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Irreversible light-induced formation of $P680^+$ and reduced cytochrome b_{559} in the D1-D2-Cyt b-559 complex at low temperature

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Abstract Cytochrome b_{559} in D1-D2-Cyt b-559 complexes from spinach can be photoreduced in the presence of DBMIB at a temperature of 180–240 K upon continuous illumination. The reduction of Cyt b-559 is accompanied by oxidation of P680. At 240 K recombination of $P680^+$ and reduced Cyt b-559 is complete in several seconds. At 220 K and below, the state $P680^+$Cyt b-559^{red} can be trapped for a long time. This indicates that the photoreduced heme is incapable of electron transfer to $P680^+$ at 220 K and below. On the other hand, the chemically reduced heme of Cyt b-559 is oxidized by $P680^+$ at 77 K. These results are consistent with the presence of two kinds of Cyt b-559 hemes in D1-D2-Cyt b-559 complexes which participate in different ways in the photochemical reactions.

Key words: Cytochrome b_{559}; D1-D2-Cyt b-559 complex; Electron transfer; Photosystem II

1. Introduction

Cytochrome b_{559} in the photosystem (PS) II reaction center (RC) complex contains two polypeptides of 9 and 4 kDa [1]. The function of the cytochrome is still debated. It is not clear whether one or two hemes of Cyt b-559 per P680 are present (see [2] for discussion). In the isolated PSII RC complex, the number of Cyt b-559 hemes per reaction center (per 2 Phe) was estimated to be close to 1 in [1–5] but 2 in [6,7].

In chloroplasts and PSII particles Cyt b-559 appears in two main redox forms with E_m = 350–400 mV (HP-form) and E_m = 60–80 mV (LP-form) [8]. Redox-titration of Cyt b-559 in the isolated RC-2 has also revealed multicomponent behaviour [3,6,7]. According to Shuvalov et al. two dominant redox transitions of Cyt b-559 are observed at pH 7.2, representing two hemes with E_m = 150 mV (pH-independent) and E_m = −45 mV [7]. The latter is split into two forms, with E_m of +40 mV and −220 mV at pH >7.7. Three redox forms were found in the potential range from 500 mV to −50 mV at pH 8 by Ahmad et al. with midpoints at 430 mV, 180 mV and 25 mV [3]. It has been suggested [7] that IP (E_m = 150 mV) and XLP (E_m = −45 mV, pH 7.2) forms of Cyt b-559 found in isolated RC-2 complexes correspond to HP and LP redox transitions of Cyt b-559 in chloroplasts, respectively.

Electron transfer in the isolated RC-2 is restricted to photoreduction of Phe due to the lack of quinone molecules in the complex [1,4,6,9,10]. When the IP heme is reduced before illumination it can be photooxidized at 77 K, resulting in the accumulation of the state Cyt b-559^+P680 Phe^- [6]. On the other hand, Cyt b-559 can be reduced reversibly by steady-state illumination in RC-2 supplemented with exogenous quinones such as DBMIB, plastoquinone-9, and decycloplastoquinone [11–13], or under anaerobic conditions, without any quinone added [14]. It is believed that exogenous quinones act at the position of Q_A and Q_B at the PSII acceptor side in isolated RC-2 [11–13,15,16].

The present work further demonstrates that the population of Cyt b-559 in the isolated RC-2 supplemented with DBMIB is heterogenous with respect to the photoinduced electron transfer. Photoreduced heme b-559 can be trapped in the state $P680^+$PheCyt b-559^{red} at temperatures below −55°C. However, heme b-559 initially reduced in the dark can be photooxidized even at 77 K.

Part of this work has been presented in abstract form at the Conference on the D1-D2-Cyt b-559 complex in Mulheim (Germany) in August 1993 [30].

2. Materials and methods

RC-2 complex from spinach was isolated as described earlier [6] using Triton X-100 for PSII particles and RC-2 solubilization. DEAE-chromatography with Fractogel TSK DEAE-650 (S) (Merck, Darmstadt) was used for the purification of RC-2. Triton X-100 was replaced by 2 mM *n*-dodecyl-β-D-maltoside (Sigma) on the column.

For anaerobic conditions 5 mM glucose, 0.05 mg/ml glucoseoxidase (Sigma) and 0.05 mg/ml catalase (Sigma) were added to the samples.

The absorbance and light-induced difference absorbance change spectra were measured at 77 K to 273 K using a Specord M-40 spectrophotometer (Carl Zeiss, Germany) and a homemade phosphoroscopic set up (sensitivity of 10^{-4} A). Optical density at 679 nm in the samples with the optical length of 2 mm was 0.9–1 A.

3. Results

Fig. 1A shows the dark absorption spectra of RC-2 at pH 7.2 (a) and pH 9.2 (b) frozen to 77 K in the presence of 100 μM DBMIB. In agreement with previous reports [6,17–20] the low temperature spectra are characterized by the mixed band of Q_y transitions of Chl and Phe molecules with two maxima at 672 nm and 679 nm, Q_x transition of Phe at 542 nm, the transitions of carotenoid molecules at 508 nm and 488 nm. As one can see from the spectrum (a), at pH 7.2 Cyt b-559 is fully oxidized, exhibiting its γ-band at 416 nm. However, at pH 9.2 Cyt b-559 is present in a partially reduced form which can be seen from the appearing α-band at 557 nm and Soret maximum at 426 nm (curve b). A partial dark reduction of Cyt b-559 is probably due

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Abbreviations: Cyt^{red}, reduced cytochrome; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; E_m, midpoint redox potential; HP, IP, LP and XLP, high, intermediate, low and extra low potential forms of hemes, respectively; P680, primary electron donor; Phe, pheophytin; PSII, photosystem II; RC-2, reaction center of PSII (D1-D2-Cyt b-559 complex); CHES, 2-*N*-cyclohexylamino]ethanesulfonic acid.

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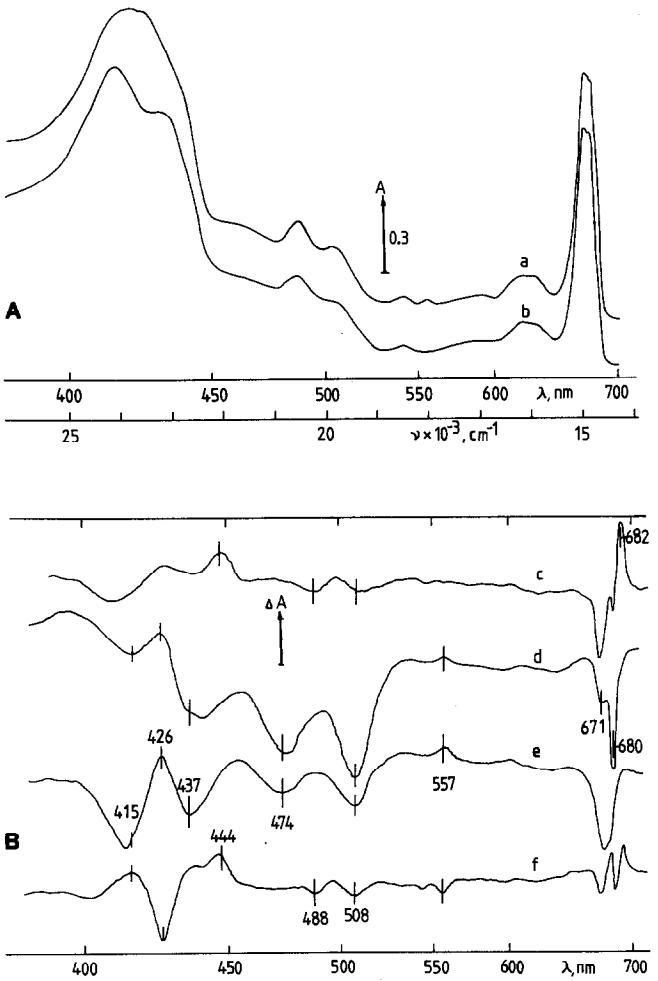

Fig. 1. Absolute dark (A) and light-induced difference (B) absorbance spectra of RC-2 at 77 K. All samples were treated under anaerobic conditions (see section 2) and contained 2 mM β-D-maltoside, 100 μM DBMIB, 60% glycerol and 50 mM Tris-HCl at pH 7.2 (a,c,d), pH 8.9 (e) or 50 mM CHES at pH 9.2 (b,f). (a,b) Samples were frozen to 77 K in the dark; (c,f) samples were illuminated at 77 K for 5 min by red light; (d) the same sample as in (c) was warmed to −60°C, illuminated for 2 min at this temperature and then frozen to 77 K upon illumination; (e) the same procedure as in (d) was done at pH 8.9, the temperature for the 2-min illumination was −40°C. For the difference spectra dark absorbance spectra of the samples at 77 K were subtracted to obtain curves c,f and the absorbance spectra of the samples illuminated at 77 K were subtracted to obtain curves d and e. The vertical arrow in Fig. 1B corresponds to 0.01 A for c, 0.04 A for d and 0.02 A for e,f.

to a shift in the ambient redox potential to more negative values at high pH.

Fig. 2 shows the kinetics of absorbance changes upon continuous illumination in the temperature range −30 to −90°C in the sample, the spectrum of which is presented in curve a of Fig. 1A. Light-induced transients were monitored in the absorption bands of P680 at 680 nm (a–c) and of the ferric Cyt b-559 at 416 nm (d–g). The reversible bleachings at 680 nm and 416 nm were observed at −30 to −40°C (curves a and d). When the temperature is lowered to −55°C or below, the changes in the Cyt b-559 band become irreversible and the P680 signal is only partially reversible (curves b, c, e–g). As can be seen from the difference spectra, the irreversible absorbance changes correspond to simultaneous oxidation of P680 and DBMIB-mediated reduction of Cyt b-559. Part of $P680^+$ which retains rereduction even at −70°C is probably due to the recombination of $P680^+$ and $DBMIB^-$ in part of the RC-2 where an electron transfer to Cyt b-559 does not occur.

Inhibition of reversibility of the light-induced signals at temperatures below −55°C enabled us to get the stable spectrum of the light minus dark absorbance changes in samples frozen to 77 K under illumination. In such experiments we found firstly that illumination of a sample at 77 K already results in some spectral changes which persist even after warming the sample to 0°C. The spectrum of such irreversible absorbance changes is shown in curve c of Fig. 1B. It is characterized by the bleachings at 408 nm, 488 nm, 508 nm and 665 nm and the main positive bands at 444 nm and 682 nm. Similar changes in the 665–682 nm range were observed earlier at 245 K as reported in [21]. The nature of the reaction is not quite clear. It could be related to the photoreduction of DBMIB (semiquinone band appears at 444 nm) accompanied by the oxidation of the Chl molecule and β-carotene [21,22]. At higher pHs (>8.6) these changes in the 665–682 nm range did not attain the full amplitude under 77 K illumination (see later).

Curve d in Fig. 1B shows the light-induced difference absorbance spectrum of the sample at pH 7.2 illuminated for 5 min at 77 K, warmed to −60°C in the dark, illuminated at this temperature for 2 min and then frozen upon illumination to 77 K. The initial absorbance spectrum of the sample illuminated at 77 K was used as a reference spectrum to subtract the irreversible absorbance changes. A similar difference absorbance spectrum in the presence of DBMIB was obtained at pH 8.9 (Fig. 1B, curve e).

The difference spectra d and e in Fig. 1B display the changes in absorption bands of Cyt b-559, P680 and carotenoids. The shift of the Cyt b-559 γ-band from 415 to 426 nm and the appearance of its α-band at 557 nm show that reduction of the cytochrome takes place. P680 undergoes photooxidation which at neutral pH appears as a characteristic spectrum with the bleachings at 438 nm and 680 nm [4,6,21,23–25]; a shoulder at 671 nm is probably due to photooxidation of an accessory Chl [23,25] (curve d). At higher pHs the longwave absorbance changes exhibit a more pronounced bleaching at 671 nm with respect to that at 680 nm (curve e). The latter is probably related to the increase in the amplitude of the light-induced spectral changes in the 665–682 nm range in the sample similar to those shown in curve c. These absorbance changes (bleaching at 665 nm and absorbance increase at 682 nm) did not attain the full amplitude under 77 K illumination and occurred when the sample was illuminated at higher temperatures. In that case they cannot be subtracted and interfere with those due to P680 oxidation. The absorbance changes in the range 470–510 nm are due to carotenoids. The observed absorbance changes throughout the whole spectrum were not significantly dependent on the presence or absence of oxygen.

The obtained trapped state characterized by the simultaneous oxidation of P680 and reduction of Cyt b-559 was stable for many hours at 77 K. When such a sample was thawed to 0°C, charge recombination between $P680^+$ and Cyt $b\text{-}559^{red}$ took place.

From the ratio of ~2.2 of the irreversible fractions of kinetic curves b and e in Fig. 2 at −55 to −60°C one can estimate the difference extinction coefficient ($\Delta\varepsilon$) of $P680^+$. Taking

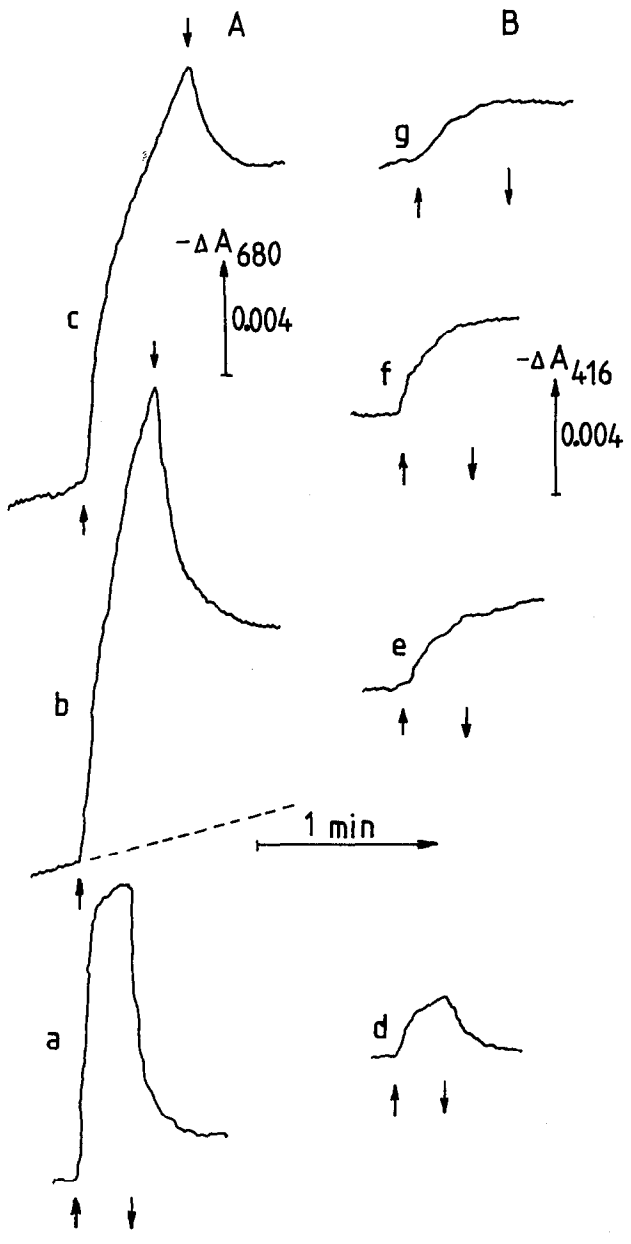

Fig. 2. Kinetics of light-induced absorbance changes coupled to the photooxidation of P680 and the photoreduction of Cyt b-559 in RC-2 at low temperatures in the presence of DBMIB. Conditions of the sample were as in Fig. 1A,a. Time-dependent absorbance changes were registered at 680 nm (A) and 416 nm (B) at different temperatures: −30°C (a); −60°C (b); −70°C (c); −40°C (d); −55°C (e); −80°C (f) and −90°C (g). Arrows show the switching on and off of the continuous illumination.

$\Delta\varepsilon$ = 54 $mM^{-1} \cdot cm^{-1}$ for reduced minus oxidized Cyt b-559 at the negative peak at 413 nm [8,26], $\Delta\varepsilon$ = 120 $mM^{-1} \cdot cm^{-1}$ for $P680^+$ at 680 nm is obtained. If one suggests that the irreversible bleachings of P680 at −60°C includes the contribution from the state $P680^+DBMIB^-$, $\Delta\varepsilon$ at 680 nm for $P680^+$ would be less.

Curve f in Fig. 1B shows the light minus dark difference spectrum of a sample containing 100 μM DBMIB at pH 9.2 (see Fig. 1A, curve b) illuminated at 77 K. Photooxidation of the initially reduced in the dark Cyt b-559 occurs in that case, which appears as a blue shift of the Soret band from 426 to 416 nm and a bleaching of α-peak of the reduced cytochrome at 557 nm. The absorption changes in the carotenoid bands (488 nm and 508 nm), at 446 nm and in the 665–682 nm range in general resemble those observed at 77 K at pH 7.2 (curve c).

4. Discussion

The spectra and kinetics of light-induced absorbance changes of RC-2 at pH 7.2 in the presence of DBMIB at low temperatures presented in Figs. 1 and 2 reflect the reduction of Cyt b-559 and oxidation of P680, in good agreement with previous reports [11–13]. However, the earlier observations did not show the stable trapping characterized by the state $P680^+$Cyt $b\text{-}559^{red}$. The latter occurs at temperatures below −55°C and demonstrates that the reduced heme is incapable of electron donation to $P680^+$ in frozen samples. Unlike this, initially reduced in the dark heme b-559 at pH 9.2 can be photooxidized even at 77 K (Fig. 1B, f; see also [6]). The heme chemically reduced in the dark represents probably the higher potential IP form, characterized by E_m = 150–180 mV [3,7] since only ~25% of Cyt b-559 was reduced at pH 9.2 (Fig. 1B).

We observed the trapped photoinduced state $P680^+$PheCyt $b\text{-}559^{red}$ in a rather small fraction of RC-2 (~10–20%), as determined from the ratio of the photoinduced absorbance changes at 680 nm to the dark peak absorbance. A similar amount was found for Cyt b-559 photooxidation and for the bleaching of the 679 nm maximum at 77 K during $^3P680$ formation (not shown).

We cannot conclude unambiguously which redox form of Cyt b-559 participates in the individual photoreactions. If the same redox form (or heme) of Cyt b-559 undergoes photoreduction and photooxidation in our experiments, the observed inhibition of electron transfer from a photoreduced heme b-559 to $P680^+$ at cryogenic temperatures may suggest that the heme oxidation is coupled to a conformational change in the protein. Interestingly in this regard, the existence of the two 'frozen' and 'relaxed' conformations was found in ferric Cyt b-559 in the EPR studies [27–29].

If the two different heme forms (IP, XLP) participate in the different photoreactions we may suppose that the IP form is photooxidizible and the XLP form is photoreducible. In the context of the suggestion that two copies of heme b-559 are present in RC-2 the obtained results are more easily understood assuming that the photoreducible XLP heme is located close to the acceptor side (pheophytin and quinone) of the RC complex, while the photooxidizible, IP heme is close to P680. The XLP heme is suggested to transfer electrons to the IP heme. The suppression of electron transfer from the photoreduced XLP heme to $P680^+$ via the IP heme may suggest that it is accompanied by a temperature-dependent (diffusional or conformational) process.

To correlate the photoreactions of Cyt b-559 with the individual redox forms of the cytochrome in the preparation the redox titration of the light-induced heme transitions is now under study.

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