Low unsaturation level of thylakoid membrane lipids limits turnover of the D1 protein of photosystem II at high irradiance

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Abstract Turnover of the D1 protein of photosystem II (PSII) was studied in mutants of *Synechocystis* sp. PCC 6803 defective in the desaturation of thylakoid membrane lipids. The lack of polyunsaturated fatty acids from membranes made PSII extremely susceptible to photoinhibition and caused a significant reduction in the D1 protein content of the thylakoid membranes at high irradiances. These results may be attributed to an impaired function in the protein synthesis machinery, most probably at the translational or posttranslational level.

Key words: D1 protein; Fatty acid unsaturation; Photoinhibition; Photosystem II; *Synechocystis*; Thylakoid membrane

1. Introduction

Similar to other multiprotein complexes functioning in photosynthetic electron transport, photosystem II (PSII) is embedded in the lipid bilayer of the thylakoid membrane. Although there is some evidence indicating that membrane lipids and specific lipid classes are involved in the function of these macromolecular complexes [1–4], the overall role of glycerolipids and the effect of the degree of unsaturation in regulative associations are far from clear. Until recently, apart from specific hydrogenation of membrane fatty acids [5,6], the effects of membrane lipid unsaturation could have been examined only non-specifically, by growing the cells at lowered or elevated temperatures. Under such conditions, however, not only the expression of membrane fatty acid desaturases but also the synthesis of many cold- or heat-induced proteins is activated. This has made it difficult to evaluate the particular effects of membrane lipid unsaturation on the functional efficiency of the photosynthetic machinery. Recent construction of mutants, differentially defective in fatty acid desaturation [7–11], has provided the tool for revealing the role of membrane lipid unsaturation without disturbing side-effects. In mutants with genetically engineered desaturases, an increased level of fatty acid unsaturation was found recently to be related to the light tolerance of PSII [12–14].

PSII is known to be vulnerable to stress induced by excess illumination, a phenomenon known as photoinhibition of PSII [15]. In this process, the D1 protein of PSII reaction center is damaged, degraded and needs to be replaced with a newly synthesized D1 protein [16–18]. This rapid turnover of the D1 protein during photoinhibition is regarded as an intrinsic feature of PSII to avoid total and energetically costly disassembly of this large, multiprotein complex. It was recently found that lack of membrane lipid unsaturation resulted in an impaired recovery of cyanobacterial PSII from photoinhibition [13]. Accordingly, it was suggested that the level of lipid unsaturation could affect the rate of repair of PSII by decreasing the rate of D1 protein turnover.

Two *Synechocystis* sp. PCC 6803 cyanobacterium mutants defective in fatty acid desaturation were used in this study. The Fad6/desA::Km^r mutant lacks the ability to desaturate monounsaturated fatty acids, whereas the Fad6 mutant is able to desaturate mono- but not di-unsaturated fatty acids [10,11], and the wild type can desaturate both mono- and di-unsaturated fatty acids. These two genetically altered mutants also differ in their susceptibility to high irradiance. In this study, we investigate the relationship between the degree of unsaturation of membrane lipids and efficacy of the turnover of PSII reaction center protein D1.

2. Materials and methods

2.1. Cyanobacterial strains and culture conditions

Wild type cells and the fatty acid mutant derivatives Fad6 and Fad6/desA::Km^r of the cyanobacterium *Synechocystis* sp. PCC 6803 [10,11] were grown photoautotrophically at 33°C under constant illumination at the PPFD of 40 μmol photons $\cdot m^{-2} \cdot s^{-1}$ (Philips TLD 36W/86 tubes) with 5% CO_2 in the air of the growth incubator (Gallenkamp). BG-11 growth medium [19] was supplementally buffered with 20 mM HEPES-NaOH (pH 7.5). The mutant Fad6/desA::Km^r was cultured in the presence of kanamycin (30 $\mu g \cdot ml^{-1}$). The cultures used for experiments were in the middle of the logarithmic growth phase.

2.2. High-light treatments of the cells

The cells were transferred to fresh BG-11 medium at a concentration of 10 μg Chl $\cdot ml^{-1}$. Cell suspensions were illuminated at the PPFD of 500, 1000 and 1500 μmol photons $\cdot m^{-2} \cdot s^{-1}$, with a slide projector, at 33°C. In some experiments, protein synthesis was blocked by adding lincomycin (Sigma) at a concentration of 400 $\mu g \cdot ml^{-1}$ to the cell suspension 5 min before the high-light treatment.

2.3. Measurements of oxygen evolution of PSII

The electron transfer activity of PSII was measured in vivo in fresh BG-11 medium (10 μg Chl $\cdot ml^{-1}$) using a Clark-type oxygen electrode. As an electron acceptor, 1 mM 1,4-benzoquinone was used with 1 mM $K_3Fe(CN)_6$.

2.4. Immunological quantification of the D1 protein

Thylakoid membranes were isolated as described earlier [20]. A serine proteinase inhibitor, phenylmethane-sulfonylfluoride (80 μM; Boehringer-Mannheim), was included in the isolation buffer. The polypeptides of the thylakoid membranes were separated by SDS-PAGE [21] with 14% acrylamide in the separation gel. After electrophoresis, the polypeptides were transferred to an Immobilon PVDF membrane

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Abbreviations: Lin, lincomycin; PAGE, polyacrylamide gel-electrophoresis; PPFD, photosynthetic photon flux density; PSII, photosystem II.

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(Millipore). Blots were probed with a D1 protein-specific antibody. Immunochemical analyses were performed using a chemiluminescence kit (Bio-Rad), and the D1 protein was quantified by scanning the immunoblots with a laser densitometer (LKB). The half-life of the D1 protein was determined by first-order kinetic fits from the loss of the D1 protein in lincomycin-treated cells.

2.5. Measurements of chlorophyll concentration

Chlorophyll concentration from intact cells was determined according to Bennett and Bogorad [22] and from thylakoid membranes in 80% acetone as reported in Arnon [23].

2.6. RNA isolation, electrophoresis and Northern blot analysis

Total RNA was isolated as described previously [20,24]. RNA was fractionated on 1.2% agarose-glyoxal gels and subsequently transferred to Hybond-N nylon membrane (Amersham). DNA probes were radiolabeled with [α-^{32}P]CTP using a multiprime DNA labeling kit (Amersham). The coding region of *psbA2* gene of *Synechocystis* sp. PCC 6803 was used as a probe. After removing this probe, the same membrane was reprobed with the *rrn* genes of *Synechococcus* sp. PCC 7942 from the pAN4 plasmid [25] to verify equal loading of the gel.

3. Results

3.1. Photoinhibition of PSII in vivo

Fig. 1 shows the in vivo photoinhibition of PSII oxygen evolution in *Synechocystis* sp. PCC 6803 mutants and in the wild type cells under the PPFD of 500 μmol photons$\cdot m^{-2}\cdot s^{-1}$. In the Fad6/desA::Km^r mutant, the oxygen-evolving activity fell 50% within 30 min of illumination, whereas in the Fad6 mutant and in the wild type, the time necessary to reach the same degree of inhibition was three and four times longer, respectively.

3.2. Effect of high irradiance on the level of the D1 protein in thylakoid membranes

To establish whether the high susceptibility of the Fad6/desA::Km^r mutant to photoinhibition is related to D1 protein turnover, the D1 protein content of the thylakoid membranes was determined under PPFDs of 500, 1000 and 1500 μmol photons$\cdot m^{-2}\cdot s^{-1}$. In the immunoblotting analyses, the D1 protein content detected corresponds to the sum of active and inactive (or damaged) D1 protein at a certain time point. At 500 μmol photons$\cdot m^{-2}\cdot s^{-1}$, only a slight reduction in the D1 protein content occurred in the wild type cells, whereas a clear decrease in the D1 protein was seen in the Fad6/ desA::Km^r

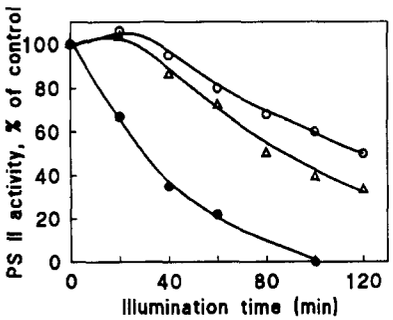

Fig. 1. Inhibition of PSII oxygen evolution upon exposure of the wild type (○), the Fad6 mutant (△), and the Fad6/desA::Km^r mutant (●) cells to a PPFD of 500 μmol$\cdot m^{-2}\cdot s^{-1}$. Control rates of oxygen evolution were 260, 270 and 310 μmol O_2 mg Chl$^{-1}\cdot h^{-1}$ for the wild type, the Fad6 mutant and the Fad6/desA::Km^r mutant, respectively. Experiments were repeated three times.

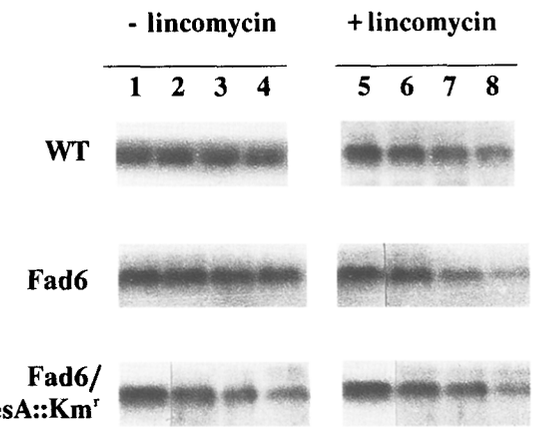

Fig. 2. Immunoblots demonstrating the loss of the D1 protein from the thylakoid membranes of the wild type, the Fad6 and the Fad6/desA::Km^r mutant strains during illumination at the PPFD of 500 μmol photons$\cdot m^{-2}\cdot s^{-1}$. The cells were illuminated for 0 (lanes 1 and 5), 40 (lanes 2 and 6), 80 (lanes 3 and 7) and 160 (lanes 4 and 8) min, either in the absence (lanes 1–4) or presence (lanes 5–8) of lincomycin. 2 μg of chlorophyll was applied to each well.

mutant (Fig. 2 and 3A–C); after 160 min of illumination, the D1 protein content fell by 14% in the wild type and 61% in the Fad6/desA::Km^r mutant cells. After 160 min illumination at the strongest light intensity, 1500 μmol$\cdot m^{-2}s^{-1}$, the Fad6/desA::Km^r mutant had lost 85% of its D1 protein, whereas the loss in the wild type cells was only 29%. In the Fad6 mutant, the loss of the D1 protein was approximately the same as in the wild type under each light intensity.

3.3. Rate of the D1 protein degradation under different light conditions

To judge whether the enhanced loss of the D1 protein of the Fad6/desA::Km^r mutant relative to the wild type is due to a faster rate of D1 protein degradation upon illumination, the photoinhibitory treatments were performed in the presence of a translation inhibitor lincomycin (Fig. 2 and 3D–F). The half-life of the D1 protein was calculated by fitting the light-induced loss of the D1 protein in lincomycin-treated cells to the first order reaction kinetics. The half-life of about 135 min for the wild type, and 117 min for the Fad6/desA::Km^r mutant was determined for the D1 protein under the PPFD of 500 μmol$\cdot m^{-2}\cdot s^{-1}$. The half-life of the D1 protein under the PPFD of 1000 μmol$\cdot m^{-2}\cdot s^{-1}$ was 84 min for the wild type and 65 min for the Fad6/desA::Km^r mutant, and under the PPFD of 1500 μmol$\cdot m^{-2}\cdot s^{-1}$ 59 min and 46 min, respectively. In the Fad6 mutant, the half-lives were very similar to those of the wild type (data not shown).

3.4. Accumulation of the psbA transcripts under high light

Since the remarkable inability of the Fad6/desA::Km^r mutant to maintain a stable level of the D1 protein under high-light treatment did not appear to be related to faster degradation of the D1 protein, the expression of the *psbA* genes at the level of transcription was studied by Northern blot analysis. Transfer of the cells from low growth-light into high light (1000 μmol photons$\cdot m^{-2}\cdot s^{-1}$) caused a rapid accumulation of *psbA* transcripts in all strains (Fig. 4). The amount of *psbA* transcripts increased three- to four-fold during the high- light treatment (30 min) both in mutants and the wild type.

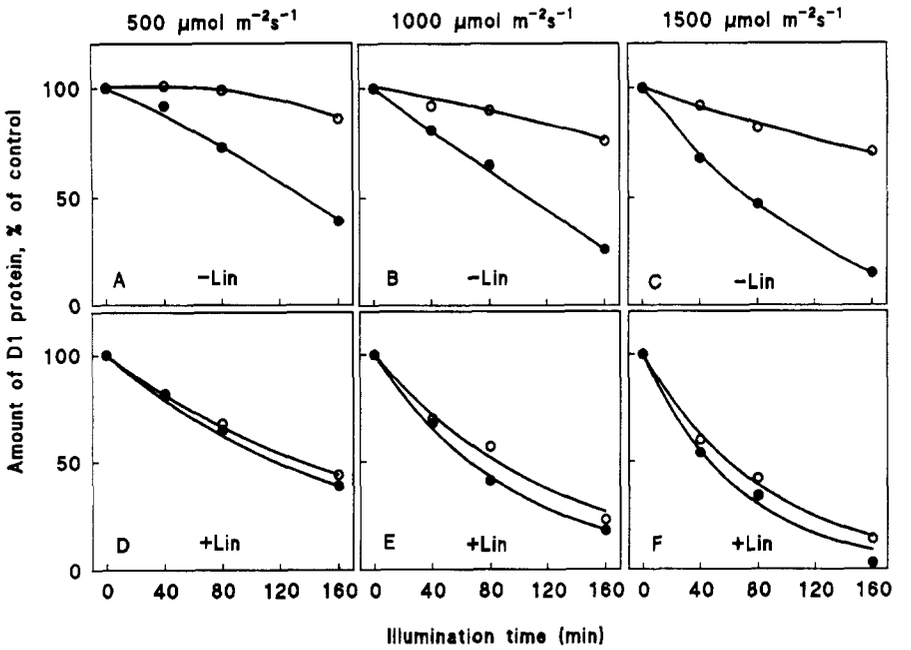

Fig. 3. Loss of D1 protein from the thylakoid membranes of the wild type (○) and the Fad6/desA::Km^r mutant (●) during illumination of intact cells at different light intensities either in the absence (A,B,C) or presence (D,E,F) of lincomycin (Lin). The values are the means of three to four independent experiments.

4. Discussion

In the present study, we have used two *Synechocystis* sp. PCC 6803 fatty acid mutants [10,11] to investigate the role of unsaturation of membrane fatty acids in the repair cycle of PSII reaction centers. PSII electron transfer activity of the Fad6/CdesA::Km^r mutant, containing only mono-unsaturated fatty acids, is extremely susceptible to high irradiance [12] (Fig. 1). Further investigation revealed that PSII inactivation, measured in the presence of the protein synthesis inhibitor, proceeded similarly in both mutants and wild type cells, suggesting that the high-light susceptibility of the Fad6/desA::Km^r mutant is mainly attributed to the impairment of the reactivation of damaged PSII centers during illumination [13]. The repair of PSII is initiated by degradation of the damaged D1 protein, which is subsequently replaced with a new D1 protein copy [17,18]. The D1 protein is first synthesized in a precursor form, and after C-terminal processing of the pre-D1 protein [26,27], the PSII complex becomes functional in electron transport.

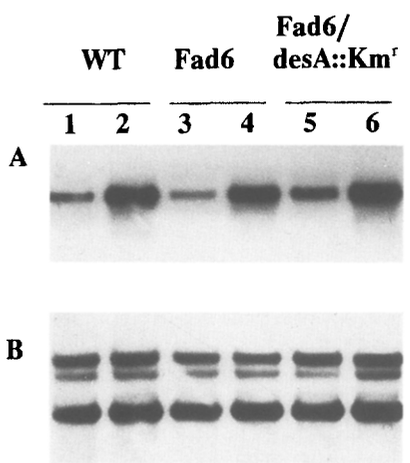

Fig. 4. Effect of high light shift on the amount of *psbA* transcripts (A) in the wild type and the mutant strains. Lanes 1, 3 and 5 = growth light; lanes 2, 4 and 6 = after 30 min at 1000 μmol·m^{-2}·s^{-1}. Equal loading of the gel was verified by reprobing the membrane with *rrn* probe (B). 5 μg of total RNA was loaded in each well.

As revealed by immunoblotting experiments, the D1 protein content decreased differentially in the course of illumination in the three strains examined (Figs. 2 and 3A–C). Only strong, long-lasting illumination induced loss of the D1 protein from the wild type membranes (Fig. 3) [20], whereas much milder conditions (lower light and shorter duration of illumination) caused significant reduction in the D1 protein in the Fad6/desA::Km^r mutant. This result clearly demonstrates that the extent of unsaturation of the thylakoid membrane fatty acids significantly affects the level of D1 protein and undoubtedly also the oxygen-evolving capacity of the cells under high irradiance. To our knowledge, this is the first time that the effect of lipid unsaturation of thylakoid membranes has been shown to alter the turnover rate of a specific polypeptide in the photosynthetic machinery.

A number of reasons can be posited for the greater, illumination-induced reduction in the D1 protein content found in the thylakoid membranes of the Fad6/desA::Km^r mutant than in the wild type or the Fad6 mutant: either the enhanced degradative capacity of photoinactivated PSII centers or, an impaired efficiency to synthesize, insert or stabilize a new D1 protein copy into the turning-over PSII complex. To study whether the first step of the repair cycle of the PSII complex, degradation and depletion of the D1 protein from this complex, is influenced by the level of lipid unsaturation, a set of D1 protein degradation experiments was performed in the presence of the translation inhibitor lincomycin (Figs. 2 and 3D–F). However, only minor differences in the half-lives of the D1 protein were re-

corded, irrespective of the drastic difference in the susceptibility of different strains to photoinhibition. This clearly demonstrates that even though the thylakoid membranes of the Fad6/desA::Km^r mutant are, due to the lower level of unsaturation, more rigid than those of the wild type, the capacity of the former to degrade the damaged D1 protein does not limit the repair of PSII centers.

Since the impairment of the efficiency of the PSII repair cycle in the Fad6/desA::Km^r mutant at high irradiances cannot be attributed to the impaired degradation of damaged D1 protein, the reason(s) for the higher susceptiblity of the Fad6/desA::Km^r mutant to photoinhibition and for reduction in the level of the D1 protein are most likely found at the level of protein synthesis. Unsaturation of membrane fatty acids did not, however, seem to affect the expression of the *psbA* genes at the level of transcription, since the transfer of low-light grown cells into high light caused a characteristic, strong accumulation of *psbA* transcripts in each strain (Fig. 4) [20,24]. Therefore, initiation of translation, translation elongation including targeting of the nascent D1 polypeptide chain into the membrane, or reassembly of the PSII complex under repair are possible phases of the repair cycle of PSII which may be affected by the decreased level of unsaturation in membrane fatty acids. However, we consider it unlikely that processing of the pre-D1 protein to mature D1 protein is involved, since no accumulation of the pre-D1 protein was observed in cells pulse-labeled with [^{35}S]methionine (data not shown). On the other hand, it should be kept in mind that PSII with unprocessed D1 protein is inactive in oxygen evolution, and is therefore extremely sensitive to donor side photoinhibition [28], which would result in rapid degradation of the newly synthesized unprocessed D1 protein.

Increased rigidity of thylakoid membranes due to a reduced level of fatty acid unsaturation may disturb cotranslational insertion of the D1 protein into the membrane, or it may increase the instability of newly assembled PSII complexes, leading to a premature loss of the D1 protein in the membrane. Alternatively, after degradation and depletion of damaged D1 protein, the remaining PSII polypeptides may become so destabilized that they are incapable of functioning as an acceptor-complex [28,29] for a new D1 protein. These alternative explanations agree with earlier studies using *Arabidopsis* fatty acid mutants which showed that defects in the desaturation of membrane lipids may impair the ability of the lipid matrix to mediate the assembly of chloroplast membrane components [30]. Although the PSII components, mainly D1 protein but also D2 protein to some extent, turn over at all light intensities [17,18], the detrimental effects of low unsaturation level of thylakoid membranes on PSII function, as shown in this study, become obvious only at high irradiances, when efficient PSII repair is required to maintain PSII activity.

In conclusion, we have shown that the first phase of PSII repair, the degradation of the damaged D1 protein, is not affected by the unsaturation level of thylakoid membrane fatty acids. Therefore, the increased susceptibility to photoinhibition and the drastic reduction in the level of the D1 protein due to the lack of polyunsaturated fatty acids in thylakoid membranes can be attributed to the impaired function of protein synthesis, most likely occurring at the level of translation or reassembly of PSII complexes under repair.

Therefore, high level of fatty acid unsaturation of the thylakoid membranes is likely to be for rapid D1 protein translation or reassembly of PSII complexes under repair.

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