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High-light-induced superoxide anion radical formation in cytochrome b_6f complex from spinach as detected by EPR spectroscopy

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Abstract

The generation of superoxide anion radical ($O_2^{\cdot-}$) in the cytochrome b_6f complex (Cyt b_6f) of spinach under high-light illumination was studied using electron paramagnetic resonance spectroscopy. The generation of $O_2^{\cdot-}$ was lost in the absence of molecular oxygen. It was also suppressed in the presence of NaN_3 and could be scavenged by extraneous antioxidants such as ascorbate, β-carotene, and glutathione. The results also indicate that $O_2^{\cdot-}$, which is produced under high-light illumination of the Cyt b_6f from spinach, might be generated from a reaction involing $^1O_2$, and the Rieske Fe-S protein could serve as the electron donor in the $O_2^{\cdot-}$ production. The mechanism of photoprotection of the Cyt b_6f complex by antioxidants is discussed.

Additional key words: Cyt b_6f; EPR; photoprotection; superoxide anion radical.

Introduction

The cytochrome b_6f complex (Cyt b_6f) is one of the three most important pigment-protein complexes (PSII, PSI, and Cyt b_6f) in the thylakoid membranes that participate in the electron transfer process of photosynthesis. A monomer of the dimeric Cyt b_6f complex (105 kDa) contains four major subunits (Cyt f, Cyt b_6, Rieske Fe-S protein, and subunit-IV), and another four low-molecular-mass subunits (Pet G, Pet L, Pet M, and Pet N). All subunits, except subunit IV, bind redox-active cofactors: Cyt f contains one c-type heme, Cyt b_6 contains two b-type hemes and one newly discovered heme named "heme x", and the Rieske Fe-S protein contains a [2Fe-2S] cluster. Subunit IV, which plays a catalytic role, is thought to be the binding site of plastoquinone (Doyle *et al.* 1989, Li *et al.* 1991). The Cyt b_6f oxido-reductase mediates electron transfer from photosystem II (PSII) to photosystem I (PSI) and the Q-cycle around PSI, through which a transmembrane proton gradient accumulates for ATP synthesis (Mitchell 1966). Furthermore, it is involved in balancing the excitation energy distribution between the two photosystems and regulates gene expression *via* redox control to maintain efficient energy conversion (Cramer *et al.* 2006, Allen 2004). Each monomer of Cyt b_6f also contains one chlorophyll a (Chl a) and one carotenoid molecule according to crystal structure (Kurisu *et al.* 2003, Stroebel *et al.* 2003). However, the necessity and functions of the two pigments remain unclear.

High-light illumination induces the Chl a bleaching of Cyt b_6f and this process is proposed to be related with the

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Abbreviations: Car – carotene; Chl a – chlorophyll a; ^{3}Chl a* – triplet excited state of chlorophyll a; Cyt b_6f – cytochrome b_6f complex; EMPO-2-ethoxycarbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide; EPR – electron paramagnetic resonance; $O_2^{\cdot-}$ – superoxide anion radical; ROS – reactive oxygen species; $^1O_2$ – singlet oxygen; TEMP – 2,2,6,6-tetramethylpiperidine; β-DM – n-dodecyl-β-D-maltoside; β-OG – n-octyl-β-D-glucopyranoside.
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production of the singlet oxygen ($^1O_2$) (Zhang *et al.* 1999). Suh *et al.*(2000) suggested, based on comparative studies of the intact and the Rieske-depleted Cyt b_6f complexes from spinach, that the Rieske subunit is responsible for $^1O_2$ production. Though $^1O_2$ is considered as the reactive oxygen species (ROS) responsible for chemically damaging Chl *a* and the surrounding proteins (Krinsky 1979), the participation of $O_2^{\cdot -}$ and other related ROS species in the high light induced photodamage to Cyt b_6f could also be possible because of the many interconversion reactions of $^1O_2$ and other ROS species (Singh 1978). Thus, the detailed $O_2^{\cdot -}$ formation mechanism in the Cyt b_6f complex of spinach is unknown, although the relevant knowledge is indispensable in understanding its photoprotective mechanism.

In the present study, we confirmed the generation of $O_2^{\cdot -}$ in the Cyt b_6f complex from spinach. We also attempted to determine the specific site and the mechanism of photoinduced formation of $O_2^{\cdot -}$ for which three different kinds of Cyt b_6f preparations, *i.e.*, the intact and the Rieske-depleted Cyt b_6f complexes, as well as the isolated Rieske Fe-S protein, have been prepared and compared by electron paramagnetic resonance (EPR) spectroscopy. Our results unambiguously showed that, for the Cyt b_6f complex from spinach, the Rieske Fe-S protein could serve as the electron donor which is needed for $O_2^{\cdot -}$ production. Furthermore, $O_2^{\cdot -}$ could also be scavenged by the antioxidants which might serve as the protection mechanism of the Cyt b_6f complex of spinach.

Materials and methods

Purification of the Cyt b_6f complex: Spinach (*Spinacia oleracea* L.) was purchased from the local market. All of the chemicals used for the Cyt b_6f preparations and for the subsequent experiments were purchased from *Sigma* and used as received: phenylmethanesulfonyl fluoride (PMSF), sodium cholate hydrate (SC), n-dodecyl-β-D-maltoside (β-DM), n-octyl-β-D-glucopyranoside (β-OG), ascorbate, glutathione, hydroxyapatite, sodium lauryl sulfate and acrylamide. 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide (EMPO) was purchased from *Alexis* (*Prod. No. 430-098-M050*). The Cyt b_6f complex was purified from spinach chloroplasts according to the procedure described previously (Yan *et al.* 2001). The final precipitate formed through ammonium sulfate fractionations, namely the purified Cyt b_6f preparation, was suspended in 50 mM Tricine–NaOH (pH 8.0) containing 30 mM β-OG or 0.2 mM β-DM, and stored at –80°C before use.

The Rieske-depleted Cyt b_6f complex and the isolated Rieske Fe-S protein were prepared following the procedures by Hurt *et al*. (1981).

EPR detection of light-induced $O_2^{\cdot -}$: Photoproduction of $O_2^{\cdot -}$ was measured by EPR spectroscopy (Lion *et al.* 1976, 1980), which is based on the observation of certain physical characteristics of active oxygen. Detection of $O_2^{\cdot -}$ was carried out with 50 mM EMPO as spin trap (Olive *et al.* 2000). For this, EPR spectrometer (*ELEXSYS E500-10/12, Bruker*, Germany) was used. The samples containing Cyt b_6f (2.5 μM cyt *f*), 50 mM EMPO and the buffer of 50 mM Tricine–NaOH (pH 8.0) with 0.2 mM β-DM, all the addition was done prior to illumination and the samples were then molecular-oxygen-saturated. A volume of approximately 80 μl was drawn into glass capillaries, sealed and then illuminated with white light (3,000 μmol m^{-2} s^{-1}) for 9 min. Both before and after illumination samples were measured at room temperature. For kinetics experiments the samples were detected after illumination for certain times. The instrumental parameters were: microwave frequency 9.78 GHz, modulation frequency 100 kHz, microwave power 10 mW, modulation amplitude 1G, time constant 40 ms, field sweep 100 G, receiver gain 60 and the operating temperature 25ºC.

The anaerobic condition, when needed, was achieved by adding 5 mM glucose, 0.1 mg mL^{-1} glucose oxidase and 0.05 mg mL^{-1} catalase to the Cyt b_6f preparations (Crystall *et al.* 1989).

EPR detection of light-induced $^1O_2$: $^1O_2$ is a strong electrophile but not a radical, however, it can oxidize 2,2,6,6-tetramethylpiperidine (TEMP) and form the stable N-oxyl radical (TEMPO) that allows the EPR detection of $^1O_2$ (Pierre *et al*. 1997). The samples (80 μL) containing Cyt b_6f (1 μM Cyt *f*) and 37.5 mM TEMP in the buffer of 50 mM Tricine-NaOH (pH, 8.0) with 0.2 mM β-DM were saturated with molecular oxygen. The samples were sealed in glass capillaries, and illuminated for 9 min with white light (1,500 μmol m^{-2} s^{-1}) filtered out with a $CuSO_4$ solution of 12 cm optical path from a 150 W halogen lamp. EPR spectra were recorded with the same parameter settings above.

Results

Light-induced formation of $O_2^{\cdot -}$ in the Cyt b_6f complex was detected by EPR spectroscopy using EMPO as the spin-trapping agent. EMPO is a better trapping agent than the commonly used DMPO as the stability of the EMPO-OOH adduct is eightfold that of the DMPO-OOH adduct (Zhang *et al.* 2000).

Fig. 1 shows the change in $O_2^{\cdot -}$ concentration in Cyt b_6f complex in the presence of EMPO under different

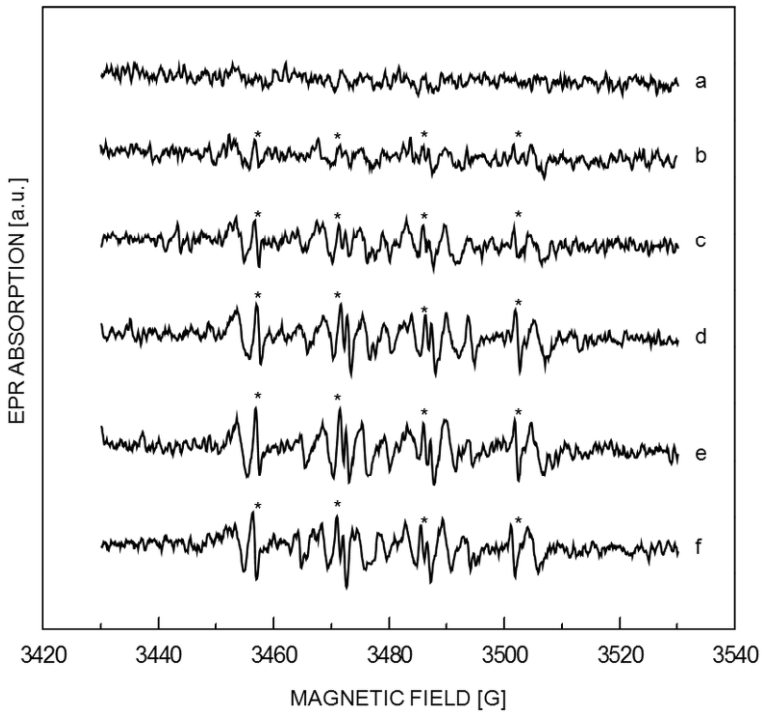

Fig. 1. Light-induced EMPO-OOH adduct EPR spectra measured in Cyt b_6f of spinach after illumination with white light (3,000 μmol m^{-2} s^{-1}) for (a) 0, (b) 3, (c) 6, (d) 9, (e) 12, and (f) 15 min. The spectra were recorded in the presence of Cyt b_6f (2.5 μM Cyt *f*), 50 mM EMPO, and 50 mM Tricine–NaOH (pH 8.0) with 0.2 mM β-DM. The samples were all molecular-oxygen-saturated. The symbol (*) indicates line component belonging to EMPO-OOH.

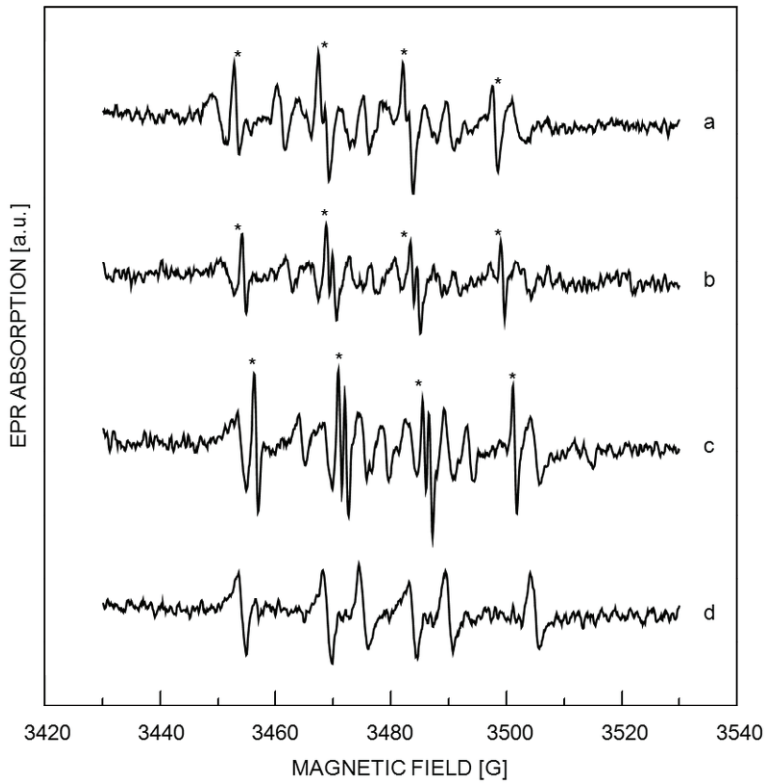

Fig. 2. EPR spectra of the EMPO-OOH adducts measured from the Cyt b_6f of spinach after illumination. The spectra were obtained after illumination of Cyt b_6f for 9 min with white light (3,000 μmol m^{-2} s^{-1}) in the presence of Cyt b_6f (2.5 μM Cyt *f*), 50 mM EMPO, 50 mM Tricine-NaOH (pH 8.0) with 0.2 mM β-DM and (a) without addition, (b) with 50 mM NaN_3, (c) with SOD (100 U ml^{-1}), and (d) under anaerobic conditions prior to illumination.

illumination times. The four-line spectra exhibited all the characteristics of the EMPO-OOH adduct EPR spectra produced by the high-light-induced reaction of EMPO with $O_2^{\cdot-}$ as reported in the literature (Zhang *et al.* 2000). No EPR signal was observed in the nonilluminated Cyt b_6f, showing that no $O_2^{\cdot-}$ was formed in the dark (Fig. 1, a), whereas the EMPO-OOH adduct EPR signal was detected when illuminated for 3 min (Fig. 1, b). Illumination with continuous white light for 3–9 min resulted in the gradually generation of the EMPO-OOH

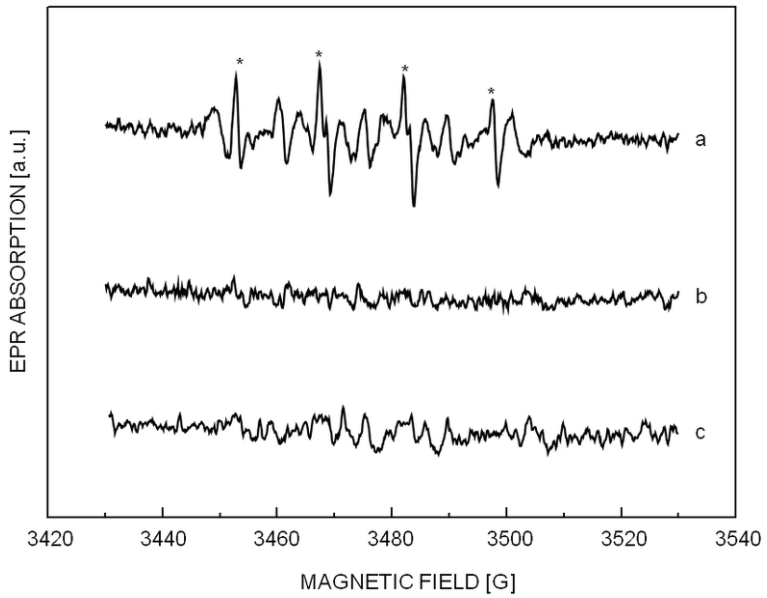

Fig. 3. EPR spectra of the EMPO-OOH adducts measured in different preparations of spinach Cyt b_6f after illumination. (a) Intact Cyt b_6f, (b) Rieske-depleted Cyt b_6f, and (c) isolated Rieske Fe-S protein. Other experimental conditions were similar to those in Fig. 2.

adduct EPR signal (Fig.1, b–d). The signal obtained after 12 min and 15 min illumination was the similar to that of 9 min, indicating that the maximal $O_2^{\cdot-}$ production was reached (Fig. 1, d). These results confirm the high-light-induced $O_2^{\cdot-}$ production in the Cyt b_6f complex from spinach.

Sources of light-induced $O_2^{\cdot-}$ formation in the Cyt b_6f complex: To detect the relationship between the generation of $O_2^{\cdot-}$ and $^1O_2$, we added 50 mM sodium azide (NaN_3), a quencher of $^1O_2$, to the Cyt b_6f complex, and the production of $O_2^{\cdot-}$ was measured. The results indicate that the intensity of the EMPO-OOH adduct EPR signal decreased by 16% (Fig. 2, b), whereas adding the same concentration of NaN_3 leads to a 23% decrease in the $^1O_2$ signal (data not shown). Approximately 69.6% of the generated $O_2^{\cdot-}$ came from the reaction, in which $^1O_2$ is one of the reactants. Interestingly, the EMPO-OOH adduct EPR signal was 1.3 times greater than the control when superoxide dismutase (SOD) was added (Fig. 2, c). An unknown reaction, which increased the EMPO-OOH adduct EPR signal, might have transpired after the addition of SOD. Furthermore, the $O_2^{\cdot-}$ formation was measured under anaerobic conditions: the absence of $O_2^{\cdot-}$ production (Fig. 2, d) demonstrates that molecular oxygen is required in the reaction to yield $O_2^{\cdot-}$.

Fig. 3 shows the production of $O_2^{\cdot-}$ in different preparations of the Cyt b_6f complex. The results illustrate that the EMPO-OOH adduct EPR signal was detected in the intact Cyt b_6f complex (Fig. 3, a) whereas no EMPO-OOH adduct EPR signal was detected in the Rieske Fe-S depleted Cyt b_6f complex (Fig. 3, b) and the Rieske Fe-S protein (Fig. 3, c). Thus, the Rieske Fe-S protein might serve as an electron donor in the $O_2^{\cdot-}$ producing reaction, however, it could not function when it was isolated from the protein environment of the Cyt b_6f complex.

Fig. 4*A* shows nearly identical signal intensities in the

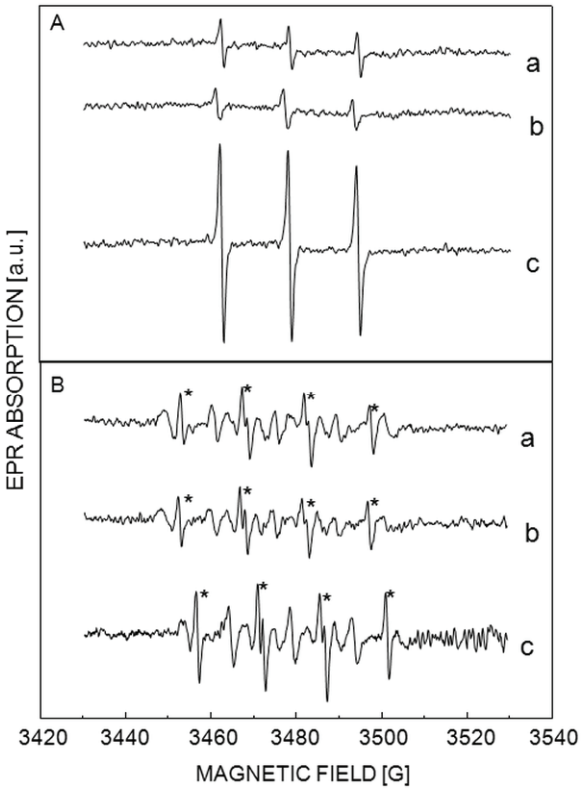

Fig. 4. *A*: High-light-induced TEMP-$^1O_2$ adduct EPR spectra measured in the Cyt b_6f of spinach suspended in different detergents. The spectra were recorded in the presence of (a) 0.375 M TEMP, 50 mM Tricine (pH 8.0), 0.02% β-DM (b) 0.375 M TEMP, 50 mM Tricine (pH 8.0), 30 mM β-OG, and (c) 0.375 M TEMP, 50 mM Tricine (pH 8.0), 1% SDS. *B*: High-light-induced EMPO-OOH adduct EPR spectra measured in the Cyt b_6f of spinach suspended in the different detergents. The spectra were recorded in the presence of (a) 50 mM EMPO, 50 mM Tricine (pH 8.0), 0.02% β-DM, (b) 50 mM EMPO, 50 mM Tricine (pH 8.0), 30 mM β-OG, and (c) 50 mM EMPO, 50 mM Tricine (pH 8.0), 1% SDS.

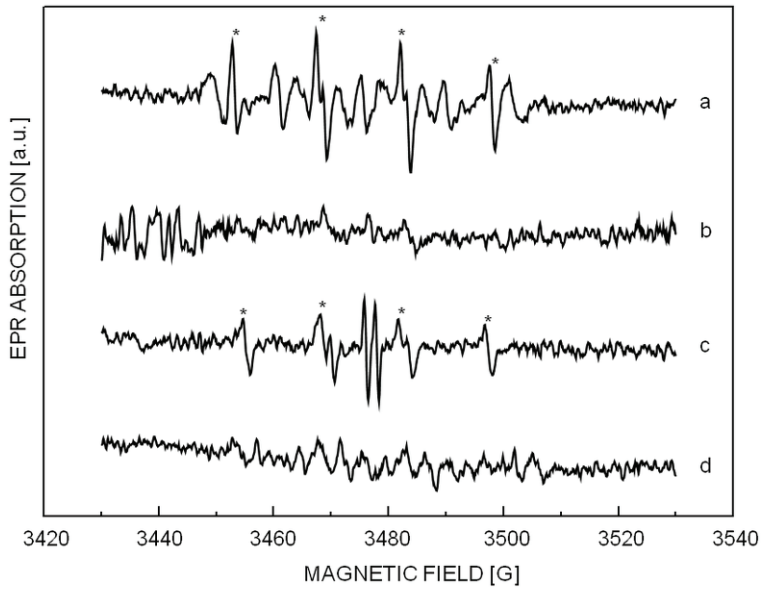

Fig. 5. EPR detection of EMPO-OOH adducts for the intact Cyt b_6f preparation from spinach as scavenged by extraneous antioxidants: (a) without scavenger (b) with β-carotene (c) with ascorbate, and (d) with glutathione prior to illumination. Other experimental conditions were similar to those in Fig. 2.

β-DM and β-OG preparations (Fig. 4*A*, a and b), whereas the signal intensity in the SDS preparation was 4.2 times higher (Fig. 4*A*, c) than those of the former two preparations. The conditions of the Cyt b_6f complex suspended in different buffers were diverse: the connection state becomes loose and the Chl might be separated from the complex suspended in SDS buffer (Chen *et al.* 2006). As a result, the free Chl *a* increased, which increased the production of $^1O_2$, Fig. 4*B* shows the production of $O_2^{\cdot-}$ by the Cyt b_6f complex suspended in the different buffers. The production of $O_2^{\cdot-}$ in the β-DM and β-OG buffers were comparable with each other (Fig. 4*B*, a and b), whereas in SDS, the production was 1.5 times higher than that produced in the complex suspended in β-DM (Fig. 4*B*, c).

Therefore, $^1O_2$ production corresponded with the $O_2^{\cdot-}$ generation in the same suspended buffers. This supports the possibility that $O_2^{\cdot-}$ was produced from an $^1O_2$ involving reaction.

Effects of scavengers on the $O_2^{\cdot-}$ production in the Cyt b_6f complex: To investigate the $O_2^{\cdot-}$ scavenging properties of ascorbate, glutathione, and β-carotene, we measured the $O_2^{\cdot-}$ production with the addition each of the scavengers and compared the results with those of the untreated control. Fig. 5 shows that the $O_2^{\cdot-}$ was 100% scavenged by β-carotene (Fig. 5, b) and glutathione (Fig. 5, d), whereas ascorbate only scavenged 45% of $O_2^{\cdot-}$ (Fig. 5, c). The characteristic EPR signal of ascorbate is shown in trace c. The results indicate that ascorbate, glutathione, and β-carotene are all effective $O_2^{\cdot-}$ scavengers.

Discussion

Thylakoid membranes, especially PSII, are capable of producing $O_2^{\cdot-}$ under illumination (Asada 2006, Pospíšil 2009). This phenomenon is called the Mehler reaction (Mehler 1951). The Mehler reaction was suggested to occur in PSI (Asada *et al*. 1974, Furbank *et al*. 1983). Some studies also show that $O_2^{\cdot-}$ is generated by PSII rather that by PSI, and pheophytin and Q_A are the main electron donors to molecular oxygen (Pospíšil *et al*. 2004). Furthermore, Cyt b_{559} is also shown as a source of $O_2^{\cdot-}$ in PSII (Pospíšil *et al*. 2006). These results demonstrate that high-light illumination of the Cyt b_6f complex of spinach leads to the production of $O_2^{\cdot-}$ (Fig. 1), and provides another site of $O_2^{\cdot-}$ generation in the thylakoid. Interestingly, the increase in the EMPO-OOH signal upon the addition of SOD (Fig. 2, c) might be due to the secondary iron signal produced by the reaction of protein and $O_2^{\cdot-}$. Further research is necessary to detect the precise reason for this phenomenon. Furthermore, the molecular oxygen requirement for the production of $O_2^{\cdot-}$ showed that O_2 is involved in $O_2^{\cdot-}$ production in the Cyt b_6f complex of spinach (Fig. 2, d), although the remaining signal is still unknown.

It is reported that the electron leakage might happen on 2Fe-2S and 4Fe-4S (Edreva 2005). No $O_2^{\cdot-}$ was detected in the preparation of the Rieske Fe-S depleted and Rieske Fe-S proteins (Fig. 3, b and c) whereas the $^1O_2$ production was detected (data not shown), which indicates that Rieske Fe-S might have participated in the formation of $O_2^{\cdot-}$. In addition, the Fe-S cluster was presumably the production site of of the superoxide anion radical in mitochondrial complex I (Genova *et al*. 2001). Our results concur with those reports. No $O_2^{\cdot-}$ was formed in the Rieske Fe-S protein because its separation from Cyt b_6f cut off the electron transfer cycle. Rieske

Fe-S protein might be an electron donor needed for the $O_2^{\cdot-}$ production. The results indicate that an intact electron transport cycle is necessary for $O_2^{\cdot-}$ production. Further study is necessary to elucidate the mechanism underlying this question. Moreover, the production of $O_2^{\cdot-}$ in the Cyt b_6f complex of spinach seems to be related with the singlet oxygen as about 69.6% of the EMPO-OOH adduct EPR signal was from the $^1O_2$ involving reaction. A study regarding the production of $O_2^{\cdot-}$ by hematoporphyrin and light indicates that by adding 1,4-diazabicyclo[2.2.2]octane (DABCO), a $^1O_2$ quencher, the intensity of the DMPO/OH spin adduct was reduced, which is consistent with the involvement of $^1O_2$ in $O_2^{\cdot-}$ production (Buettner and Oberley 1980). Our results are also consistent with these reports. Furthermore, the comparable yield of $O_2^{\cdot-}$ and $^1O_2$ in the same suspended buffer further indicates the possibility that $O_2^{\cdot-}$ might be produced from an $^1O_2$ involving reaction (Fig. 4).

Previous studies indicated that $^1O_2$ is an important factor of photodamage to the Cyt b_6f complex of spinach and the production of $^1O_2$ was detected by EPR spectroscopy (Suh *et al*. 2000). In this study, we also detected the production of $O_2^{\cdot-}$ in this complex. These results illustrate that $^1O_2$ and $O_2^{\cdot-}$ are both produced under high-light illumination of Cyt b_6f complex, and that the production of the $O_2^{\cdot-}$ might be related to the Rieske Fe-S protein. The present study has also proven that β-carotene, ascorbate, and glutathione are efficient $O_2^{\cdot-}$ scavengers, implying that the photoprotection mechanism of the Cyt b_6f in spinach might be partly implemented through various antioxidant substances in the thylakoid membranes.

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