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Charge recombination between stabilized P-680$^+$ and reduced cytochrome *b*-559 in quinone-reconstituted PS II reaction center

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Flash-induced absorbance changes in the nanosecond to millisecond time ranges have been measured in the Photosystem II reaction center complex consisting of D1 and D2 subunits and cytochrome *b*-559, in the presence of 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB). The results indicate that DBMIB largely quenches the primary radical pair (P-680$^+$ Pheo$^-$) and the formation of the triplet (^{3}P-680). Long-lived absorption signals in the red and near-infrared (bleaching at 680 nm and broad increase at 740–830 nm) and in the green (peak at 560 nm) can be attributed to the oxidation of P-680 and to the reduction of cytochrome *b*-559. These data show that addition of DBMIB induces stabilization of P-680$^+$ and a rapid (perhaps submicrosecond) reduction of cytochrome *b*-559. The signals attributed to P-680$^+$ and to reduced cytochrome decay in parallel ($t_{1/2}$ = 2 ms), showing that the cytochrome reduces P-680$^+$. The stabilization occurred also in the presence of plastoquinone-3 and (with DBMIB) at −29 °C in a viscous solution containing 60% glycerol at a low concentration of quinone, suggesting that the quinone reconstitutes the function of Q_A and thus mediates electron transport from the reduced pheophytin *a* to the intrinsic cytochrome.

Introduction

In the Photosystem II (PS II) reaction center complex consisting of D1 and D2 subunits and cytochrome *b*-559 [1], excitation by a flash of light induces the oxidation of the primary donor (P-680) and the reduction of the primary acceptor (Pheo) within picoseconds [2]. The primary radical pair (P-680$^+$Pheo$^-$) thus formed, however, recombines rapidly with a half-lifetime of 30–40 ns, because of the absence of the primary quinone acceptor (Q_A) [3,4]. The complex, on the other hand, contains a redox component, cytochrome *b*-559, whose function has not been elucidated [1]. In previous experiments (Satoh et al. in preparation, see also Ref. 5), it has been demonstrated that the cytochrome is reversibly reduced by steady-state illumination in the PS II reaction center complex supplemented with artificial quinones such as DBMIB. Reversible photoreduction of cytochrome *b*-559 in PS II reaction center has also been observed using natural quinone (PQ-9) [20]. The photoreduction was interpreted to occur by electron transfer from the reduced pheophytin (Pheo$^-$), through externally added quinone, to the cytochrome, a process which probably is accompanied by stabilization of positive charges on the oxidizing side. In this study, a flash spectroscopic analysis of the phenomenon, as suggested by Gounaris et al. [20], was conducted in order to confirm the validity of the above interpretation and to detect the stabilized species on the oxidizing side. We found that DBMIB can reconstitute Q_A function and that charge recombination takes place, in the presence of the quinone, between stabilized P-680$^+$ and reduced cytochrome *b*-559 with a half-lifetime of about 2 ms.

Materials and Methods

The PS II reaction center complex consisting of D1 and D2 subunits and cytochrome *b*-559 (and *psb* I gene product, see Refs. 6–8) was extracted with Triton X-100 from spinach grana thylakoids and purified by an isoelectric focusing as described in Ref. 6, a method which insures a much better stability than with the previous preparations. They were kept frozen at −70 °C until use. Before measurement, the complex was diluted,

Abbreviations: PS II, Photosystem II; DBMIB 2,5-dibromo-3-methyl-6-isopropylbenzoquinone; Q_A, primary quinone acceptor.

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after thawing, to an optical absorbance at 673 nm of 0.7 for an optical path of 1 cm in a 50 mM Tris-HCl (pH 7.2) buffer containing 0.2% digitonin and 20% (v/v) glycerol. When added, DBMIB was from an ethanol stock solution and the final concentration of the alcohol was 1% or less.

For flash absorption experiments, the medium with reaction centers was in a regular 10×10 cuvette, usually kept at 0 °C. Absorption changes in the time ranges of microseconds and milliseconds were measured as in Ref. 4, except that the excitation was provided by a dye laser (with a broad-band emission around 595 nm and an energy around 4 mJ) pumped by a 10 ns frequency-doubled YAG laser. For the recording of difference spectra, matching interference filters were placed before the cuvette and in front of the detector (bandwidth: 3 nm from 515 to 700 nm, 10 nm above 700 nm). Absorption changes in the time range of nanoseconds were measured as in Ref. 4, with a spacing of 6 s between excitation flashes.

Results

As previously described [4], excitation of PS II reaction center complexes with a picosecond laser flash induces an absorption increase at 820 nm, which decays essentially as a single exponential in the ns time range with a half-lifetime of 30–40 ns (Fig. 1, inset). This process is followed by a slow phase of several tens of microseconds, attributable to the decay of ^{3}P-680 formed as a product of recombination of the primary radical pair. The addition of DBMIB elicits a decrease of the initial amplitude of the signal attributable to the formation of the primary radical (Fig. 1). The slower component of the signal, estimated at the end of the traces of the nanosecond kinetics, however, was not affected drastically throughout the concentration range of DBMIB tested (up to 175 μM) (Fig. 1). If the decrease in the initial amplitude of the signal were due to the quenching effect of DBMIB on the singlet excited state of chlorophyll [10], the formation of ^{3}P-680 would be expected to decrease in parallel with the decrease in the formation of the primary radical pair. Since the absorption at 820 nm, 500 ns after the flash, is not quenched by DBMIB, another interpretation has to be proposed. A species more stable than the primary radical pair has to be invoked, the nature of which was studied by measuring the kinetics of flash-induced absorption changes in the microsecond and millisecond time ranges.

In the absence of DBMIB, the signal in the 600–870 nm region decays exponentially with a half-lifetime of about 30 μ s, which is characteristic of ^{3}P-680 measured at room temperature under aerobic conditions (see ref. 11). However, in the presence of DBMIB, new kinetic components appeared in parallel with the decrease in both the nanosecond signal (radical pair) and the ^{3}P-680

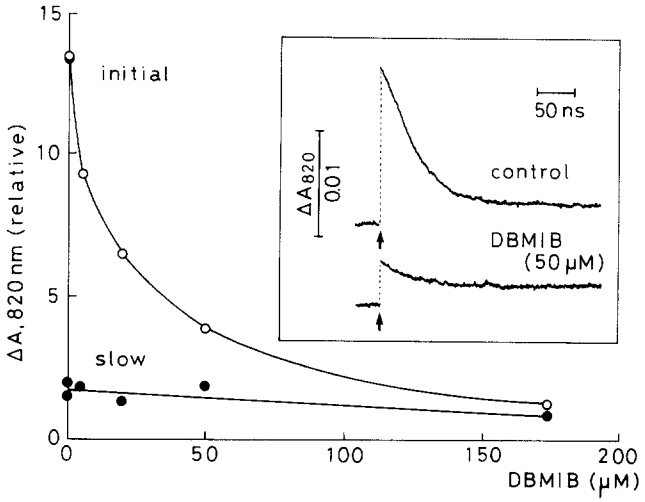

Fig. 1. Effect of DBMIB on flash-induced absorbance changes at 820 nm in the PS II reaction center complex. Open circles, initial amplitude of the nanosecond ΔA as shown by the inset (initial); closed circles, amplitude of the signal at the end of the nanosecond traces (slow). Inset, kinetic traces in the absence (control) and presence of 50 μM DBMIB (DBMIB). Temperature: +21 °C. Average of 100 flashes.

signal. Microsecond phases were attributed to rereduction of P-680$^+$ by Z [11]. A millisecond phase is shown in Fig. 2. Its half-lifetime is about 2 ms. Fig. 2 also shows the spectrum of that component formed by the flash excitation in the presence of 50 μM DBMIB in the wavelength region of 600–870 nm. The negative peak at about 680 nm and the shape around 670–820 nm are characteristic of a chlorophyll cation radical, attributable to the formation of P-680$^+$ as judged from the peak position of the bleaching band. The relative yield of the 2 ms component of P-680$^+$ was estimated, by

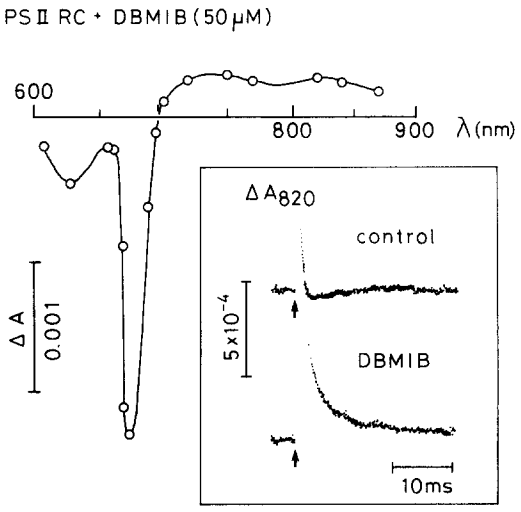

Fig. 2. Effect of DBMIB on millisecond flash-induced absorbance changes at 820 nm in the PS II reaction center complex (inset). Control, no addition; DBMIB, in the presence of 50 μM DBMIB. The difference spectrum is a plot of the size of the absorption transient which decays with $t_{1/2} = 2$ ms, in the presence of 50 μM DBMIB. Temperature: 0 °C.

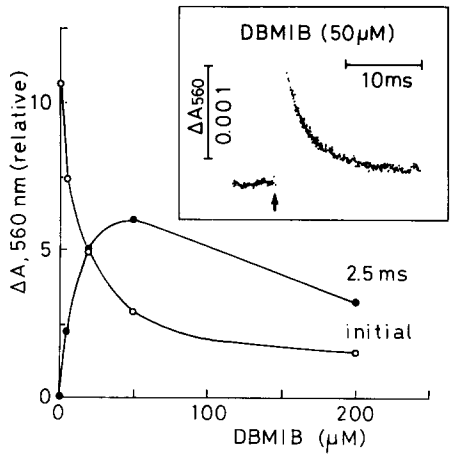

Fig. 3. Flash-induced absorbance change at 560 nm, in the millisecond time range, in the PS II reaction center complex, in the presence of 50 μM DBMIB (inset). The curve includes the results obtained at several concentrations of DBMIB. The signal is measured at the origin of the trace (initial), and at 2.5 ms after the flash (2.5 ms). Temperature: 0 °C.

using $\epsilon_{820\ nm} = 7000\ M^{-1} \cdot cm^{-1}$ for P-680$^+$ and $\epsilon_{760\ nm} = 8000\ M^{-1} \cdot cm^{-1}$ for ^{3}P-680, to be 20–25% of the triplet yield and thus calculated to be 4.5–6.0% of the primary charge separation, based on previous measurements [4].

It has previously been shown that cytochrome *b*-559 can be photoreduced in this material in the presence of DBMIB (Satoh et al., in preparation). We extended our flash kinetic study to the region of the alpha-band of cytochrome *b*-559. Fig. 3 (inset) shows a kinetic trace monitored at 560 nm after excitation of PS II reaction centers in the presence of 50 μM DBMIB. An absorption increase was observed at this wavelength, which decays with $t_{1/2} = 2$ ms. The spectrum of that kinetic component around 500–600 nm is shown in Fig. 4. It has the shape of the reduced-*minus*-oxidized spectrum of cytochrome *b*-559, indicating that the cytochrome gets rapidly reduced upon flash excitation in the presence of DBMIB. The difference spectrum shown in Fig.

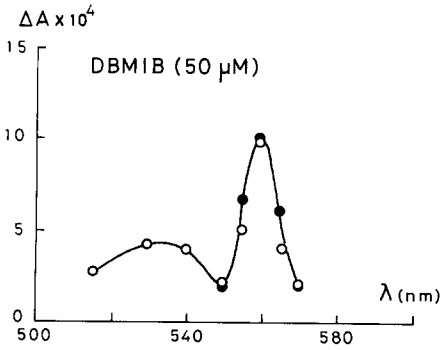

Fig. 4. Flash-induced absorbance difference spectrum of the 2 ms component in the 515–570 nm region, in the presence of 50 μM DBMIB. Temperature: 0 °C. Open and closed symboles are for two different preparations.

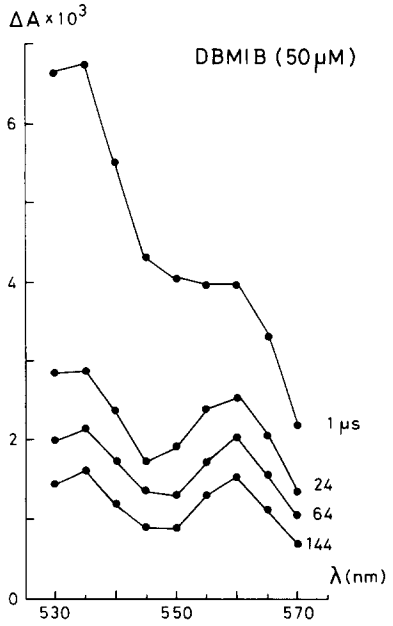

Fig. 5. A plot of the absorption change, measured at several time intervals after the excitation flash (1 μs, 2 μs, 64 μs and 144 μs), at wavelengths from 530 to 570 nm, with PS II reaction center complexes. Addition of 50 μM DBMIB. Temperature: 0 °C.

4 can obviously not be due to the oxidation of P-680 [12]. If the extent of millisecond absorbance change at 560 nm is attributed entirely to cytochrome *b*-559, and assuming a difference molar absorption coefficient of 15000 (559–570 nm) $M^{-1} \cdot cm^{-1}$ for the cytochrome [13], we find that the yield of cytochrome *b*-559 reduction is 15–22% of the yield of ^{3}P-680 formation. The amplitude corresponds reasonably to that of the 2 ms component of P-680$^+$ estimated at 820 nm using the same actinic light intensity. This, together with coincidental kinetics at both wavelengths, leads us to propose that the charge recombination is taking place between the stabilized P-680$^+$ and the reduced cytochrome *b*-559, with a half-life time of about 2 ms.

We attempted to gain some information on the kinetics and mechanisms of cytochrome *b*-559 photoreduction. With that objective, we measured absorption transients, with a microsecond time resolution, in the 530–570 nm region. As shown in Fig. 5, the absorption increase immediately after the flash has a maximum around 530–535 nm. This major signal decays with half-lifetime of 8 μs. It is also present without DBMIB and we assign it to the triplet state of β-carotene [4]. The difference spectrum at several times after the flash (Fig. 5) shows that a flat absorbance changes decays in the 200 μs domain (it probably includes contributions of residual ^{3}P-680, [14]), and that a spectrum with two broad bands (at 535 and 560 nm) remains at 144 μ s. As shown in Fig. 4, this is certainly the same spectrum which remains longer, decays with a half-life time of 2 ms, and is attributable mostly to reduced cytochrome

b-559. These features are absent when DBMIB is not added. The slowly decaying absorption at 535 nm seems too big for the β-band of cytochrome *b*-559, but it may be increased by the absorption of P-680$^+$ [12]. With that interpretation in mind it is clearly seen in Fig. 5 that the α-peak of reduced cytochrome *b*-559 is fully developed 24 μs after the flash. At 1 μs after the flash, that absorption is already present, perhaps with its full amplitude. It can thus be concluded that cytochrome *b*-559 is most probably reduced in 1 μs or less after the initial light absorption.

The stabilization of charges on P-680 and cytochrome *b*-559 can be attained by the addition of relatively low concentrations of DBMIB. As shown in Fig. 3, progressive addition of DBMIB results in a progressive decrease of the initial ΔA (we attribute this effect to the quenching of ^{3}P-680) and in a parallel increase of the slowly decaying ΔA attributed to reduced cytochrome *b*-559. At 200 μM, however, the latter signal is also decreased, perhaps because of the quenching of excited chlorophyll by DBMIB. The reduction of cytochrome *b*-559 practically saturates at about 5-times more DBMIB than the concentration of reaction centers, suggesting that the externally added quinone binds to a specific site on proteins to interact with both the intrinsic pheophytin and the cytochrome molecule. This suggestion may be verified by the fact that the stabilization of P-680$^+$ and the reduction cytochrome *b*-559 can be observed, even at $-29\,^\circ$C in the presence of 60% glycerol, as shown in Fig. 6. The spectra can be analyzed as for Fig. 5. An appreciable contribution of ^{3}P-680 is certainly present here: the extent of cytochrome *b*-559 reduction is relatively smaller, but the reduced cytochrome peak is observable at 10 μs after the flash. We also found that, at $0\,^\circ$C, the stabilization of P-680$^+$ can be attained as well by the addition of plastoquinone-3 (50 μM) or of 2,3-dichlorobenzoquinone (50 μM). We were unable to detect any effect of plastoquinone-9, possibly because of a problem of solubility.

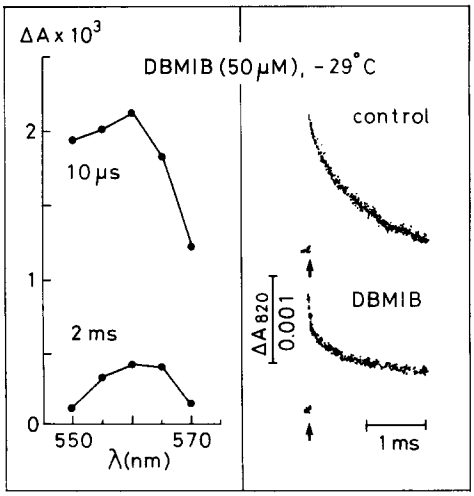

Fig. 6. Effect of DBMIB on flash-induced absorbance changes at 820 nm in the PS II reaction center complex at $-29\,^\circ$C. Control, no addition; DBMIB, in the presence of 50 μM DBMIB. The curves on the left give the absorbance change, for several wavelengths between 550 and 570 nm, immediately (about 10 μs) and 2 ms after the excitation flash, in the presence of 50 μM DBMIB, at $-29\,^\circ$C.

Discussion

A pigment-protein complex prepared by solubilizing PS II membranes with Triton X-100 carries the primary reactants of PS II, P-680 and pheophytin. The primary step of photoinduced charge separation takes place in the complex between the partners with a high quantum yield [4]. The complex does not retain the primary quinone acceptor [1], and thus the photogenerated charges recombine rapidly with a half-life time of 30–40 ns [3,4]. The complex, on the other hand, contains an additional redox component, cytochrome *b*-559, and in previous studies, it was found that the cytochrome can be photoreduced under steady-state illumination if the reaction center complex is supplemented with quinones such as DBMIB (Satoh et al. in preparation, see also Ref. 5). This phenomenon was interpreted to occur by electron transfer from the reduced pheophytin acceptor to the intrinsic cytochrome through externally added quinone bound to the protein. The stabilization of oxidized species on the donor side was also predicted to occur under these conditions (Satoh et al. unpublished data). The experimental evidence obtained by flash spectroscopy in this study is in line with the above predicition and provides a kinetic explanation to the phenomena observed during steady-state illumination, i.e., reversible photoreduction of cytochrome *b*-559. The stabilized species on the donor side with a half-life time of about 2 ms exhibits an absorption difference spectrum characteristic of the chlorophyll cation radical and the observed negative peak position in the red indicates that this component in P-680 [12]. However, another possibility might be that a monomeric chlorophyll species residing nearby the primary donor, which might supposedly be engaged in a protective cyclic electron transport around the PS II reaction center [15,16], is oxidized under our experimental conditions. Besides the primary donor of dimeric chlorophylls, the PS II reaction center complex used in this study contains only two or three chlorophyll molecules, which are expected to be organized in PS II in a similar manner to the 'accessory' (bacterio) chlorophyll molecules in the purple bacterial reaction center, although the amino-acid side-chains responsible for the binding are not conserved at the same position(s) on the D1 and D2 polypeptides [17–19]. The low-temperature absorption spectrum of the complex exhibits two peaks in the red, at about 670 and 680 nm, with an almost equal intensity [6]. Both P-680 and the primary acceptor pheophytin contribute to the

longer-wavelength peak [1,6]. Thus the absorption of the 'accessory' chlorophyll likely contributes to the shorter wavelength peak, in agreement with the EPR and optical measurements recently described [21]. These considerations seem to exclude the possibility that the oxidized species described here is a chlorophyll molecule other than P-680.

On the acceptor side, there is no direct evidence for the electron transfer sequence – pheophytin–quinone–cytochrome, since it was difficult to do flash spectroscopic analysis is the ultraviolet region. Nevertheless, the present results are best interpreted if DBMIB accepts electrons from the reduced pheophytin and then transfer them to the intrinsic cytochrome, leaving P-680 oxidized for a time much longer than the recombination time in the primary radical pair [3,4]. Similar stabilization of $P\text{-}680^+$ was attained in the presence of silicomolybdate, which also seems to accept electrons from the reduced pheophytin [20,21]. Electron transfer from the reduced quinone to cytochrome *b*-559 is very fast, as shown in Fig. 5. A similar fast oxidation of Q_A-, however, has not been reported in chloroplasts: it certainly does not constitute the main route of electron transfer under physiological conditions, although it may happen under those conditions where a cyclic electron transfer involving cytochrome *b*-559 takes place in Photosytem II [22–24]. Although the decay kinetics of $P\text{-}680^+$ contain multiple components, a large part of it has a half-lifetime of about 2 ms, which coincides well with that of the decay of the signal observed at around 560 nm, and attributable to the oxidation of reduced cytochrome *b*-559. The amplitude of the millisecond component, both at 820 nm ($P\text{-}680^+$) and at 560 nm (reduced cytochrome) also fits the hypothesis that $P\text{-}680^+$ is reduced partly by the reduced cytochrome *b*-559 in the millisecond time range in the presence of DBMIB. The physiological meaning of the electron transfer is not clear at present, but it might be associated with a protective electron transfer path around the PS II reaction center as proposed by Brudrig et al. [15,16]. The cyclic path for electrons through cytochrome *b*-559 which we have considered in this work may have some relationship with the complex redox behavior of that cytochrome in more intact systems, where a cyclic behavior has also be postulated [22–24].

The DBMIB concentration necessary to elicit photoaccumulation of reduced cytochrome in the steady-state illumination previously described (Satoh et al., unpublished data) and for the stabilization of charges on P-680 and the cytochrome *b*-559 in the flash excitation experiments described here is relatively low and is only a few times more than that of the reaction center in the mixture. This suggests that DBMIB is bound to a specific site on the protein(s), possibly in the Q_A-binding pocket on the reaction center. The suggestion is also supported by the observation that the stabilization of $P\text{-}680^+$ and the reduction of cytochrome *b*-559 takes place even at a low temperature ($-29\,^\circ C$) is a viscous solution containing 60% glycerol where diffusion-controlled reactions are expected to be severely inhibited. The specificity of quinones in the stabilization may also be another indication of the specific binding, although the natural quinone, plastoquinone-9, was inefficient in the reconstitution in this study possibly because of the secondary effects of the solubility and the folding of the quinone with a long hydrophobic side-chain.

According to recent results obtained by site-directed mutagenesis [25] and specific iodo-labeling experiments [26], the secondary electron donor, Z, is the tyrosine residue, Tyr-161 on the D1 protein. Thus Z should be observed in the purified reaction center preparation under the experimental conditions used in this study, since $P\text{-}680^+$ is largely stabilized. The functional evidence for that presence of Z was provided in a separate paper where we analyzed the microsecond kinetics of $P\text{-}680^+$ re-reduction after a flash [11]. It was concluded that Z reduces $P\text{-}680^+$ with a half-lifetime of about 5 μ s. Thus it should be expected that $P\text{-}680^+$ will be reduced by Z much more efficiently than by cytochrome *b*-559. Our results, then, are consistent only if some heterogeneity (perhaps attributable to the detergent extraction) renders Z less efficient than cytochrome *b*-559 in a fraction of the PS II reaction centers. That situation has also been observed in chloroplasts at low temperature.

Acknowledgements

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