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Biphasic reduction of cytochrome b559 by plastoquinol in photosystem II membrane fragments
Evidence for two types of cytochrome b559/plastoquinone redox equilibria

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ABSTRACT

In photosystem II membrane fragments with oxidized cytochrome (Cyt) b559 reduction of Cyt b559 by plastoquinol formed in the membrane pool under illumination and by exogenous decylplastoquinol added in the dark was studied. Reduction of oxidized Cyt b559 by plastoquinols proceeds biphasically comprising a fast component with a rate constant higher than $(10\ s)^{-1}$, named phase I, followed by a slower dark reaction with a rate constant of $(2.7\ min)^{-1}$ at pH 6.5, termed phase II. The extents of both components of Cyt b559 reduction increased with increasing concentrations of the quinols, with that, maximally a half of oxidized Cyt b559 can be photoreduced or chemically reduced in phase I at pH 6.5. The photosystem II herbicide dinoseb but not 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) competed with the quinol reductant in phase I. The results reveal that the two components of the Cyt b559 redox reaction reflect two redox equilibria attaining in different time domains. One-electron redox equilibrium between oxidized Cyt b559 and the photosystem II-bound plastoquinol is established in phase I of Cyt b559 reduction. Phase II is attributed to equilibration of Cyt b559 redox forms with the quinone pool. The quinone site involved in phase I of Cyt b559 reduction is considered to be the site regulating the redox potential of Cyt b559 which can accommodate quinone, semiquinone and quinol forms. The properties of this site designated here as Q_D clearly suggest that it is distinct from the site Q_C found in the photosystem II crystal structure.

1. Introduction

A multiprotein complex of photosystem II (PS II) operates as a water:plastoquinone oxidoreductase (see Refs. [1–4] for general reviews). Photoexcitation of the PS II reaction center drives an electron from the primary electron donor, P680, into a chain of the sequential redox carriers: accessory chlorophyll (Chl), pheothytin (Pheo), primary plastoquinone (PQ) acceptor Q_A and secondary PQ acceptor Q_B (for early stages of charge separation see work [5] and references therein). The Q_B functions as a two-electron gate, after a sequential transfer of two electrons and the two protonation events plastoquinol (PQH_2) is formed at the Q_B site [6,7] (reviewed in Refs. [8,9]). The exchange of the formed PQH_2 for a molecule of oxidized PQ promotes the cycle in the Q_B site [10]. The reservoir of free PQ and PQH_2 is the membrane quinone pool shared between several PS II complexes [11,12] (see also [13–15] and references therein). On the oxidizing side of PS II, sequential transfer of four holes from the photooxidized P680 to the oxygen-evolving complex (OEC) results in oxidation of two water molecules and a release of a molecule of oxygen. The 3D structure is known for the PS II complexes from the thermophilic cyanobacteria *Thermosynecococcus elongates* and *Thermosynecococcus vulcanus* with resolution of 1.9–3.8 Å [16–21].

Abbreviations: ADRY, acceleration of the deactivation reactions of system Y; Car, carotenoid; Chl, chlorophyll; CW, continuous wavelength; Cyt, cytochrome; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; dinoseb, 2,4-dinitro-6-sec-butylphenol; DPQ, decylplastoquinone; $DPQH_2$, decylplastoquinol; DQ, tetramethyl-p-benzoquinone or duroquinone; DQH_2, tetramethyl-p-benzoquinol or duroquinol; E_m, midpoint potential; F_v, variable fluorescence; HEPES, N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; FeCy, $K_3Fe(CN)_6$; HP, high potential; HQ, p-benzoquinol; IP, intermediate potential; K_i, inhibition constant; K_D, dissociation constant; K_{dino}, dissociation constant of dinoseb; $K_{DPQ\,H_2}$, dissociation constant of $DPQH_2$; $K^{dino}_{DPQ\,H_2}$, dissociation constant of $DPQH_2$ in the presence of dinoseb; K_{eq}, equilibrium constant; K_{50}, concentration of a reactant at a half-maximal effect; L, chemical compounds (ADRY reagents, dinoseb, DCMU (at high concentrations), TPB) that induce a concentration dependent negative shift in the E_m of HP Cyt b559; LP, low potential; MES, 2-[N-morpholino]ethanesulfonic acid; OEC, oxygen-evolving complex; ox, oxidized; PFD, photon flux density; Pheo, pheophytin; PQ, plastoquinone; PQH_2, plastoquinol; PS, photosystem; P680, primary electron donor of PS II; Q, quinone; QH_2, quinol; Q_A, primary quinone acceptor of PS II; Q_B, secondary quinone acceptor of PS II; Q_C, third quinone of PS II; Q_D, presumed bound quinone of PS II interacting with Cyt b559; red, reduced; SQ, semiquinone; TPB, tetraphenylboron; tricine, N-[2-Hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine; Tyr, tyrosine; X, quinol-ligating group in the Q_D site

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Some compounds, the PS II herbicides, inhibit the electron transport in PS II replacing PQ in the Q_B site and, thus, blocking reoxidation of the primary acceptor Q_A [10,22] (see Refs. [23–27] for reviews). The interaction of the urea herbicide DCMU with the Q_B site is characterized by a dissociation constant in the submicromolar range [22,28,29] and a slow exchange in the binding site [22,29,30]. The phenolic herbicide dinoseb impairs the secondary electron transport in PS II with an inhibition constant of 1–3 μM [29,31,32].

Cytochrome (Cyt) b559 is an integral protein of the PS II complex of higher plants and cyanobacteria. It is considered to participate in protection of PS II from photoinhibition, probably, via involvement in a cyclic electron flow around PS II [33–44], however, detailed mechanism of its functioning is not fully understood (reviewed in [45–49]). It is suggested that Cyt b559 may operate as a plastoquinol oxidase, an oxygen reductase [50–54] or a superoxide oxidase/reductase [55–58].

A distinct feature of Cyt b559 among heme proteins is its redox heterogeneity. Three forms of Cyt b559 are typical for preparations of PS II membrane fragments from higher plants: HP form with E_m of 370–400 mV, IP form with E_m of 170–250 mV and LP form with E_m of 50–140 mV [59–62]. The factors that establish the identities of the redox forms of Cyt b559 are unknown, several types of structural modifications of the heme microenvironment have been considered as possible determinants such as mutial orientation of the planes of the histidin ligands [63], the difference in the protonation and H-bonding pattern [59,64–67], polarity of dielectric environment of the heme [68] and a replacement of one of the histidin ligands [62]. The dominating redox form of Cyt b559 in PS II membrane fragments, the HP form, comprising typically 70% of total Cyt b559, is unstable towards various samples treatments and may convert into lower potential forms.

Photoreactions of Cyt b559 were intensively studied during several last decades. Reduced Cyt b559 is a terminate electron donor for $P680^{+\bullet}$ in samples with not functioning OEC [37,39,40,69–73], photooxidation of Cyt b559 is mediated by nearby molecules of Chl and Car [39,74–76]. Cyt b559 can be photoreduced via a DCMU-inhibited reaction in membrane preparations of PS II [35,36,38,41,59,77–79]. In thylakoid membranes with prephotooxidized Cyt b559 reduction of the PQ pool with a half time of 6–10 ms following saturating CW illumination largely precedes accumulation of reduced Cyt b559 occurring with a half time of ~100 ms; PQH_2 of the pool is considered as a donor for oxidized Cyt b559 [35,36]. Alternative slower routes of Cyt b559 reduction that are obviously Q_B-site independent reactions involving Pheo and/or Q_A may operate in solubilized preparations of PS II [33,43,80–82] or in membrane samples of PS II with an impaired function of the PQ pool reduction, i.e. under photoinhibiting conditions [44,57,83], in the presence of a detergent [51,53] or under conditions of dehydration [73].

Preparations of PS II membrane fragments that contain a membrane pool of PQ and are largely devoid of PS I and Cyt b_6f complexes are a convenient object to investigate the process of Cyt b559 photoreduction. In this sample type where Cyt b559 is normally predominantly reduced, light induced reduction of its oxidized lower potential forms was studied [51,53,55–58,83–85] as well as of the forms that were prephotooxidized under strong photoinhibiting illumination [44] or at higher pH [41,44]. Photoreduction of Cyt b559 that was present in its native HP redox form was investigated in samples of PS II membrane fragments containing $K_3Fe(CN)_6$ (FeCy) [59], stable reduction of Cyt b559 observed in that case only under illumination was shown to proceed via a Q_B (PQ pool)-mediated pathway.

In the study on the DCMU-insensitive redox reactions of LP Cyt b559 with the artificial plastoquinones in illuminated Triton X-100 solubilized PS II membrane fragments two more quinone binding sites were suggested in the PS II complex besides Q_A and Q_B [51]. As assumed, in one of these sites termed Q_C PQ is tightly bound in a polar environment and mediates reduction of LP Cyt b559 from Pheo via the semiquinone form while in the other site Q a weakly bound PQ that is probably in exchange with the pool participates in dark oxidation of LP Cyt b559.

It was further found that some chemical compounds, termed L, including the PS II herbicides DCMU and dinoseb, the so-called ADRY reagents [86] and the lipophilic anion tetraphenylboron (TPB), cause concentration dependent negative shifts in the E_m value of HP Cyt b559 of PS II membrane fragments [87]. This effect of the reagents L closely resembled the reversible modification of the potentiometric properties of Cyt b559 caused by extraction of PQ from the thylakoid membrane [88–90]. Accordingly, it was assumed that the PS II complex bears a quinone site different from Q_A and Q_B that interacts with Cyt b559 and regulates its redox potential [87,91]. Binding of PQ in this site named Q_C was suggested to be the factor that determines the HP form of Cyt b559.

In the recent crystallographic study carried out in PS II complexes from *T. elongates* with resolution of 2.9 Å the third bound PQ molecule was found that was called Q_C [20]. It is settled in a large cavity with an entrance from the membrane space different from the Q_B channel and its head is located at an edge-to-edge distance of 13 Å from both the head of the Q_B and the heme group of Cyt b559.

The present work addresses properties of Cyt b559-PQ redox interactions. In preparation of PS II membrane fragments with preoxidized HP Cyt b559 reduction of Cyt b559 by quinols (either light-formed inside the membrane pool or extra added to unilluminated samples) was studied. Specific features of the redox reaction of oxidized Cyt b559 with quinols were revealed and implications for the redox chemistry of Cyt b559 were considered. Some data of this work have appeared in a short form [92].

2. Materials and methods

PS II membrane fragments were isolated from sugar beet leaves according to the procedures described in Refs. [93,94]. Most of the assays were carried out in a medium containing 0.3 M sucrose, 100 mM MES (pH 6.5), 10 mM $CaCl_2$, 10 mM NaCl and 10% glycerol. For the experiments at pH 7.6 and 8.3 MES was changed for HEPES and tricine, respectively. Samples exhibited the rate of oxygen evolution of 500 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} with 0.5 mM 2,6-dichloro-1,4-benzoquinone and 1 mM FeCy as electron acceptors at pH 6.5 and 20 °C. PS II membrane fragments with a high content of oxidized HP Cyt b559 were obtained as described previously [73]. The procedure included 1 min incubation of samples in the presence of 2 mM FeCy followed by three subsequent washing steps in a buffer not containing the oxidant. Intermediate extents of reduction of Cyt b559 in preparations of PS II membrane fragments were achieved by the treatment procedure similar to one described above with a difference that the incubation medium contained 0.5 mM FeCy and variable amounts of $K_4Fe(CN)_6$. Anaerobic conditions were created with glucose/glucose oxidase system as described earlier [62]. Anaerobic redox titration of Cyt b559 in PS II membrane fragments was performed as in work [62]. Chlorophyll concentration in samples was determined as in Ref. [95] and typically was about 30–40 μg/ml.

Light induced reduction of Cyt b559 was initiated either by saturating flashes provided by a frequency-doubled Q-switched Nd:YAG laser (Nd:YAG LS-2130, 532 nm, full width at half-maximum of 11 ns, 15.5 mJ/pulse) or by red CW light obtained from a KL 1500 LSD lamp (Schott, Germany) equipped by RG and KG3 filters allowing a sharp cut-off towards wavelengths shorter than 580 nm and longer than 750 nm. The extent of reduction of Cyt b559 was estimated from the amplitude at 560 nm in the redox difference spectra of Cyt b559 registered in the interval 530–580 nm in 1-cm cuvette with a spectrophotometer Cary 4000 (Varian) supplemented with a home-built stirrer. The time of recording of the Cyt b559 redox difference spectra consisted of 10 s. The fully oxidized and fully reduced states of Cyt b559 were obtained in sample suspensions containing 50–100 μM

FeCy or small amount of dry dithionite, respectively. In the reactions of light induced and chemical reduction of Cyt b559 the extent of rapidly reduced Cyt b559 was determined from the Cyt b559 difference spectra recorded after the end of sample illumination or 7 s after addition of the exogenous reductant. The $Q_A^{-\bullet}$ concentration in samples of PS II membrane fragments was determined by using extinction coefficient of 3.5 mM^{-1} cm^{-1} at "543 minus 550 nm" in reduced minus oxidized difference spectra [41,96]. Chl fluorescence measurements were carried out with PAM-100 fluorometer (Walz, Germany).

Most of the chemicals including quinones HQ, DQ and DPQ were purchased from Sigma. Quinols $DPQH_2$ and DQH_2 were obtained from the correspondent quinones by the method described in Ref. [97] and were stored in acidified ethanol in sealed vials under an argon atmosphere, at −80 °C. $DPQH_2$ concentration was determined by using extinction coefficient of 3.4 mM^{-1} cm^{-1} at 290 nm [98].

3. Results

In dark adapted samples of untreated PS II membrane fragments used in the present investigation 60–65% of Cyt b559 population attained the reduced form. In order to study in this preparation type reduction of Cyt b559 by quinols it is worth to increase the percentage of oxidized Cyt b559. To get this, samples were shortly incubated in the presence of 2 mM FeCy that was further removed by several washing steps (see Materials and methods section). As a result, a type of PS II membrane fragments preparation was obtained that contained more than 80% of Cyt b559 in the oxidized state and, together with that, retained high activity of oxygen evolution. Redox titration of Cyt b559 in such type of samples (further called as FeCy–washed PS II membrane fragments or PS II membrane fragments with preoxidized Cyt b559) revealed the following composition of the redox forms: 76% of Cyt b559 was present in the HP form with $E_m = +380$ mV, 17% in the IP form with $E_m = +221$ mV and 7% in the LP form with $E_m = +82$ mV. This redox pattern of Cyt b559 is typical for preparations of PS II membrane fragments [60–62].

3.1. PQ pool mediated photoreduction of Cyt b559 in PS II membrane fragments

Fig. 1 shows light-induced and chemically induced absorbance changes in a sample of PS II membrane fragments with preoxidized Cyt b559. The "light minus dark" difference spectrum *a* taken following 5 s illumination by red light of PFD = 50 μmol m^{-2} s^{-1} reveals two types of spectral signals: the absorbance increase at 560 nm associated with reduction of Cyt b559 and the so-called C550 signal characterized with a trough at 550 nm and a peak at 543 nm indicative of $Q_A^{-\bullet}$ formation [96,99,100]. The amplitude of the C550 signal in spectrum *a* corresponds to reduction of $Q_A^{-\bullet}$ in about 40% of PS II centers. The photoaccumulation of $Q_A^{-\bullet}$ indicates that the endogenous pool of PQ becomes reduced upon sample illumination and reoxidation of $Q_A^{-\bullet}$ via Q_B is blocked. Further quantitative estimations in Fig. 1 reveal that 88% of Cyt b559 were oxidized in unilluminated preparation of FeCy–washed PS II membrane fragments and that only 22% of total Cyt b559 became rapidly reduced following illumination. The low yield of reduced Cyt b559 in a sample with the reduced quinone acceptor pool is striking taking into account the rather positive E_m of the predominant, HP, form of Cyt b559. The data show that 10 min incubation in the dark of the illuminated sample results in an increase in the amount of reduced Cyt b559 so that the full extent of photoreduced Cyt b559 approximately doubles (curve *b*). A partial reoxidation of reduced Q_A also occurs during dark incubation as seen from the negative sign of the C550 signal in spectrum *b*.

In the following experiments the extent of reduced Cyt b559 in shortly illuminated PS II membrane fragments was determined as a function of the time period after illumination. The amplitudes of Cyt b559 reduction were found from the correspondent difference spectra recorded at the definite time intervals after sample illumination similar to curve *a* in Fig. 1. This way of registration of the kinetic curves substantially diminished the error bar in the estimated amplitudes of the Cyt b559 redox changes due to confident corrections of baseline instabilities, however, limited the kinetic resolution to 10 s (the minimal time of spectrum recording in our measurements). Typical time curves of the Cyt b559 redox changes following short illumination of PS II membrane fragments preparations are presented in Fig. 2.

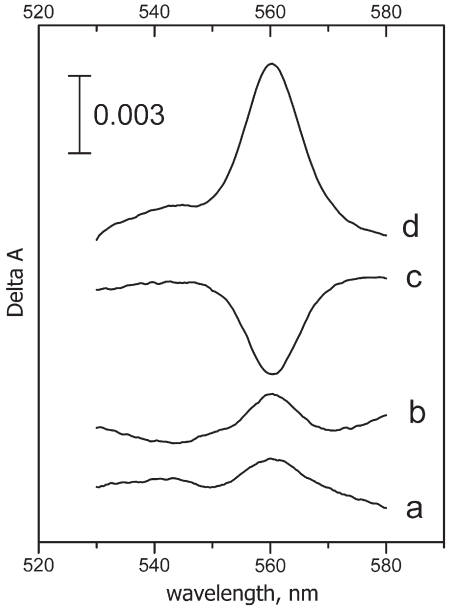

Fig. 1. Redox difference spectra of Cyt b559 in PS II membrane fragments. The difference spectra were obtained in FeCy-washed PS II membrane fragments just after 5 s illumination at 50 μmol m^{-2} s^{-1} (*a*), after 10 min incubation of an illuminated sample in the dark (*b*), after addition of 100 μM FeCy to an illuminated and dark incubated sample (*c*) and after addition of the saturating amount of dithionite to a sample with fully oxidized Cyt b559 (*d*). Chl concentration was 37 μg/ml. Smoothing of the curves was done using an Origin program.

Illumination of a sample with preoxidized Cyt b559 with a light pulse of PFD = 50 μmol m^{-2} s^{-1} results in a biphasic increase in

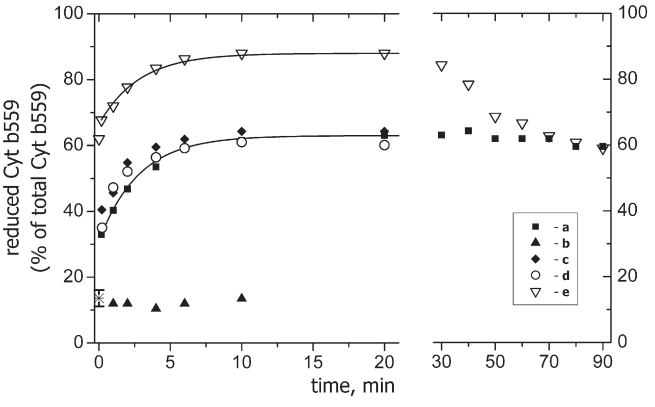

Fig. 2. Relative amplitude of reduced Cyt b559 in FeCy–washed (curves *a*–*d*) and untreated (curve *e*) PS II membrane fragments as a function of the time interval after 5 s illumination at 50 μmol m^{-2} s^{-1}(*a,b,d,e*) and 1400 μmol m^{-2} s^{-1}(*c*). Sample *b* contained 40 μM DCMU, while sample *d* was supplemented with the system glucose/glucose oxidase to maintain anaerobiosis. The star point with the error bar indicates the average extent of initially reduced Cyt b559 in samples *a*–*d*. Full lines in curves *a* and *e* represent exponential fittings of the slow phase of Cyt b559 reduction with the relative amplitudes of 31% and 20%, respectively, and the rate constant of (2.8 min) $^{-1}$. For further details, see Materials and methods section.

Table 1
Properties of light induced reduction of Cyt b559 in PS II membrane fragments of sugar beet with preoxidized Cyt b559.[a]

pH	Extent of initially reduced Cyt b559 (% of total Cyt b559)	Amplitude of phase I (% of total Cyt b559)	Amplitude of phase I (% of initially oxidized Cyt b559)	Amplitude of phase II (% of total Cyt b559)	Rate constant of phase II, $(min)^{-1}$
5.9	11	11	12	32	0.31 ± 0.01
6.5	14	19	22	30	0.37 ± 0.05
7.6	23	21	27	36	0.42 ± 0.03
8.3	23	25	32	34	0.71 ± 0.03

[a] Aerobic suspensions of FeCy-washed PS II membrane fragments were illuminated for 5 s at 50 μmol m^{-2} s^{-1}. Average values of 3–7 measurements are presented. The error bar in the estimated amplitudes of reduced Cyt b559 did not exceed ±3%.

the amount of reduced Cyt b559 (curve *a*). A fast process, in the following termed phase I, comprises photoreduction of 19% of total Cyt b559 and is already completed by the moment of registration of the first light induced difference spectrum. It is followed by a slower phase II of reduction proceeding in the dark, that is approximated by an exponent with $k_v = (2.8\ min)^{-1}$ and the amplitude of 31%. 40 μM DCMU (curve *b*) and 50 μM dinoseb (not shown) fully prevented reduction of Cyt b559 following illumination at 50 μmol m^{-2} s^{-1}. These findings indicate that the functionally active site Q_B is indispensable for both fast and slow reactions of Cyt b559 photoreduction.

Stronger actinic illumination brought about only insignificant changes in the biphasic pattern of Cyt b559 reduction. In the case of red actinic light of PFD = 1400 μmol m^{-2} s^{-1} the fast component of Cyt b559 reduction slightly increased in the amplitude at the expense of the slow component exhibiting 26% of Cyt b559 reduced in phase I and 24% in phase II (curve *c* in Fig. 2). This change was accompanied by an increase in the rate constant of phase II to $(2.1\ min)^{-1}$. Increasing of the time of illumination at 1400 μmol m^{-2} s^{-1} to 10 s did not change the pattern of Cyt b559 photoreduction (not shown). The fact that the time curve of light induced reduction of Cyt b559 is almost invariant to more than 20 fold increase in the PFD of actinic light indicates that the kinetic biphasicity observed does not stem from unsaturation of the light-dependent PS II turnover. Likewise, curve *d* shows that removal of O_2 from a sample solution does not significantly affect the pattern of Cyt b559 photoreduction suggesting that the biphasic kinetics is not a result of partial autoxidation of photoreduced Cyt b559.

The pattern of Cyt b559 photoreduction in untreated PS II membrane fragments where Cyt b559 is initially reduced by 62% (curve *e*) is characterized with a small contribution of the fast phase comprising 6% of total Cyt b559 whereas the slow component of photoreduction amounting 20% closely resembles that observed in samples pretreated with FeCy. The time curve very similar to curve *e* was obtained when a sample with preoxidized Cyt b559 once illuminated and dark adapted for 10 min was secondly illuminated (not shown). The similarity in the slow components of Cyt b559 photoreduction in an untreated sample and samples with chemically preoxidized Cyt b559 suggests that the slow rate of Cyt b559 reduction is not a feature introduced by the pretreatment with FeCy.

When the slow phase of Cyt b559 photoreduction in FeCy-washed samples was completed the concentration of reduced Cyt b559 reached 63 ± 3% and remained unchanged in the dark for at least 90 min (see curve *a* in Fig. 2). This fact demonstrates that redox equilibrium between Cyt b559 and the ambient redox potential is attained within 10 min after illumination and shows that reduced Cyt b559 is resistant to autoxidation. In contrast to the data in FeCy-washed preparations, in illuminated samples of untreated PS II membrane fragments reduced Cyt b559 attained maximally 88% and its extent slowly decreased in the dark to an equilibrium value of near 60% (curve *e*), i.e. to the same level as observed in illuminated FeCy-washed samples and unilluminated native PS II membrane fragments. These data indicate that Cyt b559 reduced over 65% is autoxidizable. The dependence of the final redox state of Cyt b559 reached in illuminated samples on the extent of initially reduced Cyt b559 is most likely attributable to a low PQ:Cyt b559 ratio in PS II membrane fragments preparations [41,101–103].

Experiments on light induced reduction of Cyt b559 in samples of FeCy – washed PS II membrane fragments were carried out at different pH. The data on the extents of phases I and II as well as on the rate of phase II of Cyt b559 reduction following 5 s illumination at 50 μmol m^{-2} s^{-1} are presented in Table 1. The data show that the amplitude of phase I increases with the rise of pH from 5.9 to 8.3. With increasing pH, the extent of reduction of Cyt b559 in unilluminated samples of FeCy–washed PS II membrane fragments also increased. Therefore, the effect of pH on the extent of phase I appears even more pronounced when the amplitude of the fast phase is determined relative to the fraction of initially oxidized Cyt b559 (see Table). At pH 7.6–8.3 about 30% of oxidized Cyt b559 get photoreduced in the fast reaction. This amount dramatically decreases at pH < 6.5 so that the data may indicate the existence of a

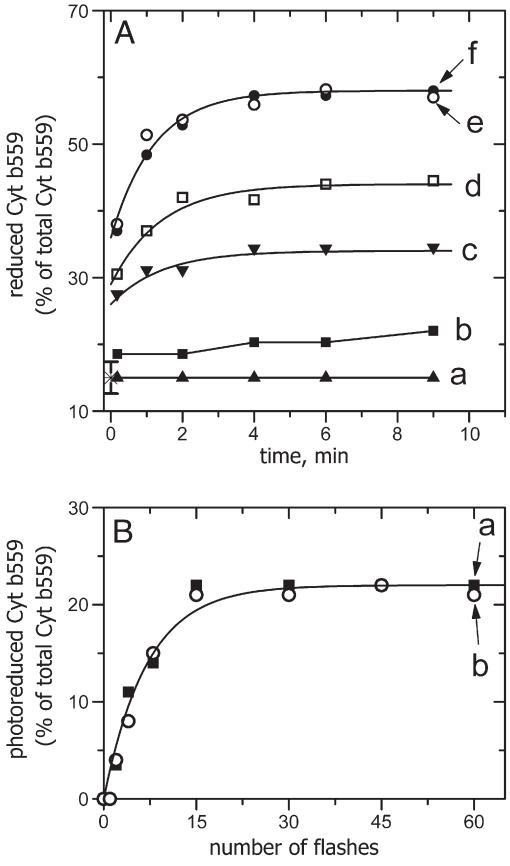

Fig. 3. Reduction of Cyt b559 in anaerobic samples of FeCy-washed PS II membrane fragments following a 5 Hz train of saturating laser flashes. (A) Relative amplitude of reduced Cyt b559 as a function of the time interval after illumination of a sample by 1 (*a*), 2 (*b*), 4 (*c*), 8 (*d*), 15 (*e*) and 30 (*f*) flashes. Full lines in curves *c*, *d* and *e* represent exponential fittings of the slow phase of Cyt b559 reduction with the relative amplitudes of 8%, 15% and 21%, respectively, and the rate constant of $(1.4\ min)^{-1}$. (B) Amplitudes of the fast (*a*) and slow (*b*) components of Cyt b559 photoreduction as functions of flash number. For further details, see legend to Fig. 2.

pK in the low pH region for the extent of the fast component of Cyt b559 photoreduction. In contrast to phase I, the amplitude of phase II was almost unaffected by changes in pH, instead, the rate of the slow component exhibited a pH dependence increasing from $(3.2\ \text{min})^{-1}$ to $(1.4\ \text{min})^{-1}$ upon a change in pH from 5.9 to 8.3.

The experiments described so far were carried out under conditions of multiple turnover irradiation that provided saturation in the extent of the reduced acceptor pool in illuminated samples. Another approach is conditions of a variable PQ/PQH_2 ratio of the pool. Fig. 3 presents data on reduction of Cyt b559 in anaerobic samples of FeCy–washed PS II membrane fragments following different number of saturating laser flashes in a 5 Hz train. Panel A displays patterns of Cyt b559 reduction induced by 1–30 flashes. As shown by curve *a*, illumination of a sample with one flash results in no increase in the amount of reduced Cyt b559. Two sequential flashes evoke only insignificant fast rise in the extent of Cyt b559 reduction followed by a small slow augment in the concentration of reduced Cyt b559 (curve *b*). In total, the increase in Cyt b559 reduction induced by two successive flashes did not exceed 10%. With progressive flash number the yield of photoreduced Cyt b559 increases and the characteristic two-component pattern clearly appears (curves *c-f*). The rate of the slow phase of Cyt b559 reduction following 4–30 flashes is approximated by an exponent with $k_v = (1.4\ \text{min})^{-1}$. The time curves of Cyt b559 photoreduction in samples illuminated with 15 and more flashes match (curves *e*,*f*) and closely resemble ones observed with CW light.

Panel B in Fig. 3 displays the amplitudes of the fast (*a*) and slow (*b*) components of Cyt b559 photoreduction as functions of flash number. The data reveal the ratio of 1 in the extents of two phases of Cyt b559 reduction following 2 and more flashes. Saturation in the amplitudes of both components at a value of 22% is reached after about 15 flashes. It is reported that full reduction of the endogenous PQ pool in preparations of oxygen-evolving PS II membrane fragments requires 12–15 saturating flashes [101,102,104]. The latter number is virtually the same as found in the experiment of Fig. 3 for the amplitudes of the two components of Cyt b559 photoreduction. This shows that the increase in the extents of both rapidly and slowly reduced Cyt b559 occurs in parallel with accumulation of PQH_2 in the membrane pool.

The data presented above revealed that in samples of PS II membrane fragments with preoxidized Cyt b559 only a fraction of Cyt b559 (about a half of photoreduced Cyt b559) could be rapidly reduced following a short illumination while the other reduced Cyt b559 was accumulated much slower in the dark. One candidate for the electron donor in phase II of Cyt b559 photoreduction could be $Q_A^{-\bullet}$ provided that the long range electron transfer (on a distance of 39 Å [20,21]) is mediated by an intermediate carrier to account for the experimentally observed reaction rates in the minutes time domain (see Ref. [105] for the rate-distance relationship). To check for a possibility of involvement of $Q_A^{-\bullet}$ in the slow photoreaction of Cyt b559, the kinetics of Chl fluorescence decay was investigated in illuminated FeCy–washed PS II membrane fragments. The decay of F_v following 5 s illumination at 50 μmlol $m^{-2}\ s^{-1}$ monitored during 10 min after the light pulse was approximated by a sum of three components with rates of $(1.1\ \text{s})^{-1}$, $(4.3\ \text{s})^{-1}$, $(33\ \text{s})^{-1}$ and a constant and relative amplitudes of 53%, 18%, 15% and 14%, respectively (not shown). The two faster F_v decay rates are attributable to charge recombinations within the states $S_2\ Q_A^{-\bullet}$ and $S_3\ Q_A^{-\bullet}$ [106,107]. The fraction of slower decaying and non-decaying $Q_A^{-\bullet}$ may originate from long living dipoles formed upon oxidation of secondary donors. The data obtained reveal no correspondence between the kinetics of $Q_A^{-\bullet}$ reoxidation and that of the slow component of Cyt b559 photoreduction. On the basis of this result we conclude that phase II of light induced reduction of Cyt b559 is not due to electron transfer from $Q_A^{-\bullet}$.

As reported, photoreduction of Cyt b559 in FeCy–washed PS II membrane fragments can be blocked by the Q_B site inhibitors DCMU and dinoseb. The experiments of Fig. 4 address the effect of DCMU on the pattern of Cyt b559 photoreduction in more detail. Curve *a* shows that DCMU promptly added to an illuminated sample of FeCy-washed PS II membrane fragments does not affect the pattern of Cyt b559 reduction. This sharply contrasts with the finding that DCMU present in a sample suspension during illumination totally inhibits reduction of Cyt b559 (Fig. 2). The data thus indicate that DCMU itself does not affect the redox reaction between oxidized Cyt b559 and the reduced quinone pool. Note that in the experiment presented by curve *a* in Fig. 4 phase I of Cyt b559 reduction is likely completed before DCMU is added to the illuminated sample taking into account the rate of the fast reaction of Cyt b559 with PQH_2 [35,36]. Since phase II of Cyt b559 photoreduction is not affected by DCMU, we conclude that the slow redox reaction of Cyt b559 is not associated with electron transfer to the oxidized heme group from the reduced plastoquinone Q_B.

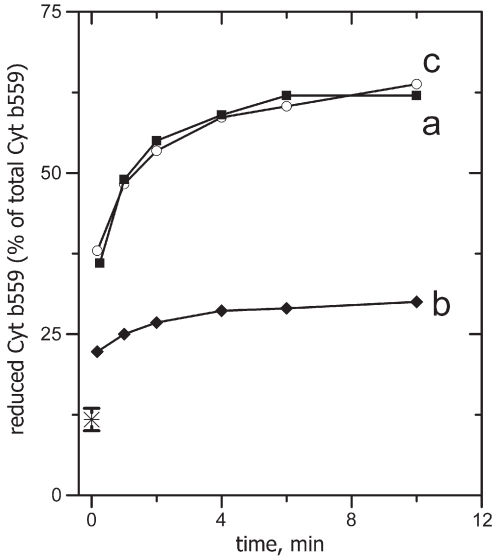

Fig. 4. Effect of DCMU on the pattern of Cyt b559 photoreduction in PS II membrane fragments with preoxidized Cyt b559. Samples were illuminated for 5 (*a*) or 10 s (*b*,*c*) at 50 μmol $m^{-2}\ s^{-1}$ (*a*,*b*) or 1400 μmol $m^{-2}\ s^{-1}$ (*c*) in the absence (*a*) or in the presence of 3 μM DCMU (*b*,*c*). In sample *a* 20 μM DCMU was added just after the end of illumination.

Curves *b* and *c* in Fig. 4 present the patterns of Cyt b559 reduction in samples of FeCy–washed PS II membrane fragments illuminated for 10 s at 50 μmol $m^{-2}\ s^{-1}$ and 1400 μmol $m^{-2}\ s^{-1}$, respectively, in the presence of 3 μM DCMU. At this DCMU concentration more than 90% of the PS II centers bind the inhibitor at the Q_B site. The data show that reduction of Cyt b559 in samples containing 3 μM DCMU is noticeably suppressed only in the case of weak actinic illumination and remains unaffected under conditions of the high PFD. We further found that in samples supplemented with 1–10 μM DCMU, unlike in a control not containing the inhibitor, the amplitudes of the fast and slow components of Cyt b559 photoreduction became functions of the illumination time within 5–10 s period (data not shown). These results demonstrate that the extent of Cyt b559 reduction reached in samples illuminated in the presence of DCMU depends on the amount of photoaccumulated PQH_2 and show that reduction of the whole PQ pool in subinhibited PS II samples may occur via the Q_B site turnover in a fraction of uninhibited PS II centers. Time curve *b* in Fig. 4 shows also that when the yield of photoreduced Cyt b559 is noticeably diminished, the pattern of Cyt b559 photoreduction remains biphasic with nearly equal in the amplitudes kinetic components. Interestingly enough to note a close similarity between the pattern of Cyt b559 reduction following illumination at 50 μmol $m^{-2}\ s^{-1}$ in the presence of 3 μM DCMU (curve *b* in Fig. 4) and that observed in a quite different sample type - uninhibited PSII membrane fragments illuminated with

4 flashes (curve *c* in Fig. 3A). The similarity in the two time curves likely suggests that similar extents of the PQ pool reduction are attained in samples following the two different experimental protocols.

The results reported in Fig. 4 are indicative of pool behavior of the reductant for oxidized Cyt b559, PQH_2. The data of Fig. 5 provide further illustration of this phenomenon. As shown in the figure, the rate of oxygen evolution (curve *a*) and the extents of phase I of Cyt b559 reduction following illumination at 50 (curve *b*) and 1400 μmol m^{-2} s^{-1} (curve *c*) are quite different functions of the concentration of DCMU. A value of 0.16 μM is obtained for the constant of inhibition (K_i) by DCMU of oxygen evolution in our preparation of PS II membrane fragments from sugar beet. This number is practically the same at obtained in the measurements on the rate of photoreduction of 2,6-dichlorophenolindophenol in spinach thylakoids [108] and is close to the values reported for K_D of DCMU at the Q_B site [22,28,29]. As follows from the data of Fig. 5, the inhibiting effect of DCMU on the extent of rapidly photoreduced Cyt b559 is substantially weaker than the effect on the rate of oxygen evolution, and, moreover, it is PFD-dependent. The latter feature was already noted in Fig. 4. Values of 2.1 and 14.1 μM are determined for the constant of inhibition by DCMU of phase I of Cyt b559 reduction following illumination at 50 and 1400 μmol m^{-2} s^{-1}, respectively (Fig. 5). One may see that even the lower from the two latter K_i values exceeds the corresponding number obtained in the oxygen evolution measurements by more than an order of magnitude.

The data of Fig. 5 show that under the experimental conditions used in this work photoreduction of Cyt b559 is a reaction mediated by the PQ pool. Our results corroborate the former conclusion that the light induced reduction of Cyt b559 in membrane preparations of PS II occurs from reduced PQ of the pool [36,38,59,78,79] and, together with that, are consistent with the results of the numerous studies in thylakoid membranes reporting a lack of sensitivity to DCMU or only a weak dependence on DCMU of Cyt b559 photoreduction [34,38,71,109–111].

3.2. Chemical reduction of Cyt b559 in PS II membrane fragments by one- and two-electron donors

The results reported above addressed properties of reduction of Cyt b559 in PS II membrane fragments by the light formed PQH_2. To obtain some insight as to a mechanism of the biphasic photoreaction of Cyt b559 it is worth to study in the same sample type the reaction of chemical reduction of Cyt b559 by exogenous quinols. Fig. 6 presents time curves of reduction of Cyt b559 in FeCy-washed PS II membrane fragments in the dark by a number of artificial reductants: HQ, DQH_2 and $K_4Fe(CN)_6$.

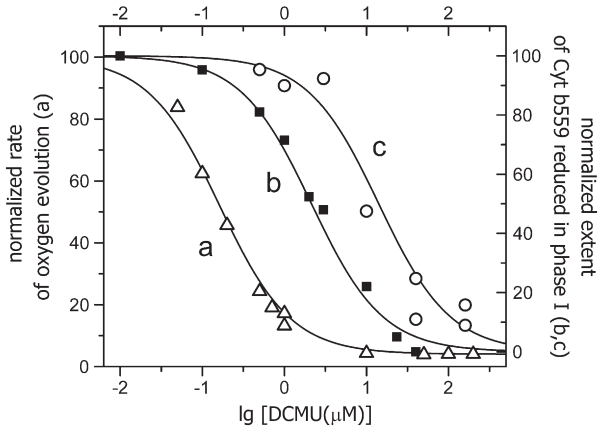

Fig. 5. Effect of DCMU on the rate of the steady-state oxygen evolution (*a*) and on the extent of phase I of Cyt b559 reduction in FeCy-washed PS II membrane fragments following 10 s illumination at 50 μmol m^{-2} s^{-1} (*b*) and 1400 μmol m^{-2} s^{-1} (*c*). In curves *b* and *c* the amplitudes of phase I were normalized relative to the maximal values observed in uninhibited samples at the correspondent PFDs.

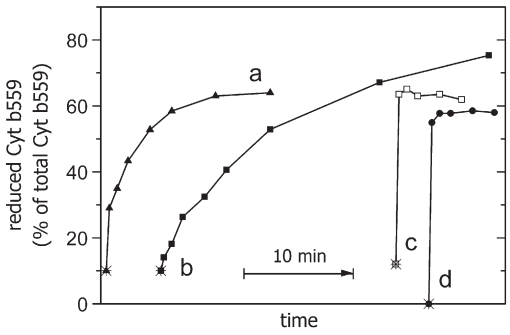

Fig. 6. Patterns of chemical reduction of Cyt b559 in FeCy-washed PS II membrane fragments following addition of 4 mM HQ (*a*,*d*), 30 μM DQH_2 (*b*) or 8 mM $K_4Fe(CN)_6$ (*c*). Sample *d* was supplemented with 0.25 mM FeCy before addition of HQ. In each curve, the star point indicates the extent of reduced Cyt b559 before addition of the reductant.

The pattern of chemical reduction of Cyt b559 by 4 mM HQ is biphasic with a fast component comprising 19% of total Cyt b559 (curve *a*), whereas reduction of Cyt b559 by 30 μM DQH_2 exhibits virtually no fast phase (curve *b*). With both quinols, prominent slow redox changes of Cyt b559 were registered in the time domain of tens of minutes resembling the slow component of Cyt b559 reduction in illuminated samples. In a sharp contrast to the reaction with quinols, the redox reaction of oxidized Cyt b559 with much weaker but one electron reductant $K_4Fe(CN)_6$ is a fast process that is completed within the experimental dead-time (curve *c*). These data nicely illustrate the remarkable difference in the rates of reduction of Cyt b559 by one- and two-electron donors. A slow rate of reduction of cytochromes by free quinols is attributed to a high energy barrier for formation of one-electron intermediate reductants – the deprotonated quinol or semiquinone [112–114].

It is worth to note that the extent of stable reduction of Cyt b559 in the presence of HQ is a parameter of the usual test for a content of HP Cyt b559 in preparations of PS II. However, as far as we are aware, a feature of slow reduction of HP Cyt b559 by HQ has not been specially noted in the literature and that circumstance was puzzling for us. Therefore, we reproduced the experimental conditions of the standard "HQ test" for the redox state of Cyt b559 in the experiment reported by curve *d* in Fig. 6, where 4 mM HQ was added to a sample of PS II membrane fragments contained 0.25 mM FeCy. In a striking contrast to the experiment with HQ alone (curve *a*), addition of HQ to a sample containing FeCy results in rapid reduction of Cyt b559, in full agreement with the numerous previous observations (see, for instance, Refs. [115,116]). An acceleration of the redox reaction of Cyt b559 with HQ in the presence of small amount of FeCy can be attributed to an involvement of one-electron reductants $K_4Fe(CN)_6$ and/or semibenzoquinone in the Cyt b559 reduction process.

Fig. 7 presents time curves of reduction of Cyt b559 in the dark by $DPQH_2$, the chemical analog of PQH_2 with a short saturated alkyl tail that is highly partitioned into the membrane phase [117]. The data were collected in anaerobic samples of PS II membrane fragments containing either no herbicides (panel A) or 50 μM dinoseb (panel B). Several features emerge from an inspection of the data of Fig. 7: (i) the pattern of chemical reduction of Cyt b559 by $DPQH_2$ is biphasic and closely resembles the pattern of Cyt b559 photoreduction, indicating that the biphasicity in the Cyt b559 reduction is an inherent property of the chemical reaction between oxidized Cyt b559 of PS II membrane fragments and plastoquinols, (ii) the extent of the fast phase of Cyt b559 reduction by $DPQH_2$ (phase I) increases with the rise in the concentration of the added quinol until the maximal value consisting of about 50% of initially oxidized Cyt b559 is reached at 13 μM $DPQH_2$ and remains unchanged with further increasing concentration of the quinol, (iii) in samples containing dinoseb the biphasic time courses of chemical reduction of Cyt b559 by $DPQH_2$

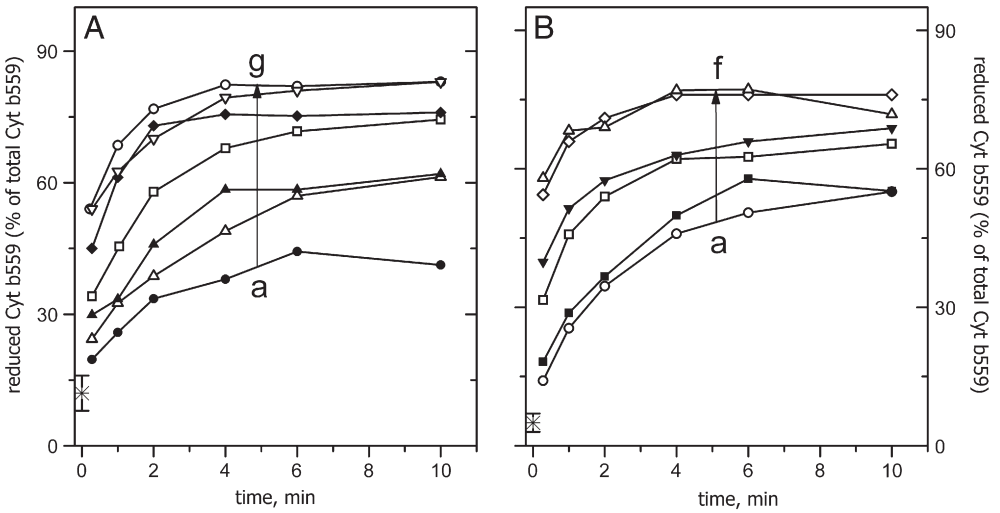

Fig. 7. Patterns of chemical reduction of Cyt b559 by $DPQH_2$ in anaerobic samples of PS II membrane fragments with preoxidized Cyt b559 containing either no herbicides (A) or 50 μM dinoseb (B). $DPQH_2$ was added to samples *a*–*g* at concentrations of 0.4, 0.65, 1.15, 2, 4.9, 13 and 78 μM, respectively (panel A) and to samples *a*–*f* at concentrations of 3.3, 9.1, 16.9, 26, 65 and 130 μM, respectively (panel B). The arrows indicate the labeling of the different curves from *a* to *g* (A) and from *a* to *f* (B).

qualitatively closely resemble ones obtained in the absence of the herbicide, (iv) dinoseb noticeably diminishes the amplitude of phase I of Cyt b559 reduction by $DPQH_2$, (v) in the presence of 50 μM dinoseb approximately ten times higher concentrations of $DPQH_2$ are necessary to provide the extents of phase I similar to that observed in samples not containing the herbicide. The data show that dinoseb impairs the efficiency of the rapid redox reaction between oxidized Cyt b559 and $DPQH_2$, however this inhibiting effect can be overcome by increasing the concentration of the quinol.

One more important result emerges from a comparison of the time curves of chemical reduction of Cyt b559 by HQ, DQH_2 and $DPQH_2$ shown in Figs. 6 and 7. It appears that the ambient redox potential established in samples in the presence of the quinols is not a factor that controls the amplitude of phase I of Cyt b559 reduction. Indeed, at the similar extents of Cyt b559 reduction attained in the presence of 30 μM DQH_2 (curve *b* in Fig. 6), 2 μM $DPQH_2$ (curve *d* in Fig. 7A) and 4.9 μM $DPQH_2$ (curve *e* in Fig. 7A) the amplitudes of phase I differ significantly in the three samples. Also, with a lower extent of reduced Cyt b559 reached in the presence of 4 mM HQ as compared to 30 μM DQH_2 (curves *a* and *b* in Fig. 6, respectively) much higher amplitude of phase I is observed in the former case. These data most likely indicate that a specific interaction of a quinol molecule with Cyt b559 determines the amplitude of the fast reduction of Cyt b559. Note that our results exclude a possibility that PQH_2 is rapidly formed in the endogenous membrane pool of PS II samples in the presence of the relatively strong reductants DQH_2 and $DPQH_2$ (the E_{m7} values of DQ and DPQ are +60 mV and +110 mV [118], respectively). This conclusion is in a correspondence with a low rate of the quinol-quinone transhydrogenase reaction in free quinones [119,120].

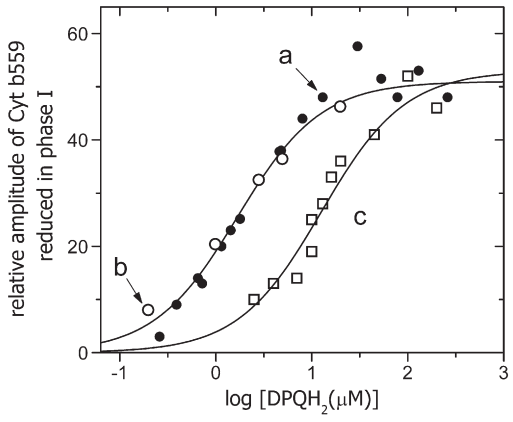

Fig. 8. Relative extent of phase I of Cyt b559 reduction by $DPQH_2$ in anaerobic FeCy-washed PS II membrane fragments containing either no herbicides (a) or supplemented with 30 μM DCMU (b) or 50 μM dinoseb (c) as a function of the $DPQH_2$ concentration. For further details, see text.

The data shown in Fig. 8 provide further support for the idea of a specific interaction between Cyt b559 and a quinol reductant in phase I. In this figure, the extent of phase I of chemical reduction of Cyt b559 by $DPQH_2$ in FeCy-washed PS II membrane fragments estimated as a fraction of initially oxidized Cyt b559 is plotted as a function of the concentration of the added quinol (in a logarithmic scale of the $DPQH_2$ concentration). An inspection of the results of Fig. 8 reveals that the amplitudes of rapidly reduced Cyt b559 in samples containing no herbicides (curve *a*) and supplemented with 30 μM DCMU (curve *b*) or 50 μM dinoseb (curve *c*) are hyperbolic functions of the $DPQH_2$ concentration and their maximal values at saturating concentrations of $DPQH_2$ are limited at 51–53%. The data show no effect of DCMU (at a concentration specific for the binding at the Q_B) on the extent of phase I of the reaction of Cyt b559 with $DPQH_2$. In contrast, dinoseb diminishes the amplitude of Cyt b559 that is rapidly reduced by $DPQH_2$ by shifting the concentration curve to higher $DPQH_2$ concentrations; together with that, dinoseb does not influence the maximal extent of phase I of Cyt b559 reduction. K_{50} values for $DPQH_2$ were determined to be 1.67 μM in the absence of the herbicides or in the presence of DCMU and 12.6 μM in the presence of dinoseb. The character of action of dinoseb on the reaction of Cyt b559 with $DPQH_2$ indicates on a competition between the quinol reductant for Cyt b559 in phase I and dinoseb for a common binding site.

As noted, Cyt b559 is redox heterogeneous in preparations of PS II membrane fragments and in samples used in this study HP Cyt b559 comprises about 75% of total Cyt b559. Further experiments were conducted to understand how the different redox forms of Cyt b559 participate in the redox reaction with $DPQH_2$. For that purpose, the time courses of Cyt b559 reduction following additions of $DPQH_2$ were monitored in samples characterized by different extents of initially reduced Cyt b559 (see Materials and methods section). It is expected that with increasing extent of initial reduction of Cyt b559 a fraction of Cyt b559 attaining the oxidized state will be enriched by the lower potential forms, IP and LP, in accordance with the redox composition of Cyt b559 in the preparation used.

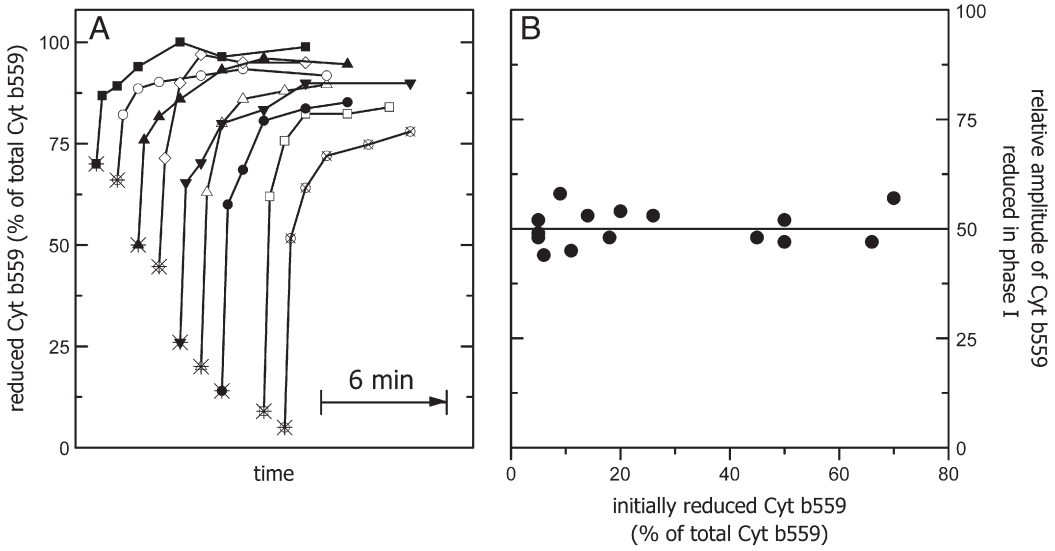

Fig. 9. Invariance of the relative amplitude of phase I of Cyt b559 reduction by 60 μM $DPQH_2$ in anaerobic samples of PS II membrane fragments to the initial redox state of Cyt b559. (A) Patterns of chemical reduction of Cyt b559 by $DPQH_2$ in samples with different extents of reduced Cyt b559. In each curve, the star point indicates the extent of reduced Cyt b559 before addition of the quinol. (B) Relative amplitude of phase I of Cyt b559 reduction by $DPQH_2$ as a function of the extent of initial reduction of Cyt b559. For further details, see text.

Panel A in Fig. 9 presents typical time courses of Cyt b559 reduction by 60 μM $DPQH_2$ in samples of PS II membrane fragments where the concentration of initially reduced Cyt b559 is varied from 5% to 70%. As follows from the figure, all the curves of Cyt b559 reduction are characterized with the biphasic patterns. Along with that property, the amplitude of phase I decreased with increasing initial reduction of Cyt b559. This feature of the redox reaction of Cyt b559 was observed also in the case of light induced reduction of Cyt b559 (see Fig. 2). In panel B of Fig. 9 the amplitude of phase I estimated relative to a percentage of initially oxidized Cyt b559 was plotted as a function of the extent of initial reduction of Cyt b559. The data clearly show that the relative fraction of Cyt b559 reduced in phase I in the presence of the saturating concentration of $DPQH_2$ is independent of the initial redox state of Cyt b559 comprising a half of oxidized Cyt b559. From this result we conclude that in the redox reaction with $DPQH_2$ all the species of oxidized Cyt b559 behave as a homogenous population.

3.3. Photoreduction of Cyt b559 in the presence of DPQ

The findings of the present study reveal close correspondence between the features of light induced reduction of Cyt b559 and chemical reduction by $DPQH_2$. Together with that, an important difference in the results gathered in the two types of the experiments was found and it consisted in the measured extents of phase I of Cyt b559 reduction. As seen from the data described above, the amplitude of the fast component of Cyt b559 reduction following saturating illumination comprises about 30% of the population of oxidized Cyt b559 (curve *c* in Fig. 2), while in the case of chemical reaction with $DPQH_2$ the maximal fraction of rapidly reduced Cyt b559 amounts 50% (Figs. 7, 8). The lower amplitude of phase I of Cyt b559 photoreduction as compared to chemical reduction is most likely a consequence of depletion of the quinone pool in preparations of PS II membrane fragments. Therefore, the photoreaction of Cyt b559 was studied in

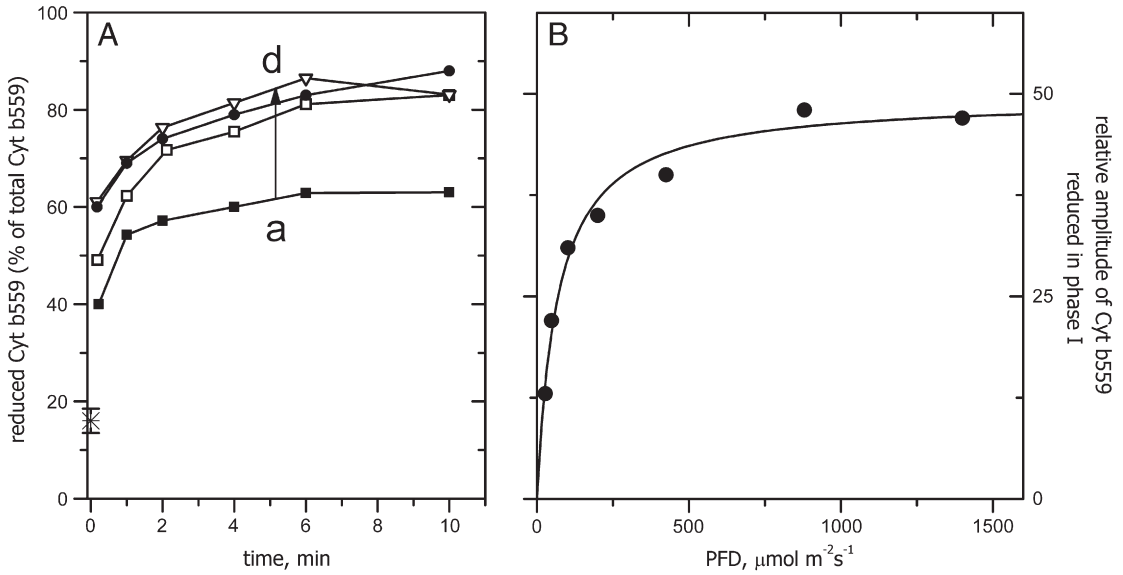

Fig. 10. Reduction of Cyt b559 in anaerobic suspensions of FeCy-washed PS II membrane fragments following 6 s CW illumination in the presence of DPQ. (A) Patterns of Cyt b559 reduction following illumination at 1400 μmol m^{-2} s^{-1} in samples containing either no further additions (*a*) or 3.5 (b), 20 (c) and 50 (d) μM DPQ. The arrow indicates the labeling of the different curves from *a* to *d*. (B) Relative amplitude of phase I of Cyt b559 photoreduction in the presence of 50 μM DPQ as a function of PFD of actinic illumination. The extent of phase I is determined relative to the amount of initially oxidized Cyt b559.

FeCy-washed samples supplemented with DPQ. The results obtained are reported in Fig. 10.

Panel A shows that reduction of Cyt b559 following illumination at 1400 μEm^{-2} s^{-1} of PS II membrane fragments containing DPQ exhibits biphasic kinetics. With increasing concentrations of the added DPQ the amplitude of phase I of Cyt b559 reduction raised until a maximal value consisting of ~50% of initially oxidized Cyt b559. Importantly to point out that the time courses of Cyt b559 photoreduction in the presence of DPQ closely resembled the kinetic curves of chemical reduction of Cyt b559 by the comparable amounts of $DPQH_2$ (see the data in Figs. 7A and 10A). This fact indicates that virtually quantitative reduction of DPQ to $DPQH_2$ occurs in illuminated samples in the experiments of Fig. 10A. An additional effect observed in preparations illuminated in the presence of DPQ is substantial increase in the full extent of photoreduced Cyt b559. Illumination of PS II membrane fragments supplemented with 20–50 μM DPQ results in almost 90% of reduced Cyt b559 suggesting that a relatively low ambient potential is attained, at a difference with samples not containing an exogenous quinone. Panel B of Fig. 10 reports the results of the experiment where the concentration of photoaccumulated $DPQH_2$ was varied via the rate of the light-driven PS II turnover. The data reveal that the fraction of oxidized Cyt b559 that becomes rapidly photoreduced in samples containing 50 μM DPQ increases with the rise in the PFD and attains a plateau at ~50% at the PFDs higher 700 μmol m^{-2} s^{-1}.

4. Discussion

4.1. The features of one-electron oxidation of quinols

Oxidation of free quinols by one-electron carriers proceeds rather slowly [112–114,121,122], the second order rate constants of 0.2 M^{-1} s^{-1} [114] and 80 M^{-1} s^{-1} [122] were found for the reaction of hydroquinone with horse heart Cyt c at pH 6 and plastocyanin at pH 7, respectively. The low rates of one-electron oxidation of quinols follow from the fact that hydroquinones are very poor one-electron reductants (E_m of higher 900 mV is evaluated for the couple $PQH_2/PQH_2^{+\bullet}$ [112]), and the actual electron donors are the thermodynamically unfavorable species – anionic quinol at pH<7 and anionic semiquinone at higher pH [112–114,121]. In proteins, the reactions of ubiquinol or plastoquinol oxidation take place in special quinone binding sites where one-electron reductants, deprotonated quinol or semiquinone, are formed much faster than in free quinols.

4.2. Reduction of Cyt b559 of PS II membrane fragments by quinols

In this work the mechanism of photoreduction of Cyt b559 and chemical reduction by exogenous quinols was investigated in PS II membrane fragments. The data on reduction of Cyt b559 following a short illumination indicate that PQH_2 accumulated in the endogenous membrane pool serves as a reductant for oxidized Cyt b559, in agreement with the general conclusion made in earlier studies in thylakoid membranes [36,38,78,79] and PS II membrane fragments [59].

A novel feature of the redox reaction of Cyt b559 with PQH_2 found in the present work is the characteristic biphasic pattern of the reaction. The first component of Cyt b559 photoreduction, phase I, most likely corresponds to the fast photoreaction of Cyt b559 observed in earlier studies in thylakoid membranes [38,77–79,109,110,123] and kinetically resolved in works in pea chloroplasts [35,36] where a half time of 100 ms for the Cyt b559 photoreaction at pH 7.8 was reported. Phase II of Cyt b559 reduction in shortly illuminated PSII membrane fragments proceeds in the dark in a scale of minutes at pH 5.9–8.3. It should be noted that a slow increase of the extent of reduced Cyt b559 under illumination or in illuminated samples in the dark has been observed in membrane preparations of PS II [41,55,56,58], however, it was not identified as a special component of the Cyt b559 photoreaction.

The data reveal similar properties for the redox reaction of oxidized Cyt b559 with PQH_2 or $DPQH_2$ formed photochemically inside the membrane pool and $DPQH_2$ added in the dark. These features are the following: (i) the redox reaction proceeds biphasically, (ii) the extents of phases I and II are functions of the quinol concentration, (iii) in both light induced and chemical reduction of Cyt b559 the rate of phase II is not significantly affected by a change in the quinol concentration, except of very low concentrations, (iv) maximally only a half of oxidized Cyt b559 can be reduced by PQH_2 and $DPQH_2$ in phase I at pH 6.5. The analysis of the results provides a view that the redox reaction between oxidized Cyt b559 of PS II membrane fragments and PQH_2 ($DPQH_2$) is characterized by two types of redox equilibria attaining in quite different time domains and corresponding to phases I and II of Cyt b559 reduction.

4.3. Assignment of phase I of Cyt b559 reduction

The properties of the Cyt b559/PQ redox equilibrium established in phase I are indicative of a reversible reduction of Cyt b559 by a bound reduced PQ. The earlier studies on Cyt b559 photoreduction pointed out to a possibility that reduced Q_B could be a fast electron donor for Cyt b559 in intact [78] and ADRY-poisoned [124,125] thylakoid membranes. Similar conclusion cannot be made in the case of the fast photoreaction of Cyt b559 observed in this work.

As found, the pattern of Cyt b559 reduction following saturating illumination in samples containing 3 μM DCMU (Fig. 4) is identical to that in a control not containing the herbicide (Fig. 2). It is known that binding interaction of DCMU at the Q_B site is characterized with quite slow dissociation of the inhibitor from the PS II complex occurring within several tens of seconds [22,30]. The later suggests that in DCMU-inhibited PS II centers (i.e. in more than 90% of the PS II complexes at 3 μM DCMU) the herbicide molecule remains bound in the Q_B site during the time period of fast photoreduction of Cyt b559. This excludes the possibility that Q_B is the donor for Cyt b559 in phase I. The latter conclusion is also corroborated by the results on chemical reduction of Cyt b559 by $DPQH_2$ where no competition between DCMU and the reductant in phase I was found (Fig. 8).

Unlike DCMU, dinoseb competed with $DPQH_2$ for binding at the site responsible for fast reduction of Cyt b559 (Figs. 7, 8). A selective affinity to the phenolic herbicide is a peculiar feature of the PQ/L binding site found in the potentiometric studies, in PS II complexes with oxidized Cyt b559 this site is characterized with K_D values of 6.6 μM and 200 μM for dinoseb and DCMU, respectively [87]. One may reasonably assume that the $DPQH_2$-binding site involved in phase I of Cyt b559 reduction and the PQ/L binding site (named Q_C in works [87,91]) is one and the same locus of PS II that can accommodate both quinone and quinol forms.

In Cyt bc_1 complex, the Q-cycle mechanism postulates a bifurcated oxidation of ubiquinol at the quinol oxidation site Q_O. The scheme of sequential oxidation of the quinol molecule suggests reduction by the deprotonated quinol of the neighboring iron sulfur center while the formed SQ acts as a reductant for the heme group of Cyt b_L (see, for instance, Refs. [126–128] for reviews). In a case of the reaction between Cyt b559 and PQH_2 bound at the quinol oxidation site a mechanism involving a SQ intermediate as a direct electron donor for Cyt b559 can be also considered. The SQ might be formed in the quinol oxidation site as a result of dismutation between the bound PQH_2 and PQ of the pool, although the probability of such kind of reaction is considerably low, or after one-electron oxidation of the bound PQH_2 by a side acceptor.

The results of the present study, however, argue against the assignment of phase I to reduction of Cyt b559 by the bound SQ. Indeed, close similarity in the features of light induced and chemical reduction of Cyt b559 by plastoquinols suggests that the fast redox process is a genuine "dark" reaction; this excludes an obligate participation of the light formed electron acceptors ($Car^{+\bullet}$, $Chl^{+\bullet}$) in the reaction mechanism. It seems also unlikely that the remote acceptor Tyr_D located at a

distance of about 38 Å from the heme group of Cyt b559 [20,21] that is known to be rereduced very slowly in illuminated PS II samples (from tens of minutes to hours, see Ref. [129] for recent review) might provide a rapid redox reaction between Cyt b559 and the bound PQH_2 via a mechanism of oxidant-induced reduction. In addition, the conclusion of the present work on rather slow electron exchange between the pool and Cyt b559 (see below) and, hence, between the pool and the quinol oxidation site interacting with Cyt b559, obviously excludes a route of the reaction where the bound SQ would be rapidly (in the timescale of phase I) formed in the site of quinol oxidation through thermodynamic equilibrium with the pool.

These arguments, together with the data on the competitive inhibiting effect of dinoseb on the extent of the fast reaction of Cyt b559 with $DPQH_2$, allow to consider that the bound quinol is the reductant for Cyt b559 in phase I and, in accordance with the common principles, the reaction proceeds via the anionic quinol intermediate. The latter species can be formed after deprotonation of the bound quinol by a presumed quinol-ligating group X. The fact that the extent of phase I of Cyt b559 reduction by $DPQH_2$ is limited at 50% (pH 6.5) most likely suggests a redox equilibrium between the oxidized Cyt b559 and the reduced quinone with an equilibrium constant $K_{eq} = 1$. The equilibria processes in the quinol oxidation site of PS II complexes with oxidized Cyt b559 at pH 6.5 are supposed as the following:

$$\text{Cyt b559}^{ox}(X) + QH_2 \overset{K_D}{\longleftrightarrow} \text{Cyt b559}^{ox}(X)QH_2 \quad (1)$$

$$\text{Cyt b559}^{ox}(X)QH_2 \overset{K_{eq}=1}{\longleftrightarrow} \text{Cyt b559}^{red}\left(XH^+\right)SQ, \quad (2)$$

where K_D denotes the dissociation constant for the quinol QH_2 in the site of quinol binding and oxidation, K_{eq} is the equilibrium constant, SQ is the bound semiquinone, and X and XH^+ are the formal designations of the deprotonated and protonated states of the quinol-ligating group, respectively. Note, that the suggested mechanism involves a long living (at least in minutes) SQ in the quinol oxidation site, implying that the presumed SQ intermediate is relatively stable towards oxidation by molecular oxygen.

4.4. Assignment of phase II of Cyt b559 reduction

Because of the substantial difference in the E_m values of the redox couples HP Cyt $b559^{ox}$/HP Cyt $b559^{red}$ and PQ/PQH_2 of the pool consisting of +380 mV (this work) and +140 mV [118] at pH 6.5, respectively, stoichiometric reduction of HP Cyt b559 is expected at equilibrium in the presence of PQH_2. According to the interpretation suggested here, the quinol bound at the quinol oxidation site is a thermodynamically weak one-electron reductant for Cyt b559 so that this reaction can account for reduction of only a half of oxidized Cyt b559 bearing the quinol at the quinol oxidation site. Therefore, phase II of Cyt b559 reduction in the presence of PQH_2 is considered as a process of slow redox equilibration between a set of the Cyt b559 redox forms and PQ/PQH_2 of the pool. When redox equilibrium between oxidized Cyt b559 and photoreduced PQ pool is fully established in illuminated samples of FeCy-washed PS II membrane fragments, the redox state of Cyt b559 is characterized with 63 % of reduced Cyt b559 corresponding to reduction of more than 80% of HP Cyt b559.

The assignment of phase II made above is in correspondence with the results of the present work indicating that $Q_A^{-\bullet}$ and reduced Q_B are not electron donors for oxidized Cyt b559 in the second phase of Cyt b559 reduction. It is also in line with the known features of redox chemistry of quinones and the kinetics found for phase II is in the range observed for one electron oxidation of free quinols [112–114,121,122]. A good illustration of the common principles is provided by our finding that the rate of Cyt b559 reduction by the strong two-electron reductant DQH_2 is markedly lower than by much weaker but one-electron donor $K_4Fe(CN)_6$. The data of the present investigation also demonstrate how a polar protein environment may accelerate the rate of one-electron oxidation of quinol. Taking the current assignments on the two components of Cyt b559 reduction and the estimations for the rates of phases I [$(100\ ms)^{-1}$] and II [$(2.4\ min)^{-1}$] at pH 7.6–7.8 from Refs. [35,36] and this work, respectively, one may infer that reduction of oxidized Cyt b559 by the PQH_2 bound in the quinol oxidation site proceeds more than 3 orders of magnitude faster than by a membrane quinol. We propose that this acceleration is due to a lowering down of the energy barrier for the quinol deprotonation step by an involvement in the reaction mechanism of the proton accepting group X.

The results of the present work suggest a low ratio PQ:Cyt b559 in samples of PS II membrane fragments and this fact is in line with the data reported in the literature. By different estimates, photoreducible pool of PQ (additionally to Q_A) in preparations of PS II membrane fragments consists of 1.3 PQ [41] or 2–3 PQ [101–103]. It is somewhat unexpected that in PS II samples that are characterized by a low content of PQ, as are PS II membrane fragments preparations, the extent of photoreduced pool increases rather gradually with progressing number of saturating flashes as observed in the measurements on the yield of oxygen evolution [101,104], the amplitude of the ms component of delayed Chl fluorescence [102] and the extent of photoreduced Cyt b559 (the present work). This feature of PS II membrane fragments preparations may be accounted for a low occupancy of the Q_B site by PQ, a presence of a fraction that is incapable of the Q_B reduction or properties of the Q_A/Q_B redox equilibrium.

In the previous study in PSII membrane fragments it was found that Cyt b559 photooxidized by strong light became slowly rereduced in the dark with a characteristic time of several minutes [41]. This kinetics is very close to that of the slow component of Cyt b559 photoreduction observed in the present work, although more pronounced dependence on pH of the rate of Cyt b559 reduction was reported in Ref. [41] as compared to our data on phase II. Together with that, one crucial difference appears between the two reactions. As shown in the former work, rereduction in the dark of photooxidized Cyt b559 was suppressed by DCMU at submicromolar concentrations and this fact allowed to consider the Q_B site bound deprotonated quinol as the slow donor for Cyt b559. This feature markedly disagrees with the properties of phase II of Cyt b559 reduction observed in our study.

4.5. Effect of dinoseb on the extent of phase I of Cyt b559 reduction by $DPQH_2$

Considering competition between dinoseb and quinol at the quinol binding site, the dissociation constant of $DPQH_2$ in PS II complexes with oxidized Cyt b559 in the presence of dinoseb, $K^{dino}_{DPQH_2}$, is given by:

$$K^{dino}_{DPQH_2} = K_{DPQH_2}(1 + [dino]/K_{dino}), \quad (3)$$

where K_{DPQH_2} and K_{dino} are the dissociation constants for $DPQH_2$ and dinoseb, respectively, in PS II complex with oxidized Cyt b559 and [dino] denotes the concentration of unbound, i.e. free, dinoseb. (Note that all the three dissociation constants $K^{dino}_{DPQH_2}$, K_{DPQH_2} and K_{dino} are considered as values measured in the presence of endogenous PQ). At the concentrations of PS II complexes and the added dinoseb consisting of ~0.2 μM and 50 μM, respectively, the approximation is valid, where [dino] = 50 μM. Using Eq. (3) and taking that the ratio $K_{DPQH_2}/K^{dino}_{DPQH_2}$ equals to the ratio of the correspondent K_{50} values determined from curves *a* and *c* in Fig. 8, we obtain a number of 7.5 μM for K_{dino}. The latter value is very close to the magnitude of 6.6 μM for K_{dino} in the PQ/L binding site of PS II complex with oxidized Cyt b559 estimated from the effect of dinoseb on the redox potential of HP Cyt b559 [87].

4.6. Relevance of the PQ-Cyt b559 interaction to the redox heterogeneity of Cyt b559

In the discussion above we consider that the quinol oxidation site responsible for phase I of Cyt b559 reduction is the same site of PS II that has been proposed from the effects of the compounds L on the redox potential of HP Cyt b559 [87,91]. This view suggests that the discussed quinone site interacting with Cyt b559 may bind PQ, SQ, PQH_2 and L.

The important finding of the present work is the fact that in the redox reaction with $DPQH_2$ oxidized Cyt b559 behaves as a homogenous population in respect with the characteristic features of the reaction, i.e. independently of the ambient redox potential established before and after addition of the quinol. This result indicates that the mechanism of oxidation of $DPQH_2$ by Cyt b559 described by Eqs. (1), (2) is valid for all the three redox forms of Cyt b559. Such a statement is difficult to rationalize if the redox heterogeneous population of Cyt b559 in PS II membrane fragments is considered as a mixture of pre-formed species characterized by different conformational and thermodynamic properties. Opposite to that view, our data suggest that in the reaction with $DPQH_2$ in the range of the redox potentials corresponding to reduction of 5-70% of Cyt b559 all the oxidized Cyt b559 behaves as one redox form characterized by the definite parameters K_D and K_{eq} in Eqs. (1) and (2). This implies that binding of quinol in the quinol oxidation site of PS II complex interacting with Cyt b559 specifies the structure of the microenvironment of the heme group of Cyt b559.

The latter conclusion raises a possibility that the redox properties of Cyt b559 in the native thylakoid membrane may be reversibly regulated by changes in a structure of the quinone binding site interacting with Cyt b559. Interestingly to note that in Cyt bc_1 the midpoint potential of the heme b_H was proposed to be affected by the redox state of the neighbor quinone Q_i [130,131]. Similar mechanism may account for the redox properties of Cyt b559. In that case an operational E_m of the Cyt b559 heme group would be dependent of the redox state of the PQ pool. Provided a negative shift in the E_m of Cyt b559 in the presence of the bound PQH_2, as discussed earlier [87], this mechanism would enable functioning of Cyt b559 as a PQH_2:O_2 oxidoreductase at high extents of reduction of the PQ pool [50–54].

4.7. Relevance to the known PS II structure

The quinone site that binds PQ/SQ/PQH_2 and the substances L (including anionic TPB and, perhaps, dinoseb and the ADRY reagents also in the anionic forms) discussed in the present work and previous studies [87,91] is expected to be located in a polar environment and likely corresponds to the polar quinone site Q_C proposed in Ref. [51]. However, in the crystal structure of the PS II complex from *T. elongates* the newly found quinone Q_C is settled in the hydrophobic environment. The crystallographic data on cyanobacterial PS II revealed a presence of a spacious intraprotein cavity filled with lipids [19–21] where the quinone Q_C is clamped within the hydrophobic surroundings formed by the tails of the bound lipids, quinone Q_B and a Car molecule [20]. No amino acid ligands interact with the carbonyl groups of the quinone Q_C.

The found structure of the Q_C binding domain makes it unlikely that this locus is the functional site regulating the redox potential of Cyt b559 which may specifically bind PQ, SQ, PQH_2 and the reagents L. The fact that the site Q_C discovered in the crystallographic studies does not bear the properties of the quinone site Q_C suggested earlier in Refs. [51,87,91] was also noted in the recent review [4]. It is quite obvious that functioning of the quinone site redox coupled with the heme group of Cyt b559 would require well defined quinone-protein interactions that are absent in the case of the third bound plastoquinone Q_C. To avoid confusion in the literature, we offer to change designation of the presumed polar quinone site of PS II that binds PQ/SQ/PQH_2/L for Q_D. The presence in PS II of two more bound quinones besides Q_A and Q_B corresponds to the original idea suggested in Ref. [51]. One of the possibilities is that the vast interior cavity found in the crystal structure accommodates two quinone binding sites, Q_C and Q_D, where either two PQ molecules are bound or the head of one bound quinone can occupy two different positions.

It can be expected that the quinone molecule Q_C that is present in the highly hydrophobic environment will favor two-electron redox transitions accompanying by the protonation events over one-electron reduction/oxidation, resembling in that sense a performance of a pool quinone. An interesting possibility then emerges, namely, that a PQ or PQH_2 molecule that is bound at the Q_C can mediate the equilibration of Cyt b559 with the pool in phase II of Cyt b559 reduction. This idea may get a support from the results of the present work showing only limited variation in the rate of phase II at rather different experimental conditions. The latter property can be rationalized assuming that the redox species that provides stabilization of reduced Cyt b559 in phase II of Cyt b559 reduction is a quinone molecule bearing the properties of a free quinol but occupying a fixed position in the neighborhood of Cyt b559. Similar mechanism of oxidation of LP Cyt b559 by artificial quinones involving a bound quinone exchangeable with the pool was suggested in former work [51]. Further studies are necessary to understand significance of the Cyt b559-PQ interactions in the Cyt b559 function.

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