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Histidine residue 252 of the Photosystem II D1 polypeptide is involved in a light-induced cross-linking of the polypeptide with the α subunit of cytochrome *b*-559: study of a site-directed mutant of *Synechocystis* PCC 6803

Lenka Lupínková [a,b], James G. Metz [c], Bruce A. Diner [c], Imre Vass [d], Josef Komenda [b,e,*]

[a] *Faculty of Biological Sciences, University of South Bohemia, 370 05 České Budějovice, Czech Republic*
[b] *Laboratory of Photosynthesis, Institute of Microbiology, Academy of Sciences, 379 81 Třeboň, Czech Republic*
[c] *Experimental Station, E. I. du Pont de Nemours and Co., Wilmington, DE 19880-0173, USA*
[d] *Biological Research Center, Hungarian Academy of Sciences, P.O. Box 521, H-6701 Szeged, Hungary*
[e] *Institute of Physical Biology, University of South Bohemia, 373 33 Nové Hrady, Czech Republic*

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Abstract

Properties of the Photosystem II (PSII) complex were examined in the wild-type (control) strain of the cyanobacterium *Synechocystis* PCC 6803 and its site-directed mutant D1-His252Leu in which the histidine residue 252 of the D1 polypeptide was replaced by leucine. This mutation caused a severe blockage of electron transfer between the PSII electron acceptors Q_A and Q_B and largely inhibited PSII oxygen evolving activity. Strong illumination induced formation of a D1–cytochrome *b*-559 adduct in isolated, detergent-solubilized thylakoid membranes from the control but not the mutant strain. The light-induced generation of the adduct was suppressed after prior modification of thylakoid proteins either with the histidine modifier platinum-terpyridine-chloride or with primary amino group modifiers. Anaerobic conditions and the presence of radical scavengers also inhibited the appearance of the adduct. The data suggest that the D1–cytochrome adduct is the product of a reaction between the oxidized residue His^{252} of the D1 polypeptide and the N-terminal amino group of the cytochrome α subunit. As the rate of the D1 degradation in the control and mutant strains is similar, formation of the adduct does not seem to represent a required intermediary step in the D1 degradation pathway.
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Keywords: Cyanobacterium; Cytochrome *b*-559; D1 polypeptide; Photoinhibition; Photosystem II; *Synechocystis* PCC 6803

1. Introduction

The membrane part of the Photosystem II (PSII) complex of higher plants, algae and cyanobacteria consists of a D1–D2 heterodimer, inner antennae CP47 and CP43 and a number of low molecular weight polypeptides including the α and β subunits of cytochrome *b*-559 (cyt *b*-559) [1]. All prosthetic groups involved in the direct electron transfer from water to plastoquinone are bound to the D1 and D2 polypeptides. In contrast, the heme moiety of cyt *b*-559, coordinated between the histidine residues of each subunit [2], seems to play a role in the cyclic electron flow around PSII protecting it against PSII photoinactivation (PSIIPI). Nedbal et al. [3] and Poulson et al. [4] obtained evidence for the function of cyt *b*-559 in preventing overreduction of the PSII acceptor side. On the other hand, Thompson and Brudwig [5] postulated its role as an auxiliary electron donor delivering electrons to oxidized chlorophylls in the PSII reaction center.

PSIIPI is also closely related to the fast turnover of the D1 polypeptide, which is believed to be a mechanism for the PSII repair and restoration of its photochemical activity [6,7]. Using in vitro systems, it has been found that PSIIPI may lead to the formation of various fragments originating

Abbreviations: Chl, chlorophyll; Cyt *b*-559, cytochrome *b*-559; DM, dodecylmaltoside; HRA, Hill reaction activity; LIN, lincomycin; PS, Photosystem; PSIIPI, photoinactivation of Photosystem II; Pt-TP, platinum (II) (2,2′ : 6′ ,2″-terpyridine) chloride; ROS, reactive oxygen species; TNBS, 2,4,6-trinitrobenzenesulfonic acid

* Corresponding author. Tel.: +420-333-721-101; fax: +420-333-721-246.
E-mail address: komenda@alga.cz (J. Komenda).

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from the cleavage of the D1 polypeptide in the external loops that join its transmembrane segments [8–10]. There is also formation of the well-defined product of cross-linking between the D1 polypeptide and the α subunit of the cyt *b*-559. This unusual polypeptide species was described for the first time by Barbato et al. [11] in illuminated reaction center complex from higher plants. More detailed characterization of the adduct [12] resulted in the hypothesis that it is formed by the cross-linking of the N-terminal residue of the cytochrome with an amino acid residue between Arg^{238} and Glu^{244} of the D1 polypeptide. However, this hypothesis was questioned by the subsequent analysis of *Synechocystis* mutants with large deletions in the DE loop of the D1 polypeptide that also included the critical region Lys^{238}–Glu^{244} [13]. Analysis showed light-induced formation of the D1–cytochrome adduct in these deletion mutants. This experiment lead to the conclusion (in contradiction to the results in Ref. [12]) that the cross-linking on the D1 polypeptide should occur at the C-terminal end of the DE loop closer to the Q_B binding site.

Also the importance of the adduct formation remains unclear. The adduct is not formed in the absence of oxygen and Okada et al. [14] suggested that it is formed as a consequence of the attack of PSII by reactive oxygen species (ROS). On the other hand, Barbato et al. [12,13] suggested that its generation during illumination is directly related to the D1 turnover and may represent a required intermediate during the D1 degradation process. Taken together, neither the mechanism of the origin nor the physiological significance of the adduct formation has as yet been determined.

Cross-linking of membrane and soluble proteins has also been observed in other biological systems including the erythrocyte membranes and myoglobin in the presence of photosensitizing substances [15,16,17]. Detailed analyses of these processes showed that an important mechanism involved in this process is the reaction of oxidized histidine residues with histidine, lysine and cysteine residues of other proteins, or with their N-terminal amino group. Examination of the primary structure of the D1 polypeptide shows that there is a conserved histidine residue in the DE loop at position 252, which could potentially be involved in the formation of the D1–cytochrome cross-link. In order to evaluate this possibility we characterized the site-directed mutant D1-His252Leu of the cyanobacterium *Synechocystis* PCC 6803 with the histidine residue 252 replaced by leucine. The results showed that indeed, the D1–cytochrome adduct is not formed in the mutant and that the mechanism of cross-link formation is based on the reaction of the oxidized His^{252} residue most probably with the free amino group of the first N-terminal serine residue on the α subunit of cyt *b*-559.

2. Materials and methods

The following strains of the cyanobacterium *Synechocystis* PCC 6803 were used in the study: (i) TC31 and TC35, two genetically identical isolates, with psbA1 and psbA2 genes replaced by chloramphenicol and kanamycin resistance cassettes and tetracyclin cassette inserted closely downstream the psbA3 gene [18]; (ii) the site-directed mutant D1-His252Leu in which the sequence of the psbA3 gene was changed in order to replace the histidine codon at position 252 by a leucine residue; and (iii) IC7, the strain with the psbH gene replaced by kanamycin resistance cassette [19].

To obtain the D1-His252Leu mutant, the site-directed mutation was introduced at codon 252 of the D1 polypeptide using 27-base mutagenic oligonucleotides according to the procedure described by Nixon et al. [18]. A new restriction site (*Rsa*I) was introduced close to the mutated codon as a marker to facilitate screening of the putative mutants. The host strain used was *Synechocystis* TD41 in which were deleted psbA1, psbA2 and all but the first 110 codons of psbA3. Plasmid pTC3 was used to introduce the site-directed replacements or the wild-type sequence (strains TC31 and TC35) into the restored psbA3.

The strains were grown in BG-11 medium supplemented with 10 mM glucose (photomixotrophic growth). On agar plates, BG-11 plus glucose contained in addition 10 mM Tes/NaOH, pH 8.2, 1.5% agar and 0.3% sodium thiosulfate [20] and, in the case of the D1-His252Leu and the IC7 mutants, 25 $\mu g\ ml^{-1}$ kanamycin and 5×10^{-6} M atrazine. Liquid cultures (100 or 1000 ml) in conical flasks were shaken using a rotary shaker and irradiated with 50–70 $\mu E\ m^{-2}\ s^{-1}$ of white light at 29 °C.

The culture was diluted every day to maintain the chlorophyll concentration at about 6–8 $\mu g\ ml^{-1}$. Before the experiments with the whole cells, the culture was adjusted to a chlorophyll concentration of 6 $\mu g\ ml^{-1}$ and placed in 18-mm-thick optical cuvettes in a temperature-controlled bath and bubbled with air containing 2% CO_2. Illumination was provided by tungsten filament bulbs. When the inhibitor lincomycin (LIN; 100 $\mu g\ ml^{-1}$ final concentration) was used, the cells were incubated for 5 min before the start of the light treatment. Each experiment was repeated two to four times.

The light-saturated (3500 $\mu E\ m^{-2}\ s^{-1}$) steady-state rate of oxygen evolution in cell suspensions was measured at 30 °C using a temperature-controlled chamber [21] equipped with a Clark-type electrode (YSI, USA). For measurement of photosynthesis, 10 mM sodium bicarbonate was added to the suspension; for measurement of Hill reaction activity (HRA) artificial electron acceptors *p*-benzoquinone (0.5 mM final concentration) and potassium ferricyanide (1 mM final concentration) were added just prior to measurement.

The initial (F_0), maximum (F_M) and variable ($F_V = F_M - F_0$) components of fluorescence were measured using a modulation PAM101 fluorometer (Walz, Germany) with a ED-101 US cuvette for cell suspension measurements. Cells were incubated for 5 min in the dark at 30 °C prior to fluorescence measurements. F_0 was subsequently determined using a red LED at 1.6 kHz. For measurement of

Table 1
Photosynthetic parameters of TC31 and D1-His252Leu strains grown in the presence of glucose

	F_V/F_M[a]	Photosynthesis (μmol O_2 mg^{-1} of (Chl) h^{-1})[a,b]	HRA (μmol O_2 mg^{-1} of (Chl) h^{-1})[a,c]
TC31	0.57 ± 0.02 (100)	230 ± 30 (100)	660 ± 50 (100)
D1-His252Leu	0.48 ± 0.03 (84)	12 ± 3 (5)	160 ± 30 (24)

[a] Numbers represent mean of four measurements ± S.E.; numbers in parentheses mean percent of the particular parameter taking value in TC31 as 100%.

[b] Photosynthesis means light-saturated oxygen evolving activity measured in cells in the presence of 10 mM bicarbonate.

[c] HRA means light-saturated oxygen evolving activity measured in cells in the presence of 0.5 mM *p*-benzoquinone and 1 mM potassium ferricyanide.

F_M, the PSII inhibitor (DCMU, 10^{-5} M final concentration) was added to the suspension which was then illuminated using a white light source with an output of 500 μE m^{-2} s^{-1} until the signal reached the maximum. This protocol for variable fluorescence measurement was employed instead of a saturating flash protocol to eliminate state transition effects on F_V values [22].

The rate of Q_A^- reoxidation after a single saturating flash was measured with a double-modulated fluorometer FL-100 (P.S.I., Czech Republic). Short, non-actinic pulses of blue light were used as the measuring light and F_M, reflecting fully reduced Q_A, was elicited by the strong saturating red flash. Cells were incubated for 5 min in the dark before the measurement began. The rate of Q_A^- reoxidation after a series (1.67 Hz) of five saturating 2-μs xenon flashes was measured as described in Ref. [23]. The cells were suspended at an OD_{730} of 0.9 in BG-11 medium and treated for 10 min in the dark with 0.2 mM *p*-benzoquinone and 0.2 mM $K_3Fe(CN)_6$ plus 50 mM Hepes/NaOH pH 7.5 and 10 mM $NaHCO_3$ prior to the measurement using the flash detection spectrophotometer. The time points of measurement were at 50, 100, 200 and 500 μs, and 1, 2 and 5 ms after each actinic flash. Each actinic flash was separated by 600 ms.

Thermoluminescence glow curves were obtained using a home-built apparatus described previously [24] with cells resuspended at 50 μg of (Chl) ml^{-1}. The cells were first illuminated with continuous white light for 30 s at 20 °C. After 5 min of dark adaptation at 20 °C, a single saturating flash was given at −5 °C (in the absence of DCMU) or at −10 °C (in the presence of DCMU) which was followed by fast cooling to −40 °C.

Thylakoid membranes were prepared by breakage of the cells with glass beads (150–200 μm in diameter) at 4 °C followed by differential centrifugation. For a small scale preparation (approximately 150 μg of Chl) the cells were washed and resuspended in 150 μl of 25 mM Tris/HCl buffer, pH 7.5, containing 1 mM aminocaproic acid. The beads (200 μl) were added to the suspension and the mixture was vortexed twice for 1 min with 2-min interruption for cooling on ice. The beads were then washed four times with 200 μl of buffer. Aliquots from the washings were pooled and spun at 3000 × *g* for 1 min to remove unbroken cells. The thylakoids were collected from the supernatant by a second centrifugation at 20,000 × *g* for 10 min. The final pellet was resuspended in 25 mM Tris/HCl buffer, pH 6.8, containing 1 M sucrose (final concentration 400–600 μg of (Chl) ml^{-1}) and stored at −75 °C. A large scale preparation of thylakoids (approximately 10 mg of Chl) was performed according to Tang and Diner [25] using a Beadbeater for breaking the cells.

Photoinhibition experiments with solubilized thylakoids were performed after the resuspension in 35 mM Hepes/NaOH, pH 8.0, and addition of dodecylmaltoside (DM/Chl = 20, w/w). After sedimenting unsolubilized material, the concentration of chlorophyll was adjusted to 150 μg of (Chl) ml^{-1} and the solubilized thylakoids were illuminated using white light at 1000 μE m^{-2} s^{-1} at 30 °C in optical cuvettes (optical path, 3 mm) with occasional mixing.

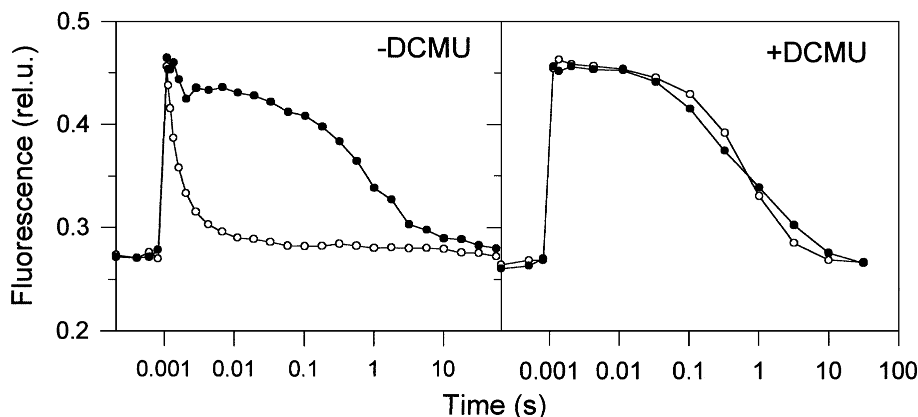

Fig. 1. Relaxation of the variable fluorescence after a single saturating flash given to cells of the control strain TC31 and the D1-His252Leu mutant strain. Cells of TC31 (empty circles) and D1-His252Leu (closed circles) at 3 μg of (Chl) ml^{-1} were dark-adapted for 5 min and then the variable fluorescence was followed before and after a saturating flash using the P.S.I. double modulated fluorometer in the absence (left panel) or presence of 10 μM DCMU (right panel).

Histidine residues in thylakoid proteins were modified according to the procedure of Ghirardi et al. [26]. Thylakoid membranes were resuspended in 20 mM Mes buffer, pH 6.5, containing 400 mM sucrose and 20 mM NaCl to a final concentration of 120 μg of (Chl) ml^{-1} and incubated with 100 μM platinum (II) (2,2′ : 6′ ,2″-terpyridine) chloride (Pt-TP) for 1 h at room temperature in the dark. The reaction was terminated by the addition of 2 volumes of ice-cold buffer supplemented with 10 mM histidine. Modification was followed by the absorption difference spectra of modified minus unmodified membranes according to Ref. [26]. Covalent binding of Pt-TP to histidyl residues is characterized by a $\Delta A_{342}/\Delta A_{328}$ ratio of 1.32. After modification the membranes were washed, solubilized and used for photoinhibition experiments.

Primary amines in thylakoid proteins were modified using 2,4,6-trinitrobenzenesulfonic acid (TNBS). Briefly, thylakoids were resuspended in the reaction buffer (0.1 M $NaHCO_3$, pH 8.6) to a final concentration 250 μg of (Chl) ml^{-1}. After the addition of TNBS to a final concentration of 1 mM, the thylakoids were incubated for 60 min at room temperature in the dark. After incubation the membranes were washed, resuspended in 35 mM Hepes/NaOH, pH 8.0, solubilized and used for photoinhibition experiments.

The protein composition of the thylakoids was determined by SDS-PAGE using a 12–20% linear gradient polyacrylamide gel containing 7 M urea [27]. The thylakoids were solubilized in 60 mM Tris/HCl, pH 9, containing 2% SDS (w/v) and 2% dithiothreitol (mass/vol) at 20 °C for 60 min. Sample lanes were loaded with the equal amounts of chlorophyll (1–2 μg of Chl) and the proteins in the gel were transblotted onto nitrocellulose membrane (0.2 μm, Schleicher-Schuel, Germany) by semi-dry blotting. The nitrocellulose membrane was incubated with specific antibodies and then with goat anti-rabbit secondary antibody–alkaline phosphatase conjugate. The polypeptides were visualized by a colorimetric reaction using BCPIP–NBT system. The antibodies used in the study were raised in rabbits against residues 58–86 of the spinach D1 polypeptide (D1 Ab) and the isolated α subunit of cyt *b*-559 from *Synechocystis* PCC 6803 (cyt *b*-559 Ab).

To measure the chlorophyll concentration, the cells were sedimented by centrifugation and extracted with 100% methanol. The concentration of chlorophyll was calculated from the absorbance of the extract at 666 and 720 nm using the extinction coefficients of Wellburn and Lichtenthaler [28].

3. Results

Table 1 shows several parameters that characterize the photosynthetic performance of the D1-His252Leu mutant in comparison with the control strain TC31. Despite rather similar values of F_V/F_M indicating functionality of the PSII reaction center of the mutant, further analysis showed strongly reduced rate of oxygen evolution. When measured in the presence of an artificial electron acceptor, the rate of oxygen evolution in the mutant did not exceed 30% of that in the TC31 strain dropping to 5% in the presence of 10 mM bicarbonate only. Figs. 1–3 provide an explanation for the inhibition of the photosynthetic oxygen evolution in the mutant. It is apparent that reoxidation of the reduced PSII electron acceptor Q_A^- is extremely slow, nearly identical to

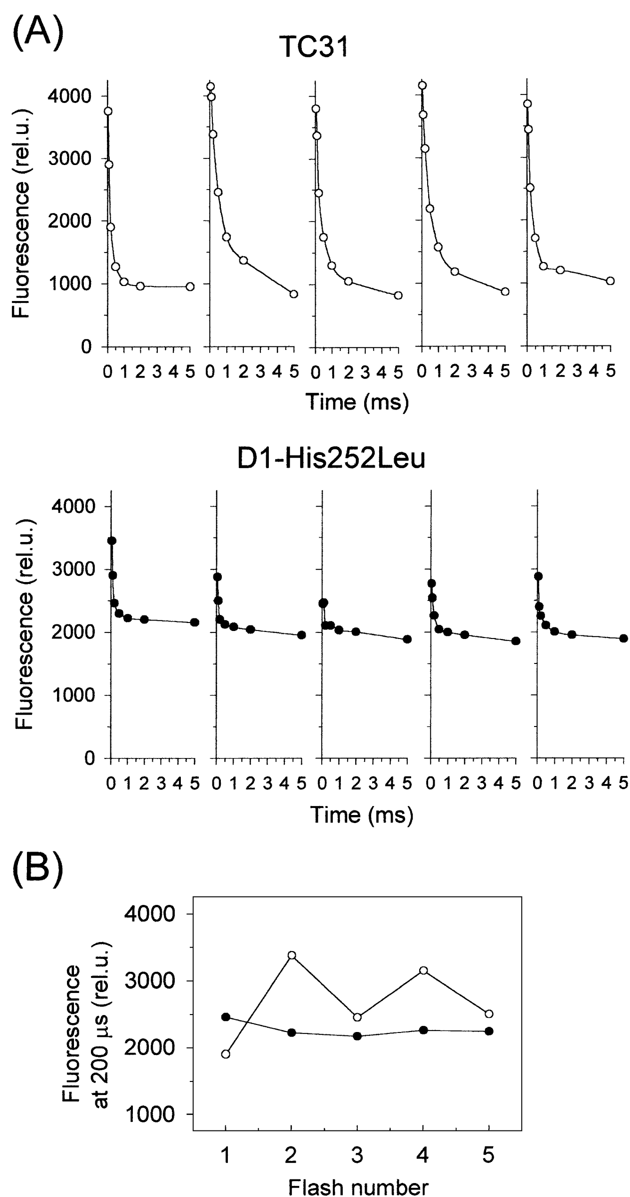

Fig. 2. Relaxation of the variable fluorescence (A) and oscillation of fluorescence at 200 μs (B) after a series of five saturating flashes given to cells of the control strain TC35 and the D1-His252Leu mutant strain. The cells of TC35 (empty circles) and D1-His252Leu (closed circles) were suspended at an OD_{730} of 0.9 in BG-11 medium and treated for 10 min in the dark with 0.2 mM *p*-benzoquinone and 0.2 mM $K_3Fe(CN)_6$ plus 50 mM Hepes/NaOH, pH 7.5, and 10 mM $NaHCO_3$ prior to the measurement using the flash detection spectrophotometer.

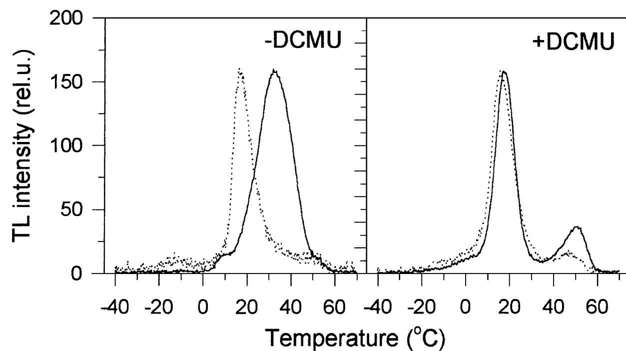

Fig. 3. Flash-induced thermoluminescence glow curves from cells of the TC31 and D1-His252Leu strains in the absence and presence of the PSII inhibitor DCMU. Cells of TC31 (solid line) and D1-His252Leu (dotted line) at 50 μg of (Chl) ml^{-1} were excited with one flash either at 5 °C in the absence (left panel) or −10 °C in the presence of 10 μM DCMU (right panel).

that observed in TC31 in the presence of the inhibitor DCMU (Fig. 1). The measurement of the fluorescence decay after five consecutive flashes in TC35 was characterized by an oscillation of period two of the fluorescence yield at 50–500 μs after the actinic flash (Fig. 2). The oscillation, which was most marked at the third measurement time point (200 μs after the actinic flash, Fig. 2B), arises from a faster reduction of Q_B than Q_B^- by Q_A^-. There was no such oscillation in the mutant. The reduction of Q_B and of Q_B^- are slow, both likely due to an impairment of coupled protonation steps. In addition, the quenching of the fluorescence after the second and third flashes likely reflects the influence of the S_2 and S_3 states, though there may also be some contribution arising from centers with incompletely assembled Mn clusters. Additional support for the slow electron transfer between the quinone acceptors comes from the measurement of thermoluminescence (Fig. 3). The B bands reflecting charge recombination between the reduced electron acceptor Q_B^- and the $S_{2,3}$ states on the donor side of PSII exhibited a typical maximum at 32–36 °C in the TC31 strain. In the presence of the PSII inhibitor, DCMU, the maximum of the Q band originating from the charge recombination between reduced electron acceptor Q_A^- and $S_{2,3}$ states was found at 16–18 °C. In contrast, the TL glow curve in the D1-His252Leu strain had a maximum at 16–18 °C both in the absence and presence of DCMU confirming the inhibition by the mutation of electron transfer between Q_A and Q_B.

Exposure of the cells of TC31 and D1-His252Leu to strong illumination in the presence of the protein synthesis inhibitor lincomycin resulted in the decline of PSII activity and a degradation of the D1 polypeptide (Fig. 4). The relative loss in activity was only slightly slower in the mutant strain and the rate of D1 degradation was very similar in both strains. Thus, the D1 degradative pathway in the mutant was not significantly affected by the mutation.

No adduct of the D1-polypeptide and the α subunit of cyt *b*-559 was detected in illuminated cells of either the TC31 or the D1-His252Leu strains. However, when thylakoids isolated from the TC31 strain and solubilized by dodecylmaltoside were exposed to strong light, the adduct was clearly detected (Fig. 5A). In contrast, no adduct was detected in the thylakoids from the D1-His252Leu strain (Fig. 5B). This result suggests that the histidine residue H252 of the D1 polypeptide could be involved in the formation of the adduct.

Derivatization of the histidine residues using a chemical modifier provides further support for the implication of a histidine residue in the formation of the adduct. In this case and in the following experiments with amino group modi-

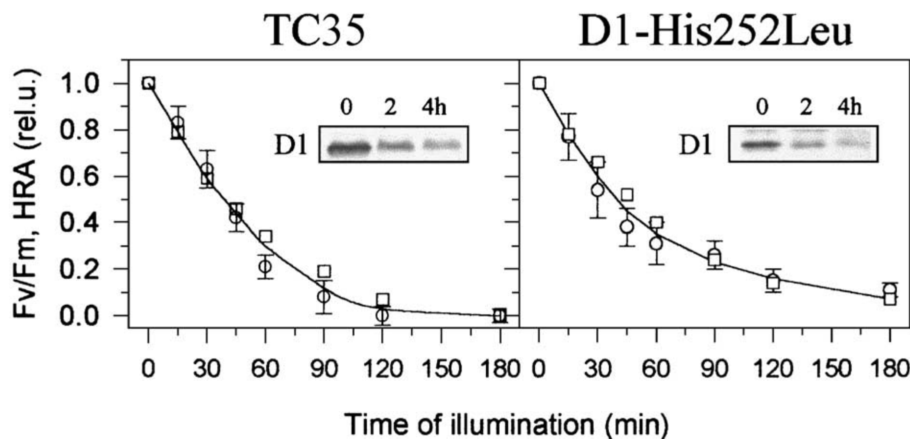

Fig. 4. Time course of the PSII photoinactivation and the D1 degradation in the cells of the TC31 and D1-His252Leu strains. The cells of the TC31 (left panel) and D1-His252Leu (right panel) strains were illuminated at 1000 μE m^{-2} s^{-1} in the presence of lincomycin (100 μg ml^{-1}) and aliquots of the suspensions were taken at the times indicated; F_V/F_M (circles) and HRA (squares) were assayed in whole cells as described in Materials and methods. Values in the plot represent mean of three measurements; only the standard errors for measurement of HRA are shown; in the case of F_V/F_M they did not exceed 8% in both strains. Initial values of F_V/F_M and HRA were in the range as shown in Table 1. Inserts: Western blot of polypeptides from cells taken at 0, 120 and 240 min of illumination using anti D1 antibody.

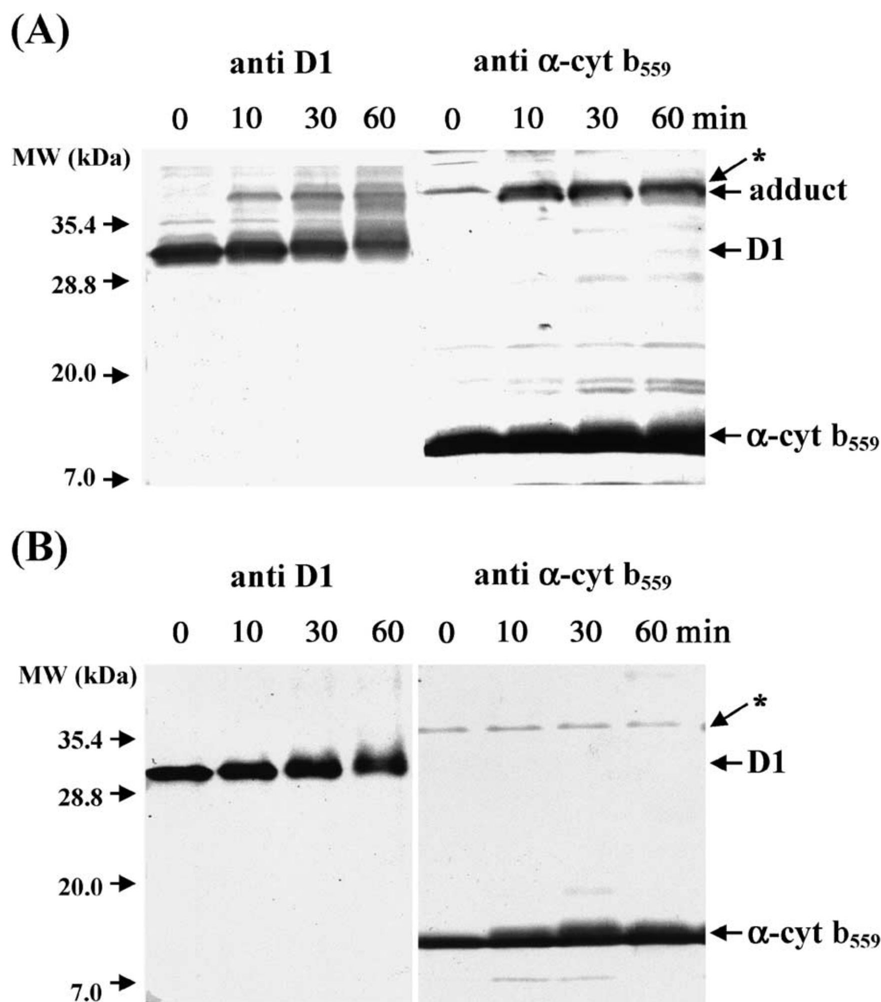

Fig. 5. The adduct of the D1 polypeptide and the α subunit of cyt *b*-559 is formed in solubilized and illuminated thylakoids of the control strain but not in those isolated from the D1-His252Leu strain. Thylakoids of the control strain TC31 and the D1-His252Leu mutant were subjected to high irradiance of 1000 μE m^{-2} s^{-1} for the indicated times and then analyzed by SDS-PAGE. The D1 polypeptide and the α subunit of cyt *b*-559 were detected on Western blots by specific antibodies. Polypeptide band indicated by * with slightly slower mobility than the D1–cyt adduct was present in the nonilluminated control thylakoids and represents an artifactual signal arising from nonspecific cross-reaction of the anti cyt *b*-559 antibody.

fiers and scavengers of ROS we used the strain IC7, the psbH deletion mutant of *Synechocystis*, in which the formation of the adduct is even more pronounced than in TC31 and the effect of modifiers or scavengers could be better evaluated. As can be seen in Fig. 6A, illumination of the modified thylakoids resulted in the loss of the D1 polypeptide but formation of the D1–cyt adduct was inhibited. Using measurement of variable fluorescence in the presence of DCMU and hydroxylamine [23], we found that the modification did not significantly influence the PSII charge separation activity in thylakoids and therefore its inhibition cannot account for the observed effect of the modifier (data not shown).

Based on microsequencing of the polypeptide, Barbato et al. [12] proposed that the N-terminal serine residue of the cyt *b*-559 α subunit might be involved in cross-linking to D1. In order to confirm this suggestion, we also analyzed the effect of pretreatment with chemical reagents that block free amino groups. Fig. 6B demonstrates that the preincubation of thylakoids from IC7 with TNBS reduced the appearance of the adduct during the subsequent illumination. Similar results were obtained also with other amino group modifiers citraconic anhydride, methylacetimidate and hydroxysuccinimide acetate (data not shown). As in the case of the histidine modification, there was no indication of an effect of the modifiers on the photochemical activities of the PSII complex as judged from the measurement of variable fluorescence.

Previous data on the mechanism of adduct formation were in line with that in erythrocyte membranes postulated as a reaction of histidine residues oxidized by ROS, and amino groups of neighbor proteins [15,16]. In order to confirm the dependence of adduct formation on the action of ROS (see also Ref. [14]), illumination of IC7 thylakoids was performed in the presence of several scavengers of ROS (Fig. 6C). In the presence of histidine, a scavenger of singlet

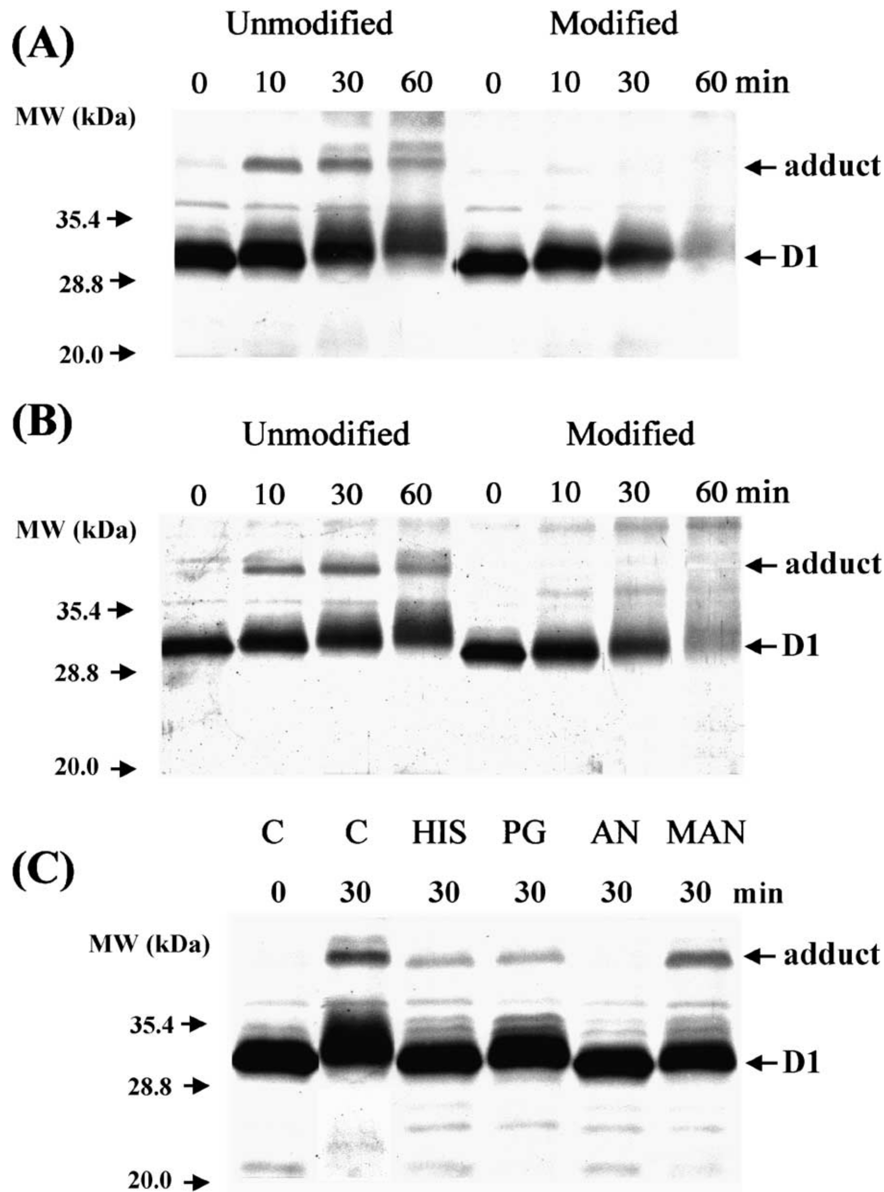

Fig. 6. Effect of protein modifications and presence of radical scavengers on the light-induced formation of the D1 – cyt adduct in solubilized thylakoids of the IC7 strain. (A) Thylakoids of the IC7 strain were pretreated with the histidine modifier platinum-terpyridine-chloride (100 μM) as described in Materials and methods; (B) thylakoids of the IC7 strain were pretreated with the amino group modifier TNBS (1 mM) as described in Materials and methods; and (C) following scavengers of ROS were added to the suspension of IC7 thylakoids prior to illumination: HIS—50 mM histidine; PG—10 mM propylgallate; AN—10 mM glucose, 50 U ml^{-1} glucose oxidase and 1000 U ml^{-1} catalase; MAN—50 mM mannitol. Thylakoids were then subjected to a high irradiance of 1000 μE m^{-2} s^{-1} for the indicated times. Thylakoid proteins were afterwards separated by SDS-PAGE and after transfer from the gel onto nitrocellulose membrane the D1 polypeptide was detected by specific antibody.

oxygen, the amount of the adduct was reduced, but a shift in the electrophoretic mobility of the D1 polypeptide, another phenomenon observed during illumination, was largely eliminated. Also propyl gallate, scavenger of alkoxyradicals, inhibited formation of the adduct but it did not affect the shift of electrophoretic mobility. In the absence of oxygen, both the mobility shift and the adduct formation were eliminated. Finally, mannitol, a scavenger of hydroxyl radicals, showed only a limited effect on the mobility of the D1 polypeptide.

4. Discussion

The purpose of this study was to examine the influence of the site-directed mutation D1-His252Leu on the formation of the D1 – cyt b559 adduct. Characterization of the mutant in vivo revealed a block of the electron transfer between the PSII electron acceptors Q_A and Q_B, which probably represents the primary reason for the inability of the strain to grow photoautotrophically. This block was also found in strains with His^{252} replaced by Gly, Lys and Glu, indicating a

general requirement of the histidine residue in the position 252 for the proper function of the PSII acceptor side.

A value of the F_V/F_M ratio of around 0.5 in the D1-His252Leu mutant demonstrates a high quantum efficiency of the PSII reaction center for primary charge separation comparable with the control strain TC31. Also an intensity of the D1 band on Western blot obtained with samples containing the same chlorophyll content suggests only slightly lower content of PSII in the thylakoids of D1-His252Leu strain compared to TC31.

Western blot analysis of changes in the D1 and cytochrome content during illumination of thylakoids showed that the D1 polypeptide from the D1-His252Leu strain could not undergo a cross-linking reaction with the cytochrome subunit. One possibility is that the block in the electron transfer is responsible for the loss of the cross-link. However, we consider this possibility as improbable for the following reasons. In the solubilized thylakoids, Q_B is either not bound in or is very quickly released from its binding site in PSII during illumination. In addition, solubilization makes other plastoquinone molecules unavailable for PSII electron transfer. This implies that there is minimal or no electron flow coming out of PSII in thylakoids of both control and mutant strains. Therefore, the effect of the mutation on the cross-linking reaction cannot be explained by its inhibitory influence on the electron transfer between Q_A and Q_B. In agreement with this argument, the presence of the PSII inhibitor DCMU did not affect the appearance of the adduct in the control thylakoids.

Previous data by Okada et al. [14] as well as our own experiments with scavengers of ROS provide evidence regarding the participation of these species in the process of adduct formation. Therefore, another explanation for the absence of the adduct in the illuminated thylakoids of the mutant is that the generation of ROS in PSII is blocked due to the mutation. However, the ROS generation is closely related to the PSIIPI [29] and our data showed only small difference in the rate of this process between the control and mutant strains.

We consider as the most likely explanation for the above-described observations that the residue H252 is directly involved in adduct formation. Firstly, the residue lies outside the region R225–V249 that was shown to be dispensable for the adduct formation [13]. Secondly, in line with this idea, histidine modification by the specific chemical modifier blocked the formation of the adduct. Thirdly, cross-linking of proteins via oxidized histidine residues has also been demonstrated in other biological systems [16,17].

Barbato et al. [12] proposed participation of the N-terminal serine residue of the α subunit of cyt *b*-559 in adduct formation as judged by the inability to get any sequence information by Edman degradation of the adduct. The effect of amino group blockers supports this conclusion. Again, reaction of the free amino group with the oxidized histidine residue has been documented during photosensitizer-induced cross-linking of proteins [15,16]. The data are in agreement with the following scenario of cross-linking: the histidine residue 252 of the D1 protein is oxidized by singlet oxygen (partially scavenged by histidine), and thus the generated histidine-alkoxyradical (partially scavenged by propylgallate) reacts with the amino group of the cytochrome subunit.

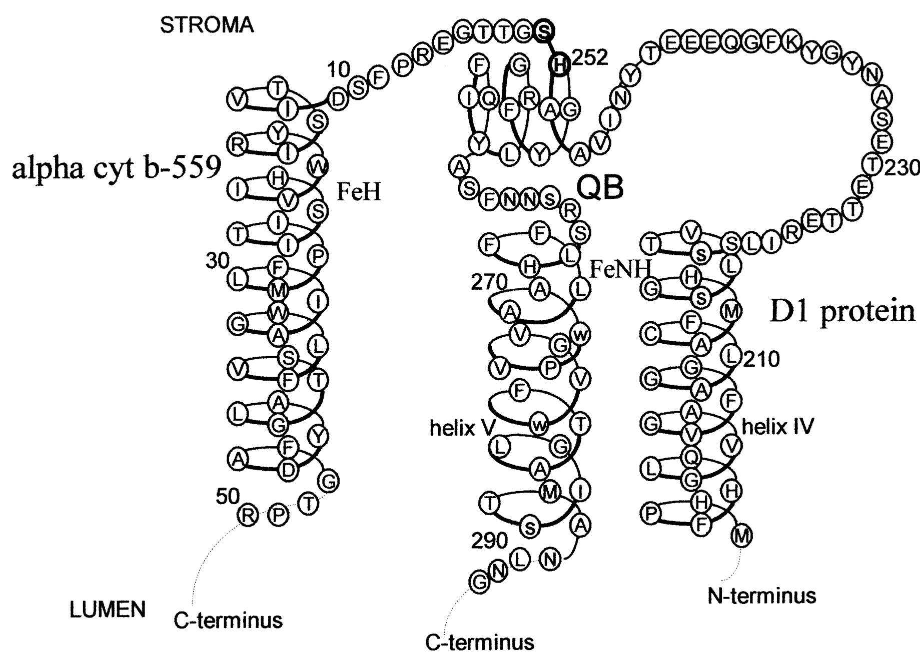

Fig. 7. Folding model of the IV and V helixes of the D1 polypeptide and N-terminal part of the α subunit of cyt *b*-559 showing the location of the cross-link (bold letters). Helixes of the D1 polypeptide and the α subunit of cyt *b*-559 are shown together with approximate location of non-heme iron (FeNH), quinone acceptor Q_B and heme moiety of the cyt *b*-559 (FeH).

The fact that the rate of the D1 degradation is very similar in the cells and thylakoids of the control and mutant strains shows that the main degradative pathway(s) of the polypeptide is not significantly changed by the mutation. We assume that the formation of the adduct is not a common step of this pathway and, as judged from the intensity of its band, only a small fraction of the oxidized D1 polypeptide undergoes a reaction with the cytochrome subunit. The oxidized D1 polypeptide alone or after reaction with the cytochrome is a target of proteolysis [30, 31]. However, oxidation does not seem to be a prerequisite for D1 degradation. This has been documented for the cyanobacterium *Synechococcus* PCC 7942, in which replacement of the high-light form D1:2 for D1:1 upon the transfer of the cells from high to low light occurs in functional PSII complexes in which polypeptide oxidation is improbable [32]. All of these data reinforce the idea of the existence of multiple pathways leading to the degradation of this polypeptide [30–32].

The identification of the cross-linking site between the D1 polypeptide and the cytochrome subunit (for model see Fig. 7) can also be important from the point of view of cyt *b*-559 function. Some studies (e.g. Ref. [33]) have indicated the possibility of the electron transfer between Q_B and cyt *b*-559. However, as judged from the 3.8 Å structural model of PSII [34], the heme moiety is distant from the Q_B binding pocket. However, proximity of the N terminus of the cytochrome subunit to the Q_B binding pocket, as judged from the present study, may indicate that this part of the cytochrome subunit could function as a molecular wire, facilitating electron transfer from the heme to the Q_B quinone.

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