Donor-side photoinhibition in photosystem II from *Chlamydomonas reinhardtii* upon mutation of tyrosine-Z in the D1 polypeptide to phenylalanine

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Abstract **When tyrosine-Z of the D1-polypeptide of the photosystem II from *Chlamydomonas reinhardtii* was changed to phenylalanine, the rapid donor to P_{680}^+ was lost, and P_{680}^+ accumulated on illumination. The rapid donation from tyrosine-Z was replaced by a slow electron transfer from an endogenous donor. Spectrophotometric measurements showed that carotenoids and chlorophylls were bleached by the P_{680}^+ either directly or indirectly upon illumination. The carotenoid bleaching was inhibited in the presence of SOD or catalase, but the reaction did not require molecular oxygen as an electron acceptor. These observations led us to conclude that active oxygen radicals, possibly hydroxyl radicals, take part in the destruction of carotenoids in the Y161F mutant. Possible mechanisms for the destruction are discussed.**

Key words: Photosystem II; Photoinhibition; Carotenoid; Tyrosine-Z; Active oxygen

1. Introduction

Photoinhibition is the debilitating effect of high light intensities on the photosynthetic capability of green plants. The light effect is mainly targeted to photosystem II (PS II) and leads to damage and degradation of the reaction center D1 polypeptide and bleaching of the chromophores (for reviews, see ref. [1] and [2]). Two types of photoinhibition have been defined [1]: (1) 'acceptor-side photoinhibition' could occur when the bound quinone acceptor Q_A is reduced to an abnormal, doubly reduced, protonated state, $Q_A H_2$, and further electron-transfer is inhibited, and (2) 'donor-side photoinhibition' could occur when the donation of electrons from water does not keep up with electron transfer from P_{680} to the acceptor side.

The 'donor-side photoinhibition' has previously been studied in vitro by using isolated PS II reaction centers or thylakoid membranes which have an inhibited or inactive water

oxidizing complex. Several light-induced events have been observed including degradation of D1 and D2 polypeptides [2,3], and irreversible oxidation and destruction of accessory pigments [4–7].

In this study, we generated a donor-side photoinhibition system by using genetic engineering to substitute a redox inactive phenylalanine for the primary donor, Tyr-161 (Y_Z), in the D1 polypeptide of the photosynthetic reaction center from *C. reinhardtii*. The corresponding mutation in *Synechocystis* PCC6803 was recently studied for its effect on turnover of the D1 protein [8]. Vermaas et al. [8] observed that D1 protein in this mutant was degraded in the light faster than in wild type and inferred that this was due to generation of the oxidized primary donor, P_{680}. We have examined the functional consequences of the mutation by measuring flash-induced changes in fluorescence yield (ϕ_f), and absorbance changes upon illumination under a variety of conditions. We observed a significant irreversible photobleaching corresponding to carotenoid and chlorophyll bleaching, which occurred when oxidized P_{680} accumulated. Since the donor-side photoinhibition is apparently a pleiotripic phenomenon, and it is not yet clear how each event is related, we focussed on the light-induced carotenoid bleaching to study a donor-side photoinhibition in a Y_Z-less mutant. We showed that the carotenoid bleaching was inhibited in the presence of SOD or catalase. Anaerobicity inhibited the carotenoid bleaching, but this inhibition was reversed by addition of ferricyanide. These results indicate that active oxygen radicals produced on the acceptor side are probably not involved but that a mechanism for production of oxygen radicals on the donor side, which does not require di-oxygen, is the probable source of the species responsible for the photooxidative damage. Possible mechanisms for the destruction of carotenoids are discussed.

2. Materials and methods

The tyrosine-161 residue (Y_Z) in the *psbA* gene from *C. reinhardtii* was replaced by phenylalanine via site-directed mutagenesis by means of PCR using an oligonucleotide primer (5′-TTCTTAGTTTTCCC-TATCGGC-3′). The mutagenesis, sequencing, and transformation were facilitated by using an intron-free *psbA* gene on a plasmid pBA155, which was described previously [9]. A *psbA* deletion mutant, ac-u-ε (from the Chlamydomonas Genetic Center, Duke University), was used as a host strain for the transformation. A wild-type control strain was made by transforming the ac-u-ε strain with the intron-free *psbA* gene on plasmid pBA155 which codes for the wild-type D1 polypeptide. The entire nucleotide sequence of the gene cassette between the restriction sites *Pst*I and *Sal*I was determined to contain the mutation Y161F and no unexpected mutations. The mutant cells were grown in dim light (≈ 1 μE/m^2/s) in a liquid TAP medium [10]. Flash-

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Abbreviations: P_{680}, primary electron donor chlorophyll(s); PS II, photosystem II; Q_A, primary quinone acceptor in PS II; Y_Z, tyrosine-161 residue on the D1 protein; *C. reinhardtii, Chlamydomonas reinhardtii*; SOD, superoxide dismutase; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethyl urea; Car, carotenoid

induced changes in fluorescence yield were measured using whole cells in the growth medium at room temperature. The time-course of changes in absorption spectra was measured at room temperature using thylakoid membranes (5 μg chlorophyll/ml) prepared by disrupting cells twice using a French press at 4000 psi. After separation by differential centrifugation, the thylakoids were suspended in a buffer containing 0.3 M sucrose, 25 mM HEPES, 25 mM MES, 5 mM MgCl$_2$, and 10 mM NaCl (pH 7.5). Fluorescence and absorbance kinetics were measured by a kinetic fluorimeter and a spectrophotometer, respectively, which were constructed in-house [11,12]. To achieve photoinhibitory conditions, samples were illuminated by a bank of red LEDs (65 μE/m^2/s) at room temperature. This relatively low intensity was sufficient to cause significant bleaching in the donor-side impaired centers. Anaerobic conditions were achieved by blowing a gentle stream of Ar gas over the surface of the sample solution stirred in a closed cuvette for 30 min, or incubating the sample in a closed cuvette for 5 min with 0.1 mg/ml glucose oxidase (Sigma), 0.1 mg/ml catalase (Sigma), and 5 mM glucose at room temperature. CuZn-SOD (bovine erythrocyte) was purchased from Sigma.

3. Results

3.1. Effects of removal the tyrosine-Z on P_{680}^+ re-reduction

The primary electron donor to oxidized P_{680}, tyrosine-161 (Y_Z), was replaced by phenylalanine through site-directed mutagenesis. The transformants with the Y161F mutant gene were unable to grow photoautotrophically. In order to examine effects of the mutation on the PS II photochemistry, we first measured the flash-induced fluorescence yield rise in whole cells in the presence of a Q_B-site inhibitor, DCMU. The fluorescence rise kinetics in the < 1 μs range seen in normal cells reflects the rapid re-reduction of P_{680}^+ (a quencher) by Y_Z; a phase with $t_{1/2} \sim 30$ μs reflects the thermodynamic phase of an equilibrium between states $Y_Z^+.P$ and $Y_Z.P^+$, and the kinetic phase of re-reduction of Y_Z^+ by the S-states (for reviews see ref. [13]). In the mutant strain, the fluorescence yield was low after the first actinic flash, showing that no rapid donor was present (Fig. 1). This result is consistent with that found previously using the corresponding mutant, Y160F, in *Synechocystis PCC 6803* [14], verifying
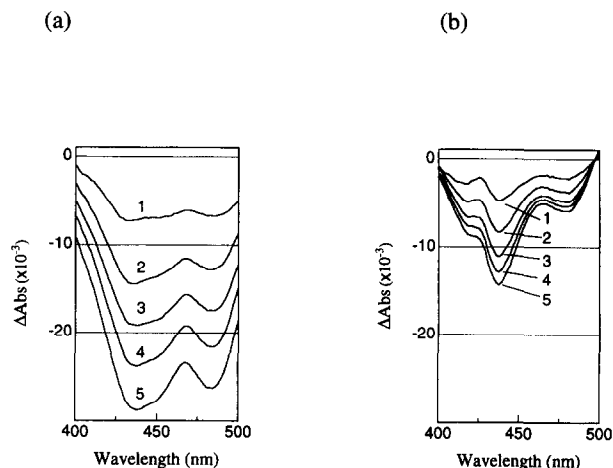
Fig. 2. Difference spectra upon illumination. Thylakoid membranes (5 μg Chl./ml) of the Y161F mutant (a) and wild-type control (b) were illuminated by red LEDs (65 μE/m^2/s) at room temperature. Spectra 1–5 are difference between spectrum after illumination for 1–5 min, respectively, and a spectrum in the dark.

that tyrosine-161 in *C. reinhardtii* is working as the primary electron donor, Y_Z, in a similar way.

When a train of actinic flashes was given, however, a high fluorescent state was accumulated, indicating that slow electron donor(s) were present, which competed with the back reaction ($t_{1/2} = 0.1$–1 ms, ref. [14]) for the reduction of P_{680}^+ (Fig. 1). This accumulation of a high fluorescent state has been observed previously in the cyanobacteria mutant [14]. Mets et al. [14] suggested that the high fluorescent state could be achieved by re-reduction of P_{680}^+ by hydroquinone generated in situ from *p*-benzoquinone, but this is unlikely in the present study because no *p*-benzoquinone was added. Therefore, we conclude that the lifetime of P_{680}^+ (a quencher of fluorescence) in the Y161F mutant is longer than that in the wild type, and that there is slow electron donation from endogenous donor(s) to P_{680}^+ in the mutant.

3.2. Photobleaching of carotenoids

In order to identify the slow electron donor to P_{680}^+, we measured absorbance changes of thylakoid membranes from the Y161F mutant upon continuous illumination. As is shown in Fig. 2a, several absorption bands including chlorophyll(s) (435 nm) and carotenoid(s) (488 and 450 nm) were found to be bleached. The bleached species had a half-time in the hours range, and the bleaching was essentially irreversible. Only minor bleaching species were observed in the wild-type control (Fig. 2b). Since the bleaching effects were induced by removal of the normal electron donor to P_{680}', we expected that an exogenous donor should compete with the endogenous slow donor. A donor hydroquinone was therefore added at 2 mM in the mutant thylakoid membrane samples. As shown in Fig. 3, the photobleaching at 488 nm was absent in the presence of hydroquinone, verifying that oxidized P_{680} is required for carotenoid bleaching. This is further confirmed by the observation that the bleaching was also absent in the presence of 10 μM DCMU which blocks Q_A^- to Q_B electron transfer, and therefore restricts the accumulation of P_{680}^+ to that formed in one turn-over (Fig. 3).

Photobleaching of carotenoids has been observed previously as one of the phenomenon diagnostic of photoinhibi-
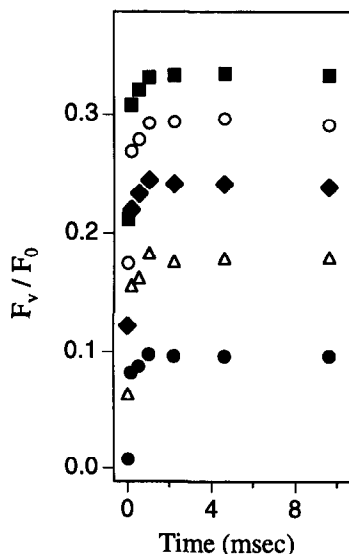

Fig. 1. Flash-induced kinetics of fluorescence yield changes in the presence of DCMU. DCMU was added at 10 μM to whole-cell samples of the Y161F mutant in growth medium (5 μg Chl./ml). Normalized fluorescence yield after the first (●), second (△), third (◆), fourth (○), and fifth (■) actinic flash are shown.
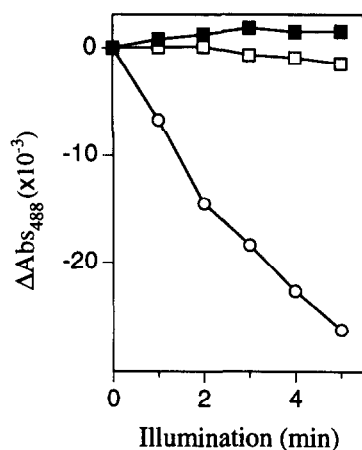
Fig. 3. Kinetics of the 488 nm bleaching in the presence of hydroquinone or DCMU. Kinetics of the photobleaching at 488 nm in the thylakoid membrane samples of the Y161F mutant in the presence of 2 mM hydroquinone (■) or 10 μM DCMU (□), and without additions (○) are shown. Thylakoid membrane samples were illuminated by red LEDs (65 μE/m²/s) at room temperature.

tion in chloroplasts [5] or PS II membranes [6] after removal of the Mn cluster, or in isolated reaction centers [7]. In this study, illumination of a mutant lacking Y_Z in the D1 polypeptide was also shown to be accompanied by irreversible photobleaching of carotenoid(s) upon illumination. Such a result strongly supports the generally held view that an accumulation of highly oxidizing radical leads to an inactivation of PS II [1].

3.?. Effects of active reduced oxygen species

It has been reported previously that degradation of the D1 polypeptide in Cl^--depleted or Tris-washed thylakoids [2], or in isolated reaction centers [3], was accelerated under either aerobic or anaerobic conditions, while Hill-reaction activity in hydroxylamine-extracted PS II membranes showed that anaerobiosis, oxyradical scavengers or SOD suppressed the photodamage [15]. Chen et al. [15] proposed a reaction mechanism for the donor-side photoinhibition in which a superoxide molecule reacts with a Y_Z^+ radical. In order to examine the effects of active oxygens on the irreversible carotenoid bleaching in the Y161F mutant, we illuminated samples in the presence of histidine, SOD, or catalase. While histidine, a singlet-oxygen scavenger, did not protect the bleaching in the range of concentration 1–25 mM, 20 μg/ml SOD or 330 μg/ml catalase markedly protected against the bleaching (Fig. 4). These results suggest that the bleaching of carotenoids in donor-side photoinhibition is caused by reaction with reduced oxygens, whereas singlet oxygen, which was shown to play an essential role in the acceptor-side photoinhibition [1], is not involved in photodestruction in the Y161F mutant. We measured absorbance changes at 488 nm under anaerobic conditions (Fig. 5), and observed that the bleaching no longer occurred in the absence of molecular oxygen. This seems to imply a requirement for oxygen to yield active oxygens. However, addition of 2 mM potassium ferricyanide to the sample under anaerobic conditions reversed the anaerobic inhibition of the photobleaching (Fig. 5), suggesting that the loss of bleaching under these conditions could be attributed to loss of an electron acceptor function at the acceptor side; the bleaching could not to be attributed to a specific role of oxygen in the produc-

tion of superoxide. We therefore conclude that active oxygen species are more likely to be formed by oxidation of water.

4. Discussion

Three plausible sites of oxygen radical formation were previously suggested: (1) oxygen reduction at the acceptor side of PS I [16] (Mehler reaction), (2) oxygen reduction at the acceptor side of PS II [17] and (3) water-oxidation at the donor side of PS II [18]. In the present study, we used a mutant of C. reinhardtii in which the oxygen evolving system is nonfunctional, making whole-chain electron transfer to PS I impossible. In addition, thylakoid membranes from C. reinhardtii are known to be broken during the process of disruption by a French press, causing soluble electron-transfer components, including plastocyanin, to be lost from the samples. Oxygen radicals are, therefore, unlikely to be formed at the acceptor side of PS I in the mutant.

The second possible site for oxygen radical formation is the acceptor side of PS II [17] and there are reports which support it in particular circumstances [15,19]. However, the reduction of oxygen at the site, if any occurs, has nothing to do with the carotenoid bleaching in this mutant, since addition of ferricyanide to the sample under anaerobic conditions reversed the anaerobic inhibition of the photobleaching (Fig. 5).

The third possibility is that active oxygen species are formed at the donor side of PS II. While oxygen radicals are not formed at the donor side in the intact PS II [20], H_2O_2 formation does occur in the Cl^--depleted Mn cluster as a product of OH^- oxidation [18], presumably by reaction with the strong oxidants available as Y_Z^+ and P_{680}^+ accumulate. In the Y161F mutant, the quenching of fluorescence indicates that P_{680}^+ accumulated, and likely provided a suitable oxidant. A major effect on Mn binding was detected in this mutant [21], with loss of a high-affinity site for Mn donation. The high-affinity site has previously been implicated in photoactivation of the Mn complex [22], and it seems possible that formation of the cluster is perturbed when no active tyrosine-Z is present to transfer oxidizing equivalents to the site, leaving a disrupted complex similar to that following Cl^--deple-
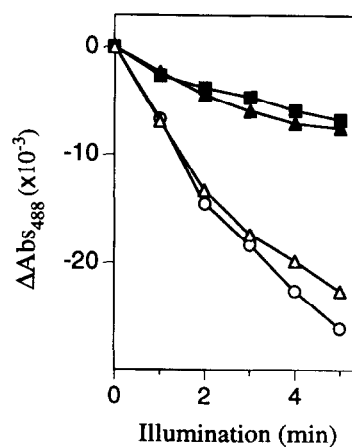

Fig. 4. Kinetics of the 488 nm in the presence of histidine, SOD or catalase. Kinetics of the photobleaching at 488 nm in the thylakoid membrane samples of the Y161F mutant in the presence of 2 mM histidine (△), 20 μg/ml SOD (▲) or 330 μg/ml catalase (■) and without additions (○) are shown. Thylakoid membrane samples were illuminated by red LEDs (65 μE/m²/s) at room temperature.
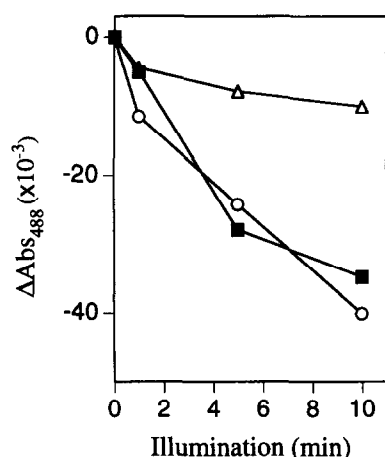
Fig. 5. Kinetics of the 488 nm bleaching under anaerobic conditions. Kinetics of the photobleaching at 488 nm in the thylakoid membrane samples of the Y161F mutant under aerobic conditions (O), under anaerobic conditions (△), or under anaerobic conditions in the presence of 50 mM potassium ferricyanide (■) are shown. Thylakoid membrane samples were illuminated by red LEDs (65 $\mu E/m^2/s$) at room temperature.

otenoids usually act as quenchers of excited triplet and singlet states and are not normally redox active. However, those in PS II were reported to be oxidized under photoinhibitory conditions (ref. [7] and this study). This seems to be a unique feature of PS II.

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tion or other inhibitory treatments; H_2O_2 could then potentially be formed at the site. The inhibition of the bleaching by SOD as well as catalase suggested that H_2O_2 itself was not the damaging species, since SOD catalyzes a disproportionation reaction of O_2^- and produces H_2O and H_2O_2. Together with the notion that H_2O_2 is not a very destructive species, but instead its decomposed product, hydroxyl radical (OH•), is more toxic [23], and the result that catalase protected the bleaching by catalyzing the reaction ($2 H_2O_2 \rightarrow O_2 + 2 H_2O$), it seems more likely that OH• is the species leading to damage in the mutant. While we inferred from our own results that the hydroxyl radical is the active species causing carotenoid bleaching, further information on the role of this oxygen radical was previously obtained by Hideg et al. [24]. They used spin-trap EPR experiments to demonstrate that hydroxyl radicals dominated under donor-side photoinhibition conditions. We reached the same conclusion and have provided an independent line of evidence for a role of hydroxyl radicals on the donor-side photoinhibition. Since SOD is known to inhibit the 'iron-catalyzed Haber-Weiss reaction' or 'superoxide-driven Fenton reaction' ($H_2O_2 + Fe^{2+}$ complex $\rightarrow$ Fe^{3+} complex $+ OH• + OH^-$, $H_2O_2 + Fe^{3+}$ complex $\rightarrow O_2^- + 2 H^+ + Fe^{2+}$ complex) [25], a protective role of SOD on the carotenoid bleaching can be accounted for by its inhibition of hydroxyl radical generation.

Accordingly, we think that the donor side of the PS II is the only plausible site for formation of the active oxygen species damaging carotenoids in the Y161F mutant. We postulate a three-step mechanism for the photodamage of carotenoid: (1) a P_{680}^+ is re-reduced via a nearby carotenoid, forming Car^+, (2) a water molecule is oxidized by re-oxidized P_{680}^+ formed after the second turnover, forming H_2O_2, and (3) a decomposed product of H_2O_2, OH•, destroys Car^+ irreversibly. Car-