formation of a charge-transfer complex gives rise to a new absorption band (sometimes two bands) in the near UV or visible regions the energy of which is determined by the ionization potential of the donor and the electron affinity of the acceptor. It is reasonable to assume that the new broad band at 465 nm, which appeared upon addition of imidazole to DDQ (Fig. 5A), represents the charge-transfer complex formed in the solution. The formation of a DDQ-Im complex is also accompanied by the appearance of a stable EPR signal from the DDQ anion-radical, similar to other paramagnetic charge-transfer complexes of DDQ [39]. Thus, imidazole like other hetero-aromatic compounds can efficiently function as a \pi-donor with respect to DDQ, one of the strongest oxidizing quinones. The DDQ-imidazole complex is stable only in weakly polar solvents, like CH\textsubscript{2}Cl\textsubscript{2}, while no complex formation was observed in DMSO. Although the stoichiometry of the complex was not determined, it is likely to be 1:1, similar to other DAC. The observed dissociation of the hemin-imidazole complex upon DDQ addition (Fig. 5B) is the consequence of a competition reaction between quinone and imidazole to form a charge-transfer complex. The spectral changes show that, under the given relative concentrations of the components, the following equilibrium exists between the ‘free’ and \textit{bis}-imidazole coordinated heme iron(III):

\[
[\text{Fe}^{III}\text{Por Im}_2]^+ + 2 \text{DDQ} \leftrightarrow [\text{Fe}^{III}\text{Por}]^+ + 2 \text{DDQ}^*\text{Im}
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where the ‘free’ complex [Fe\textsuperscript{III}Por\textsuperscript{+}] actually contains Cl\textsuperscript{–} as a fifth ligand. The possibility of mono-imidazole complex [Fe\textsuperscript{III}PorIm\textsuperscript{+}] formation upon DDQ treatment can not be completely excluded, though such complexes are normally not stable in the case of Fe(III) porphyrins, including iron(III) protoporphyrin IX [36]. The EPR results are in good agreement with the conclusions derived from the absorption studies. Indeed, the spin-state of iron(III) porphyrins is dependent on the strength of the axial ligation (L) at the fifth and sixth positions [40, 41]. It is generally believed that the HS state (\(S = 5/2\)) corresponds to a five-coordinate complex of Fe\textsuperscript{III}PorL which has a square pyramidal structure where the Fe(III) ion is above the plane of the porphyrin. Most six-coordinate octahedral Fe\textsuperscript{III}PorL\textsubscript{2} complexes have low spin (\(S = 1/2\)). In complexes where the axially ligating group is of a weak field, a transition occurs between HS and LS, or such complexes are fully HS. Iron(III) protoporphyrin IX, being HS in CH\textsubscript{2}Cl\textsubscript{2} solution, is converted to the LS state upon the addition of imidazole (Fig. 6A and B). This is similar to previously observed spin changes of iron porphyrins (including hemin) in the presence of imidazole [31, 34-37, 42]. Upon subsequent treatment of the imidazole-hemin complexes with DDQ, the LS EPR spectrum vanishes, while the EPR spectrum characteristic of a HS state appears again (Fig. 6C). The mechanism of DDQ-induced spin change must be associated with the removal of at least one of the two imidazoles from the axial positions of the porphyrin ring. The model studies may serve to explain the cyt \textsubscript{b559} spin-state changes in photosystem II membranes upon treatment with DDQ. In untreated membranes the majority of cyt \textsubscript{b559} is LS (Fig. 1) similar to the previous reports (reviewed in refs. [3, 27]). Low-temperature (77 K) photochemical oxidation of PSII membranes or chemical oxidation with K\textsubscript{2}IrCl\textsubscript{6} or DCBQ at room temperature does not change the spin-state of cyt \textsubscript{b559} [12, 28]. Treatment of PSII membranes with potassium ferricyanide leads to cyt \textsubscript{b559} oxidation (monitored by changes in the absorbance at 559 nm) with concomitant appearance of EPR signals around \(g = 6.0\) and \(g = 8.0\), which disappear upon illumination at 77 K (data not shown). These signals do not originate from cyt \textsubscript{b559} but are attributed to the oxidized form of the non-heme iron on the acceptor side of PSII. In addition to ferricyanide treatment [43, 44], the non-heme HS signals could also be induced by molecular oxygen [43-45] and exogenous quinones [44, 46, 47]. These signals are completely different from the presently observed HS cyt 64
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signals. In contrast to the latter, the non-heme signals are high-potential (E_m \approx 400 \text{ mV}) and are photo-reducible at low temperatures [43, 44, 47, 48]. Thus, room temperature oxidation of cyt b_{559} itself does not induce the appearance of HS cyt b_{559}. Instead, it is reasonable to assume that DDQ, except being a strong chemical oxidant capable to oxidize cyt b_{559}, also forms a charge-transfer complex with at least one of the histidine imidazoles ligated to cyt b_{559} heme iron, as was observed in the model systems. Displacement of the histidine side chain from the axial position changes the molecular symmetry from octahedral to square pyramidal in which the iron

Figure 7. (A) Schematic representation of cyt b_{559} heme (thick vertical line) in PSII in the native LS-state before interaction with DDQ. The heme group is coordinated to the two histidine imidazole rings. (B) Donor-acceptor interaction (shown by arrow) of DDQ with one of the histidine imidazole results in the ligand displacement from the axial position, forming the HS state.
atom protrudes from the porphyrin ring (Fig. 7). This would then induce low- to high-spin conversion of cyt \( b_{559} \). In the present study we showed that treatment of PSII membranes with DDQ in the dark causes not only oxidation of the HP cyt \( b_{559} \), but partially converts it into the HS state. A number of other exogenous quinones are widely used in PSII studies including DCCBQ and PpBQ but their possible influence on the spin-state of heme iron was not studied in detail. The only well established effect of quinones is light-induced oxidation of non-heme iron resulting in the HS signal mentioned above [44, 46, 47]. Compared to DDQ these quinones are less powerful oxidants, consequently less capable of forming DACs, although recently it was found that weakly oxidizing \( \alpha \)-tocopherol quinone can cause a LS to HS cyt \( b_{559} \) spin conversion [49]. Thus, the existence of the HS state of the HP cyt \( b_{559} \) in PSII membranes inferred from the optical spectra [29], as well as found by EPR in chloroplasts, PSII membranes and purified RCs [20-24] may be related to the presence of different quinones (including DDQ) during the experiments. High concentrations of DDQ and at least 10 minutes incubation at 0 °C are required to produce the HS state of membrane cyt \( b_{559} \). For the experiments shown in Fig. 1 the [Chl]:[DDQ] molar ratio is 2.2:1.0. Assuming 200-240 Chl molecules per RC in the PSII BBY-type membrane fragments, gives [DDQ]:[cyt \( b_{559} \)] ≈ 100:1 or 50:1 for one and two cyt \( b_{559} \) molecules per RC, respectively. In model systems, ratios of [DDQ]:[Im] ≥ 1 already yielded a spin change in \([\text{Fe(III)PorIm}]+\) (Fig. 5B and 6C). The need for higher ratios of DDQ to induce the spin change in PSII, can be explained by the shielding of the cyt \( b_{559} \) heme moiety by the protein matrix [8, 9], thus preventing DDQ-access, in contrast to the model system where the hemin-imidazole complex can readily come into contact with DDQ. Another plausible explanation is that in the native photosynthetic system the interaction of DDQ with the protein imidazole may be weaker as compared to the situation in organic solvents. The shape of the resulting HS EPR spectrum is not identical for the model compound and for cyt \( b_{559} \). For \([\text{Fe}^{III}\text{Por}]^+\) Cl both \( g_x \) and \( g_y \) values were the same \( (g_x = g_y = 5.95) \) (Fig. 6A) which corresponds to a strictly tetragonal symmetry. For the HS state of native cyt \( b_{559} \) the x- and y-components are not completely equivalent: the EPR line at \( g \approx 6 \) splits into \( g_x = 6.19 \) and \( g_y = 5.95 \) peaks (Fig. 1). This probably reflects deviation from tetragonality as was observed previously for a number of other heme proteins [30].
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The $g_{\parallel} (= g_z = 2.00)$ line cannot be detected in the photosynthetic system due to the other strong EPR signals present in this region. The observed pH dependence of the cyt $b_{559}$ HS-state (Fig. 2) is in line with the proposed mechanism. The pH interval of the HS signal formation (pH 5-7) corresponds to the range where protonation of histidine imidazole normally takes place (the macroscopic dissociation constant for ImH$^-$ in histidine is pK$_a$ = 6.0 [50]). Protonation of the histidine ligand would facilitate its displacement from the coordinated Fe(III). The weak HS signal that appears in the absence of DDQ at acidic pH (pH 5.5-6.0) (Fig. 2) may be caused by water acting as one of the axial ligands. The LS state of cyt $b_{559}$ is photochemically active both with respect to photochemical oxidation and reduction [51]. Reduced cyt $b_{559}$ serves as an alternative donor to P$_{680}$ in PSII membranes at temperatures < 100 K when the Mn-complex is not functional [12, 28]. In previous studies the involvement of the HS cyt $b_{559}$ with OH$^-$ as the sixth ligand in low-temperature photochemistry was reported [20, 22]. Illumination at 80-140 K resulted in a disappearance of the $g = 6.8$ shoulder (ascribed to the heme ligated with OH$^-$) with concomitant increase of $g = 5.8$ and 6.1 signals (ascribed to heme without the sixth ligand). It was explained as a removal of OH$^-$ from heme Fe(III) after its light-induced oxidation to an OH$^+$ radical. Our studies showed that the HS cyt $b_{559}$, being characterized by a split signal at $g = 6.19$ and $g = 5.95$, is not photochemically active upon continuous illumination at 77 K and 200 K as well as upon subsequent dark adaptation at 0 °C. Thus, dark reduction of the oxidized HS cyt $b_{559}$ does not occur as was reported for the LS cyt $b_{559}$ [51]. It is likely that under our conditions in the presence of excess of DDQ accumulation of possible reductants of the HS cyt $b_{559}$ (like reduced Q$_A$ and Q$_B$) is impossible. The three-point redox titration (hydroquinone, ascorbate, dithionite) revealed that partial reduction of the cyt $b_{559}$ HS signals could be achieved only by addition of the strongest reductant. This suggests that the HS state is characterized by a low $E_m$ ($E_m \approx 0$ mV or even lower). In PSII membranes cyt $b_{559}$ is present in different redox forms: HP ($E_m = 370-430$ mV); IP ($E_m = 150-250$ mV) and LP ($E_m = 0-100$mV) with relative contents of about 45-60 %, 25-30 % and 20-25 %, respectively [28, 29]. Although by its redox characteristics the HS cyt $b_{559}$ is LP, it is obtained via conversion of both LP and HP forms of initial LS cyt $b_{559}$ (Fig. 1A and B). Thus, there is no preference for DDQ interaction with histidine bound to the heme either in
the LP or in the HP form. The fact that the HS state is characterized by the low value for $E_m$ demonstrates that the LP form of cyt $b_{559}$ may originate from the weak heme-histidine interaction. The idea that the mode of histidine ligation (histidine plane orientation and hydrogen bonding to histidine ligands) is essential for determining the redox state of cyt $b_{559}$ is discussed in the literature [3, and references therein]. Another aspect of PSII membrane treatment with DDQ is the damaging effect of this quinone on the $O_2$-evolution process. When DDQ is used as electron acceptor (instead of DCBQ or PpBQ), $O_2$ evolution is completely inhibited. In the presence of DDQ ($5$-$250$ µM) with concomitantly added PpBQ as exogenous acceptor a significant decrease of the $O_2$ evolution rate is observed (Fig. 3). It is possible that similar to other inhibitors (DCMU, 6-phenanthroline) the inhibitory effect of DDQ is due to its strong binding to the Q$_B$ site. However, in addition to this potential inhibitory effect, DDQ causes irreversible damage to the Mn$_4$ cluster as observed by the appearance of the Mn$^{2+}$ signal (Fig. 1B). The observed Mn release may be caused by a mechanism similar to the one we suggest for the formation of HS cyt $b_{559}$, i.e. the replacement of histidine residues ligated to Mn$_4$ cluster [52] upon DDQ treatment. In conclusion, the DDQ-induced HS state of cyt $b_{559}$ represents its non-native form which appears due to the modification in bis-histidine ligation to the heme iron (Fig. 7). The similar effect could be expected upon treatment of PSII membranes with other high-potential compounds (including many quinones) capable to form charge-transfer complexes with the imidazole moiety of protein histidine. At low pH and a high ligand concentration inorganic weak field ligands that are known to form complexes with iron(III)/(II) protoporphyrin IX (F$^-$, Cl$^-$, OH$^-$, H$_2$O and SCN$^-$), can also disturb the heme-imidazole interaction, thus causing the conversion of cyt $b_{559}$ from the low to the high spin form. The effect of DDQ on PSII membranes obviously may not be limited only to histidine ligated to the cyt $b_{559}$ heme iron, but could also be expected for all other histidine moieties including those involved in metal coordination or in hydrogen-bonds to other key components (e.g. quinones and tyrosines). The general consequence of DDQ-histidine interactions is the possible changes in the native binding sites of many photosynthetic cofactors which makes application of this quinone, especially at high concentrations, undesirable.
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