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Membrane Lipid Unsaturation Modulates Processing of the Photosystem II Reaction-Center Protein D1 at Low Temperatures[1]

Eira Kanervo, Yasushi Tasaka[2], Norio Murata, and Eva-Mari Aro*

Department of Biology, University of Turku, FIN-20014 Turku, Finland (E.K., E.-M.A.); and Department of Regulation Biology, Institute for Basic Biology, Myodaiji, Okazaki 444, Japan (Y.T., N.M.)

The role of membrane lipid unsaturation in the restoration of photosystem II (PSII) function and in the synthesis of the D1 protein at different temperatures after photoinhibition was studied in wild-type cells and a mutant of *Synechocystis* sp. PCC 6803 with genetically inactivated desaturase genes. We show that posttranslational carboxyl-terminal processing of the precursor form of the D1 protein is an extremely sensitive reaction in the PSII repair cycle and is readily affected by low temperature. Furthermore, the threshold temperature at which perturbations in D1-protein processing start to emerge is specifically dependent on the extent of thylakoid membrane lipid unsaturation, as indicated by comparison of wild-type cells with the mutant defective in desaturation of 18:1 fatty acids of thylakoid membranes. When the temperature was decreased from 33°C (growth temperature) to 18°C, the inability of the fatty acid mutant to recover from photoinhibition was accompanied by a failure to process the newly synthesized D1 protein, which accumulated in considerable amounts as an unprocessed precursor D1 protein. Precursor D1 integrated into PSII monomer and dimer complexes even at low temperatures, but no activation of oxygen evolution occurred in these complexes in mutant cells defective in fatty acid unsaturation.

The fatty acid composition of membrane lipids plays a major role in the low-temperature acclimation of plants and cyanobacteria. Chilling-sensitive plants are known to contain high levels of saturated molecular species of phosphatidylglycerol in their thylakoid membranes, whereas chilling-resistant species have the majority of their phosphatidylglycerols in the unsaturated form (Murata, 1983). Many plant species can vary their level of membrane fatty acid unsaturation in response to environmental conditions, e.g. by increasing the level during low-temperature acclimation. Similar changes in the extent of fatty acid unsaturation have also been found to occur in the thylakoid membranes of cyanobacteria (Sato and Murata, 1980; Wada and Murata, 1990). Low temperature, however, may modify the protein composition of the membranes as well as the carbohydrate status of the cells, thus making it difficult to establish whether a change in fatty acid unsaturation is of primary importance in the acclimation of the cells to low temperatures. Only recently have techniques become available that enable the lipid and protein composition of membranes to be separately manipulated and studied (Somerville, 1995).

Recent constructions of plant and cyanobacteria mutants with specific changes in membrane lipid composition and in the level of fatty acid unsaturation have offered new possibilities for the direct study of the physiological importance of these membrane properties with respect to chilling tolerance and low-temperature-induced photoinhibition (Murata and Wada, 1995; Somerville, 1995). Several recent studies have succeeded in demonstrating increased susceptibility of cyanobacterial mutant cells to low-temperature-induced photoinhibition when the unsaturation level of membrane lipids was reduced by genetic manipulation of fatty acid desaturase genes (Wada et al., 1990; Gombos et al., 1992, 1994). Furthermore, recovery from photoinhibition at low temperature was shown to be remarkably impaired in such a cyanobacterial mutant (Gombos et al., 1994). In another study, tolerance for chilling-induced photoinhibition was shown to be altered in transgenic tobacco plants containing increased amounts of disaturated phosphatidylglycerol (Moon et al., 1995). Both of these studies suggest that events in the PSII repair cycle may be involved in the response of plants and cyanobacteria to low-temperature-induced photoinhibition.

The molecular mechanisms controlling photoinhibition and PSII repair have been under intense study for more than a decade (Prasil et al., 1992; Aro et al., 1993). An interesting feature of photoinhibition and the subsequent repair of PSII is the exceptionally rapid turnover of the heterodimeric reaction-center protein, D1, which is constantly degraded and resynthesized in a light-intensity-dependent fashion (Tyystjärvi and Aro, 1996). The repair process has been shown to require a replacement of a damaged D1 protein with a newly synthesized protein copy (Ohad et al., 1984; Mattoo et al., 1988). The D1 protein is known to be synthesized on thylakoid-bound ribosomes (Minami and Watanabe, 1984; Herrin and Michaels, 1985) as a precursor form with a 9- to 16-amino acid carboxyl-

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[2] Present address: Research Institute for Biological Sciences Okayama (RIBS) 7549–1 Yoshikawa, Gayon-cho, Jouhou-gun, Okayama, Japan 716–2.

*Corresponding author; e-mail evaaro@utu.fi; fax 358–2–333–8075.

Abbreviations: Chl, chlorophyll; pD1, D1-protein precursor.

terminal extension (Reisfeld et al., 1982; Marder et al., 1984; Takahashi et al., 1988). After insertion into the thylakoid membrane, pD1 is processed at its carboxyl-terminal end by a specific lumenal protease (Reisfeld et al., 1982; Taylor et al., 1988; Inagaki et al., 1989; Taguchi et al., 1993; Fujita et al., 1995) that cleaves the extension at amino acid 344, resulting in a mature D1 protein of equal size in both plants and cyanobacteria.

The importance of pD1 processing in the establishment of the PSII oxygen-evolving complex has been demonstrated in several studies (Diner et al., 1988; Bowyer et al., 1992; Nixon et al., 1992). However, little is known of how low-temperature photoinhibition affects D1-protein synthesis and maturation. In the present paper we have studied the relationship between D1 protein synthesis and low-temperature-induced photoinhibition. To further investigate how changes in the level of thylakoid-membrane lipid unsaturation may affect D1-protein synthesis under varied conditions of temperature and light, we used the fatty acid double mutant *desA*$^-$/*desD*$^-$ of the cyanobacterium *Synechocystis* sp. PCC 6803, in which the extent of fatty acid unsaturation was reduced by inactivation of desaturase genes (Tasaka et al., 1996). We demonstrate that the low level of fatty acid unsaturation drastically impairs D1-protein turnover at lowered temperatures by exerting its effects specifically on the processing of the pD1 protein in the thylakoids, thereby preventing recovery from photoinhibition.

MATERIALS AND METHODS

Cyanobacterial Strains and Culture Conditions

The fatty acid double mutant *desA*$^-$/*desD*$^-$ of *Synechocystis* sp. PCC 6803 contains *desA* and *desD* genes disrupted with kanamycin- and chloramphenicol-resistance gene cartridges, respectively (Tasaka et al., 1996). The fatty acid double mutant Fad6/*desA*::Km^r was constructed as described previously (Wada et al., 1992). Both the mutant and the wild-type strains were grown photoautotrophically at 33°C under constant illumination at a PPFD of 40 μmol photons m^{-2} s^{-1}. BG-11 growth medium (Stanier et al., 1971) was supplementally buffered with 20 mM Hepes-NaOH, pH 7.5. The mutant *desA*$^-$/*desD*$^-$ strain was cultured in the presence of kanamycin (10 μg mL^{-1}) and chloramphenicol (5 μg mL^{-1}), and the mutant Fad6/*desA*::Km^r was cultured in the presence of kanamycin (30 μg mL^{-1}). Cultures in the middle of the logarithmic growth phase were used in the experiments.

Measurement of Oxygen Evolution of PSII

The electron-transfer activity of PSII was measured in vivo in fresh BG-11 medium (10 μg Chl mL^{-1}) using an oxygen electrode (Hansatech Instruments Ltd., King's Lynn, UK). 1,4-Benzoquinone (1 mM) was used as the electron acceptor with 0.8 mM $K_3Fe(CN)_6$.

In Vivo Labeling of the Proteins

For preparation of the cells for labeling, cells were transferred to fresh BG-11 medium at a concentration of 10 μg Chl mL^{-1}. [^{35}S]L-Met (1000 Ci $mmol^{-1}$, Amersham) was added to the culture to a final concentration of 1.5 μM, and labeling was carried out at 40 or 1000 μmol photons m^{-2} s^{-1} at a temperature of either 33 or 18°C. During the labeling period samples for electrophoresis were taken at different times, nonradioactive L-Met was added to a final concentration of 1 mM, and the samples were cooled rapidly on ice. The cells were harvested by centrifugation at 8000*g* for 10 min at 4°C.

For low-temperature photoinhibition experiments, the cells were first transferred to fresh BG-11 medium at a concentration of 10 μg Chl mL^{-1}, and then subjected to photoinhibitory illumination at 1000 μmol photons m^{-2} s^{-1} at either 18 or 15°C for 1 h. Immediately after high-light illumination, [^{35}S]L-Met (1000 Ci $mmol^{-1}$) was added to the cultures to a final concentration of 1.5 μM, and the cells were labeled at 40 μmol photons m^{-2} s^{-1} at 33, 26, 18, or 15°C. After the labeling period, the cells were treated as described above.

SDS-PAGE, Autoradiography, and Immunological Quantification of the D1 Protein

Thylakoid membranes were isolated as described previously (Gombos et al., 1994), and the polypeptides were separated on linear SDS-PAGE (Laemmli, 1970) with 12% acrylamide and 4 M urea in the separation gel. After electrophoresis, the gels were either prepared for autoradiography or blotted onto Immobilon-P membranes (Millipore) for immunodetection. For autoradiography the gels were fixed (25% 2-propanol, 10% acetic acid) for 20 h, dehydrated (35% ethanol, 2% glycerol) for 90 min, treated with Amplify (Amersham) for 20 min, dried, and exposed to radiography films. Autoradiograms were scanned with a laser densitometer (Ultroscan XL, LKB, Bromma, Sweden).

For protein blotting, a semi-dry transfer apparatus (Pharmacia) was used. Blots were probed either with an amino-terminal or a DE-loop-specific commercial D1-protein antibody. The commercial antibodies (Research Genetics, Huntsville, AL) were raised against the oligopeptides TTTLQQRESASLWEQF and NYGYKFGQE, containing amino acids 2 to 17 from the amino terminus and amino acids 234 to 242 from the loop between the α-helixes D and E of the D1 protein of *Synechocystis* sp. PCC 6803, respectively. Immunochemical analyses were performed using a chemiluminescence kit (Bio-Rad), and the membranes were exposed to radiography films. The D1 protein was quantified by scanning the fluorograms with a laser densitometer.

Deriphat-PAGE

Protein complexes of the thylakoid membranes were separated by nondenaturing Deriphat-PAGE, as originally described by Peter and Thornber (1991) with the modifications of Barbato et al. (1995). For Deriphat-PAGE gels a 5 to 16% (w/v) acrylamide gradient was used at an acrylamide: bisacrylamide ratio of 48:1.5. The thylakoid samples (25 μg of Chl per well) were solubilized with 2% β-D-dodecyl maltoside on ice for 8 min, centrifuged in a microfuge for 2 min, and immediately applied to the gel. Electrophoresis

was carried out using 0.1% Deriphat-160 (Henkel Corp., Hoboken, NJ) in the running buffer (upper container). For second-dimension SDS-PAGE, the green bands representing PSII monomer and dimer complexes were cut out and incubated in the solubilizing buffer (Laemmli, 1970) on ice for 3 h, and run as described above.

High-Salt Washing of the Thylakoid Membranes

Thylakoid membranes were washed with 2 M (or 4 M) NaBr using a slightly modified method of Nelson (1980). A solution of 5 M NaBr was added to the membrane suspension (1 μg Chl μL^{-1}) in 0.4 M Suc, 10 mM NaCl, 5 mM DTT, and 10 mM Tricine (pH 8), to give a final concentration of 2 M (or 4 M) NaBr. After incubation at 0°C for 30 min, an equal volume of water was added, and the suspension was centrifuged for 15 min. The pellet was resuspended in 10 mM Tricine (pH 8.0) at 10 times the original volume, and centrifuged for 15 min. The resulting pellet was homogenized in a medium containing 50 mM Tricine (pH 7.5), 0.6 M Suc, 30 mM $CaCl_2$, and 1 M Gly betaine.

Measurement of Chl Concentration

The Chl concentration of the intact cells was determined according to the method of Bennet and Bogorad (1973), and that of the thylakoid membranes in 80% acetone was determined according to the method of Arnon (1949).

RESULTS

Accumulation of the pD1 Protein during Low-Temperature Photoinhibition Treatment

To investigate whether the D1 protein undergoes turnover during low-temperature photoinhibition, the D1 protein was immunoassayed following high-light treatment (1000 μmol photons m^{-2} s^{-1}, 18°C, 60 min) performed in the presence and absence of the protein-synthesis inhibitor lincomycin. During high-light treatment at 18°C in the presence of lincomycin, total D1 protein content decreased approximately 20% in both wild-type and *desA*$^-$/*desD*$^-$ mutant cells, whereas in the absence of lincomycin no loss of D1 was observed (Fig. 1). This would seem to indicate that at least limited D1-protein turnover can also occur at low temperature, with no difference in the degradation of the D1 protein being found between the wild-type and the fatty acid mutant strains.

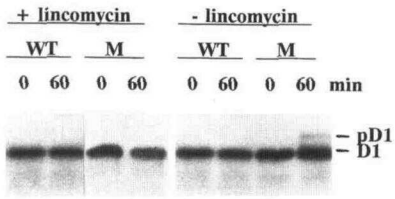

Figure 1. D1-protein immunodetection during low-temperature photoinhibition treatment. An immunoblot demonstrating the total D1-protein content in thylakoid membranes of the wild type (WT) and the *desA*$^-$/*desD*$^-$ mutant (M) of *Synechocystis* sp. PCC 6803 before (0 min) and after (60 min) the photoinhibition treatment at 1000 μmol photons m^{-2} s^{-1}, 18°C, in the presence and absence of lincomycin. Thylakoid membranes were isolated, followed by separation of polypeptides with linear urea-SDS-PAGE. Polypeptides were then transferred to an Immobilon-P membrane, immunoassayed with a DE-loop-specific D1-protein antibody, and quantified with a laser densitometer. Samples equivalent to 0.75 μg of Chl were applied in each well.

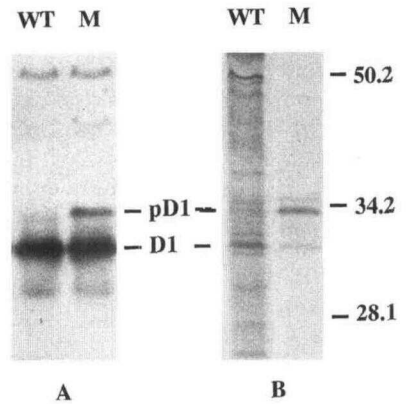

Figure 2. Identification of pD1. The cells were pulse-labeled with [^{35}S]L-Met at 1000 μmol photons m^{-2} s^{-1} for 30 min at 18°C. Thylakoid polypeptides were separated with urea-SDS-PAGE and then transferred to an Immobilon-P membrane. After exposure to radiography film, the membrane was further used for immunodetection of the D1 protein with a DE-loop-specific antibody. A, Immunoblot; B, autoradiogram. WT, Wild type; M, *desA*$^-$/*desD*$^-$ mutant. Molecular mass markers in kD are indicated to the right of the autoradiogram. Samples of 1.25 μg of Chl were applied in each well.

During high-light incubation at 18°C in the absence of lincomycin, an extra polypeptide band was recognized by the D1-specific antibody in the cells of the fatty acid mutant *desA*$^-$/*desD*$^-$ (Fig. 1). The molecular mass of this band, approximately 33.5 kD, corresponded to that of the precursor form of the D1 protein. Its identity as unprocessed pD1 was confirmed on the basis of the following evidence: (a) the antibodies raised against both the amino-terminal end and the DE-loop of the D1 protein recognized the 33.5-kD protein; (b) no immunoresponse at the 33.5-kD region could be obtained if lincomycin was present during low-temperature photoinhibition treatment (Fig. 1), indicating that the 33.5-kD protein is newly synthesized; and (c) the pulse-labeling experiments also revealed the appearance of the same 33.5-kD protein in the mutant cells under low-temperature photoinhibition conditions (Fig. 2). It will be shown below that the newly synthesized 33.5-kD protein disappeared concomitantly with the appearance of the mature D1 protein, not only in the mutant but also in the wild-type cells, clearly behaving as a pD1 protein (Reisfeld et al., 1982).

Impaired D1-protein processing and the accumulation of the pD1 protein in the thylakoid membranes could, in theory, result from the strict temperature regulation of the processing reaction itself. An alternative reason could simply be that there is a lack of the processing enzyme on the lumenal side of the thylakoid membrane, possibly because of its rapid turnover and temperature-sensitive import mechanisms to the thylakoid lumen. To distinguish between these two possibilities, the processing of pD1 accu-

mulated during low-temperature photoinhibition was followed at different temperatures in the presence of the protein-synthesis inhibitor lincomycin. The cells were first incubated under high-light conditions (1000 μmol photons m^{-2} s^{-1}) at 18°C for 60 min, after which lincomycin was added and the cells were transferred to low light at either 18 or 33°C (Fig. 3). At 18°C the amount of the pD1 protein in the *desA*$^-$/*desD*$^-$ mutant cells remained practically unchanged in the presence of lincomycin (Fig. 3), whereas at 33°C pD1 diminished rapidly from the cells of the *desA*$^-$/*desD*$^-$ mutant. It therefore seems that the processing enzyme is relatively stable on the lumenal side of the thylakoid membrane, whereas the processing reaction appears to be highly temperature-dependent. This temperature dependence can be attributed to either the catalytic process itself or to low-temperature-induced modifications in the availability of the substrate, the carboxyl terminus of the D1 protein.

Synthesis of the D1 Protein after Low-Temperature Photoinhibition

Cells of wild-type *Synechocystis* sp. PCC 6803 and of the double mutant *desA*$^-$/*desD*$^-$ were first incubated at 1000 μmol photons m^{-2} s^{-1} at 18°C for 60 min to induce photoinhibition of PSII, and were subsequently transferred to 40 μmol photons m^{-2} s^{-1} at either 18 or 33°C, with concomitant pulse-labeling with [^{35}S]L-Met. During incubation at 18°C, the label was incorporated not only into the D1 protein but also into some other proteins in the wild-type cells (Fig. 4). In sharp contrast to the wild-type cells, the fatty acid mutant accumulated radioactivity predominantly into an approximately 33.5-kD polypeptide, which was identified above as pD1. At 33°C, after a short pulse period of 7 min, the majority of the label in the mutant cells was detected in pD1 (Fig. 4), whereas with prolonged pulse periods (30 and 60 min), the radioactivity in this polypeptide gradually diminished, and the label in the mature D1 protein concomitantly accumulated (Fig. 4). This behavior in the incorporation of radioactivity into these two polypeptides provides strong support for the interpretation that the 33.5-kD polypeptide is indeed the pD1 protein.

Impaired D1-protein processing at low temperature in the fatty acid mutant *desA*$^-$/*desD*$^-$ was accompanied by

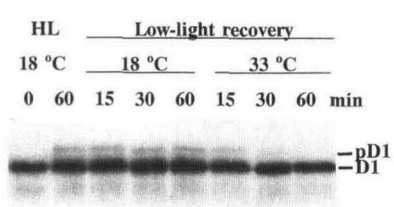

Figure 3. An immunoblot demonstrating the low-temperature retention of the pD1 protein in the *desA*$^-$/*desD*$^-$ fatty acid mutant of *Synechocystis* sp. PCC 6803 after low-temperature photoinhibition in the presence of lincomycin. The cells were incubated at 1000 μmol photons m^{-2} s^{-1} (HL) for 60 min at 18°C. Lincomycin was added, the cell suspension was transferred to low light at either 18 or 33°C, and the D1-protein was immunoassayed after 15, 30, and 60 min. Samples of 0.75 μg of Chl were applied in each well.

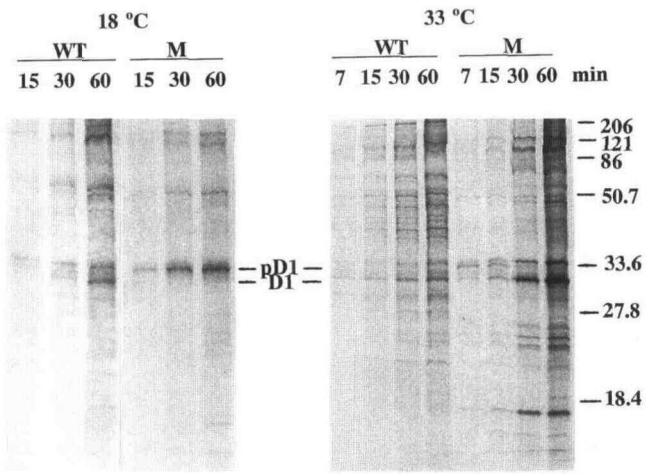

Figure 4. In vivo synthesis of the D1 protein after low-temperature-induced photoinhibition in the wild type (WT) and the fatty acid mutant *desA*$^-$/*desD*$^-$ (M). Cells were incubated at 1000 μmol photons m^{-2} s^{-1} for 60 min at 18°C and subsequently pulse-labeled with [^{35}S]L-Met under low light at either 18 or 33°C for different periods of time. Thylakoid polypeptides were analyzed with linear urea-SDS-PAGE followed by autoradiography. Samples of 1.25 μg of Chl were applied in each well. The molecular mass markers in kD are indicated to the right of the autoradiogram.

the inability to restore PSII oxygen evolution under low light at 18°C (Fig. 5A). On the contrary, the wild-type cells were able to process the pD1 protein, since it was shown not to accumulate under similar conditions. Thus, the wild-type cells were also able to restore PSII oxygen evolution at 18°C after low-temperature photoinhibition (Figs. 4 and 5A). At 33°C both the wild-type and mutant strains were able to recover from low-temperature-induced photoinhibition (Fig. 5B).

Accumulation of pD1 protein was not, however, unique to the fatty acid mutant cells. When the recovery temperature was further decreased to 15°C (the photoinhibitory pretreatment of the cells was also performed at 15°C), wild-type cells also started to accumulate pD1 in their thylakoid membranes (Fig. 6). Accordingly, the wild type had a clearly lower threshold temperature for maturation of the pD1 protein than the *desA*$^-$/*desD*$^-$ mutant. Figure 7 shows the temperature dependence of the accumulation of label into the pD1 protein, expressed as a percentage of the label in total D1, during 30 min of recovery under low-light conditions (40 μmol photons m^{-2} s^{-1}). Decreasing the recovery temperature from 33 to 26°C, and especially from 26 to 18°C, drastically increased accumulation of pD1 in the fatty acid mutant, whereas in the wild-type cells only a slight increase was seen in the accumulation of pD1 under similar conditions. At 15°C, however, the wild type also underwent major perturbations in the processing of the pD1 protein, with approximately 70% of the newly synthesized, labeled D1 appearing in its precursor form after 30 min of recovery (Figs. 6 and 7). We thus conclude that it is not only the temperature, but also the fluidity of the membranes that is modulated by the level of thylakoid lipid

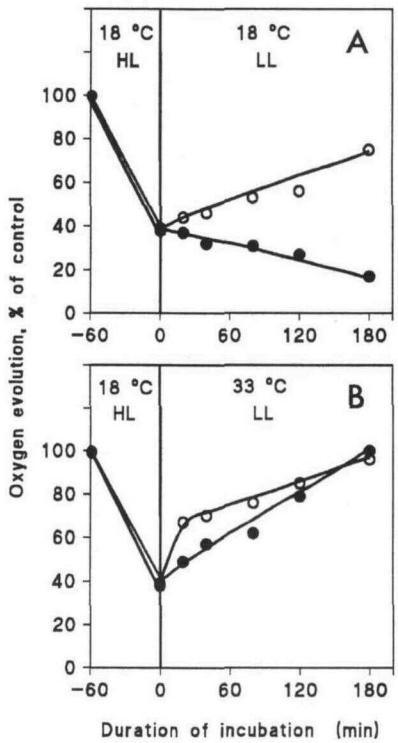

Figure 5. Restoration of PSII oxygen evolution in *Synechocystis* sp. PCC 6803 cells during recovery from low-temperature photoinhibition. The cells were first photoinhibited at 1000 μmol photons m^{-2} s^{-1} (HL) at 18°C for 60 min and subsequently allowed to recover at 40 μmol photons m^{-2} s^{-1} (LL) at either 18°C (A) or 33°C (B). The oxygen-evolving activity of PSII was measured from the cells in vivo under saturating light at 32°C with an oxygen electrode. Values represent the means of four independent experiments, with SD values not exceeding 10% of the mean. The control rates of oxygen evolution were 263 and 270 μmol O_2 mg^{-1} Chl h^{-1} for the wild type (○) and the *desA*$^-$/*desD*$^-$ mutant (●), respectively.

unsaturation, that determines the processing of the pD1 protein.

Is pD1 Stably Inserted into the Thylakoid Membranes?

Since *Synechocystis* sp. PCC 6803 cells accumulated the pD1 protein at low temperature, we next resolved to determine whether pD1 was properly inserted into the thylakoid membrane, or whether it was just loosely bound to the membrane. In the latter case, the pD1 should have been easily removed from the membranes by high-salt washing. However, repeated washes with 2 or 4 M NaBr did not release the newly synthesized, labeled pD1 from the thylakoid membranes (data not shown). It could thus be concluded that the newly synthesized pD1 protein had become integrated into the thylakoid membranes and was also protected against immediate proteolytic attack.

Is pD1 Incorporated into the PSII Complexes?

Since mutant cells were found to accumulate newly synthesized pD1 that was stably inserted into the thylakoid membrane, we next tested whether the accumulated pD1

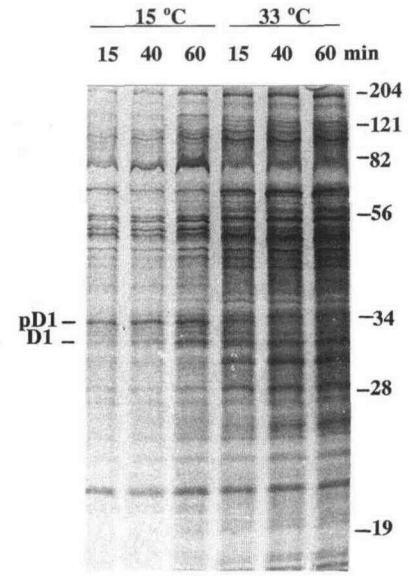

Figure 6. Accumulation of the pD1 protein also occurs in the wild-type cells when temperature is further decreased to 15°C. The cells were photoinhibited at 1000 μmol photons m^{-2} s^{-1} at 15°C for 60 min, and subsequently pulse-labeled with [^{35}S]L-Met under low light at either 15 or 33°C for different periods of time. Thylakoid polypeptides were analyzed with linear urea-SDS-PAGE followed by autoradiography. Molecular mass markers in kD are indicated to the right of the autoradiogram. Samples of 1.25 μg of Chl were applied in each well.

had also been integrated into the PSII complex. PSII complexes of cyanobacterial membranes can be separated with Deriphat-PAGE into two oligomeric states: PSII monomers and PSII dimers (Barbato et al., 1995). To study the integration of the accumulated pD1 protein into the PSII complexes, the cells were first photoinhibited as described above (1000 μmol photons m^{-2} s^{-1}, 18°C, 60 min) and thereafter labeled with radioactive Met under low-light conditions (40 μmol photons m^{-2} s^{-1}) at 18°C for 30 min. The thylakoids were briefly solubilized with a nonionic detergent, β-D-dodecyl maltoside, and the pigment-protein complexes were separated with nondenaturing Deriphat-

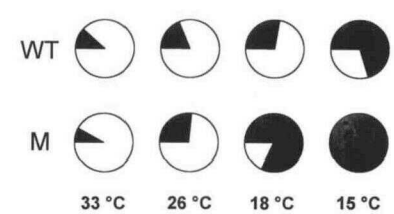

Figure 7. Accumulation of the label in pD1 under low-light conditions at different temperatures in the wild-type (WT) and *desA*$^-$/*desD*$^-$ mutant (M) cells. The cells were first photoinhibited at 1000 μmol photons m^{-2} s^{-1} at 18°C for 60 min and subsequently pulse-labeled with [^{35}S]L-Met under low light (40 μmol photons m^{-2} s^{-1}) at different temperatures for 30 min. Thylakoid polypeptides were analyzed with linear urea-SDS-PAGE followed by autoradiography. pD1 and D1 proteins were quantified by scanning the autoradiograms with a laser densitometer. Accumulation of pD1 (black sector) is expressed as a percentage of label in total D1 (pD1 + D1); $n = 3$. SD values did not exceed 10% of the mean.

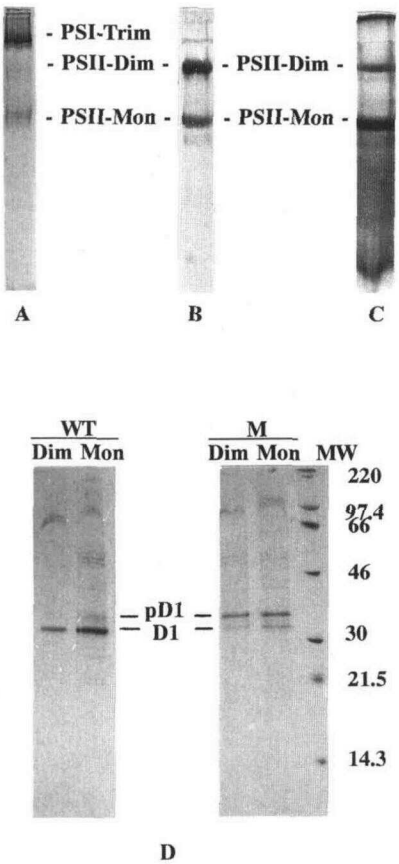

Figure 8. Electrophoretic analysis demonstrating the incorporation of the newly synthesized D1 protein into PSII monomer and dimer complexes during low-temperature recovery. Wild-type and *desA*$^-$/*desD*$^-$ mutant cells were photoinhibited at 1000 μmol photons m^{-2} s^{-1}, 18°C, for 60 min and subsequently pulse-labeled with [^{35}S]L-Met under low light at 18°C for 30 min. Thylakoid membranes were isolated and solubilized with β-D-dodecyl maltoside followed by separation of protein complexes with Deriphat-PAGE. The gel was loaded on a Chl basis (25 μg of Chl per well). A, B, and C represent a green gel, an immunoblot with a D1-protein-specific antiserum, and an autoradiogram, respectively, run from the wild-type cells and taken from the same gel. The locations of the PSI trimer (PSI-Trim), the PSII dimer (PSII-Dim), and the PSII monomer (PSII-Mon) are indicated. In D the PSII dimer (Dim) and monomer (Mon) complexes from the wild-type (WT) and the *desA*$^-$/*desD*$^-$ mutant (M) cells were cut out from the green Deriphat gel, solubilized, run on SDS-PAGE, and prepared for autoradiography. ^{14}C-labeled molecular mass markers (MW) in kD were run on the right side of the gel.

PAGE. A lane of a green gel after Deriphat-PAGE is shown in Figure 8A. Immunoblotting of the green gel with D1-specific antisera recognized two bands representing the PSII monomer (molecular mass around 240–250 kD) and the PSII dimer (molecular mass around 400–500 kD) complexes (Fig. 8B). These two complexes also became predominantly labeled during a 30-min pulse with [^{35}S]L-Met in the wild type, as shown in the autoradiogram (Fig. 8C). The same complexes were also labeled in the *desA*$^-$/*desD*$^-$ mutant, but to a somewhat lesser extent.

Second-dimension SDS-PAGE of PSII monomer and dimer complexes followed by autoradiography were performed to determine whether the newly synthesized D1 protein in these complexes was present as a precursor or as a mature processed form. As shown in the autoradiogram (Fig. 8D), both the PSII monomer and dimer complexes contained D1 protein predominantly in its pD1 form in the *desA*$^-$/*desD*$^-$ mutant, whereas in the wild type most of the D1 protein in these complexes was in the mature, processed form. These results demonstrate that the pD1 protein, which accumulated under low-temperature recovery conditions in the fatty acid mutant *desA*$^-$/*desD*$^-$, was not free in the thylakoid membranes but had been incorporated into the PSII monomer and dimer complexes.

Is Accumulation of the pD1 Protein Specifically Linked to Photoinhibition and Repair of PSII at Low Temperature?

To determine whether pD1 accumulation is specific to low-temperature photoinhibition, or possibly reflects a more general light- or temperature-induced phenomenon linked to the level of fatty acid unsaturation, we studied D1 protein synthesis under high- and low-light conditions without any photoinhibitory pretreatment of the cells. Incorporation of the [^{35}S]L-Met label into the D1 protein was examined under high light (1000 μmol photons m^{-2} s^{-1}) and low light (40 μmol photons m^{-2} s^{-1}) at either 18 or 33°C. In these experiments two fatty acid double mutants, *desA*$^-$/*desD*$^-$ and Fad6/*desA*::Km^r, were used. The results of these experiments are summarized in Figure 9. At 18°C under both low- (Fig. 9, A–C) and high-light (Fig. 9A) conditions, 60 to 80% of the labeled D1 protein in the fatty acid mutants was in the pD1 form after a 30-min pulse,

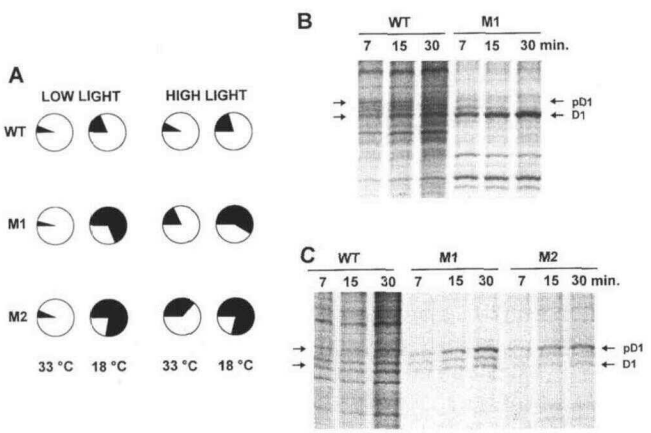

Figure 9. Accumulation of the label in the precursor form of the D1 protein in *Synechocystis* sp. PCC 6803 without a prior low-temperature photoinhibition. The cells were pulse-labeled for 30 min under low- and high-light conditions at both 33 and 18°C. Thylakoid polypeptides were analyzed with linear urea-SDS-PAGE followed by autoradiography. Samples of 1.25 μg of Chl were applied in each well. The autoradiograms were quantified with a laser densitometer. A, Graphic representation of the relative amount of the newly synthesized pD1 (black sector) after a 30-min pulse under low light (40 μmol photons m^{-2} s^{-1}) or high light (1000 μmol photons m^{-2} s^{-1}) at either 33 or 18°C; $n = 3$. SD values did not exceed 10% of the mean. B and C, Typical autoradiograms demonstrating in vivo synthesis of the D1 protein under low light at 33 and 18°C, respectively. WT, Wild type; M1, *desA*$^-$/*desD*$^-$ mutant; M2, Fad6/*desA*::Km^r mutant.

whereas pD1 accounted for only about 20% of the labeled D1 protein in the wild type under similar conditions. When the labeling was carried out under high-light conditions at 33°C, the mutants still had 20 to 40% of their newly synthesized D1 protein in the unprocessed form after a 30-min pulse (Fig. 9A), whereas hardly any pD1 was discernible during low-light treatment at 33°C (Fig. 9, A and B). These results indicate that accumulation of the pD1 protein may not be specifically linked to low-temperature-induced photoinhibition, since low-temperature stress was also found to severely impair the processing of the pD1 protein at growth light (Fig. 9C). Moreover, even relatively short high-light stress at growth temperature induced perturbations in D1-protein processing in the mutant cells (Fig. 9A). These results provide further evidence that pD1 processing may form a rate-limiting factor in the turnover of D1 protein under stress conditions, when such rapid turnover would be needed for optimal functioning of PSII.

DISCUSSION

In this study we sought to provide more detailed information concerning the relationship between membrane lipid unsaturation and low-temperature-induced perturbations of PSII function (Williams, 1994; Somerville, 1995). Earlier studies have shown that low temperature modulates the extent of PSII photoinhibition, not only by reducing the utilization of excitation energy in carbon fixation, but also by lowering the rate of concomitant recovery from photoinhibition (Greer et al., 1986). During the recovery process, the D1 protein is first proteolytically degraded and subsequently replaced by a new, de novo-synthesized D1 protein (Prasil et al., 1992; Aro et al., 1993). To study the relationship between membrane lipid unsaturation and the temperature dependence of the photoinhibition repair cycle, we used a mutant strain of *Synechocystis* sp. PCC 6803 deficient in desaturating 18-carbon fatty acids in its thylakoid membrane lipids (Tasaka et al., 1996).

Our results indicate that PSII inactivation and D1-protein degradation at low temperatures proceeded independently of the level of membrane lipid unsaturation in *Synechocystis* sp. PCC 6803 cells. However, the subsequent recovery from photoinhibition was greatly modulated in the fatty acid mutant at low temperatures (Fig. 5), which is consistent with the findings of Gombos et al. (1994). The reduced level of membrane lipid unsaturation in the mutant strain significantly retarded the repair of PSII centers at low temperatures (Fig. 5A), whereas at growth temperature both the mutant and the wild type completely restored their photosynthetic activity (Fig. 5B). An interesting molecular mechanism was found to explain the poor recovery capacity of PSII oxygen evolution in the fatty acid mutant: low temperature dramatically impaired the processing of pD1 in mutant PSII reaction centers, resulting in the accumulation of pD1 (Figs. 4, 7–9). Posttranslational carboxyl-terminal processing of the pD1 protein, occurring on the lumenal side of the thylakoid membrane, is known to be an absolute requirement for activation of PSII oxygen evolution (Bowyer et al., 1992; Nixon et al., 1992).

Accumulation of the pD1, however, was not specific to the mutant strain, since further decreasing the temperature also exerted severe stress on the wild-type cells, leading to the accumulation of the pD1 protein (Figs. 6 and 7). Despite this impairment of pD1 processing, overall membrane-protein synthesis in the wild type was only slightly affected by lowering the temperature from 18 to 15°C. This implies that a posttranslational modification, the carboxyl-terminal processing of the pD1 protein, is a phase in D1-protein turnover that is extremely sensitive to chilling temperatures. The threshold temperature at which perturbations in pD1 processing started to emerge was clearly lower in the wild type than in the fatty acid mutant. This difference in the threshold temperatures is also seen as a reduced overall synthesis of the thylakoid proteins in the fatty acid mutant at 18°C, the D1 protein still being preferentially synthesized. These results suggest that the extent of thylakoid membrane lipid unsaturation, which modifies membrane fluidity but not the temperature, per se, is crucial for post-translational pD1 processing.

Low temperature as the only stress factor (Fig. 9, A and C) also impeded the pD1-protein processing. Comparable results have been reported by Campbell et al. (1995) for *Synechococcus* sp. PCC 7942 cells. These cells typically exchange the form of the D1 protein (from D1:1 to D1:2) in PSII centers under stress conditions, and when chilled (25°C), are found to accumulate a higher-molecular-mass D1:2 protein. It is not yet known whether this protein also represents a precursor form or is a modified form of the mature D1:2 protein. Low temperature, however, was not the only stress factor impeding pD1 processing. Even relatively short high-light stress was sufficient to impair D1-protein processing to some extent, especially in thylakoids with a low level of fatty acid unsaturation (Fig. 9A), and may also limit the repair of photodamaged PSII centers under prolonged light-stress conditions (Kanervo et al., 1995).

A reduced level of thylakoid-lipid unsaturation could have caused perturbations in the incorporation of the newly synthesized pD1 protein into monomeric PSII complexes under chilling stress. However, neither the assembly of PSII nor the dimerization of PSII monomers became affected by the reduced level of fatty acid unsaturation, as demonstrated by two-dimensional gel electrophoresis of in vivo-labeled thylakoid polypeptides (Fig. 8). Earlier studies have suggested that functional PSII has a dimeric organization (Mörschel and Schatz, 1987; Santini et al., 1994; Boekema et al., 1995), and that the D1 protein can integrate into the PSII in its precursor form (Taylor et el., 1988; Adir et al., 1990). Furthermore, our present results indicate that even PSII dimerization can occur without a prior processing of pD1. Therefore, PSII dimers cannot be regarded as indicators of functional PSII centers. Considering the restoration of PSII function under low-temperature conditions (Fig. 5A), it is likely that the unsuccessful pD1 processing with a failure in the activation of oxygen evolution is the rate-limiting step in the PSII repair. Importantly, the threshold temperature at which the pD1 processing starts to limit PSII repair is critically dependent on the level of fatty acid unsaturation.

The significance of the carboxyl-terminal extension in the D1 protein is not known. It has been speculated that regulation of D1 protein processing would offer a mechanism for preventing the migration of PSII complexes from the nonappressed membranes to the appressed membranes before ligation of an oxygen-evolving complex has taken place, at least in those organisms possessing such differentiated membrane regions (Bowyer et al., 1992). However, although thylakoid membranes of cyanobacteria lack this membrane differentiation (Gantt, 1994), they still have preserved the C-terminal extension in the pD1 protein. Although PSII assembly and activation can also occur in the absence of the carboxyl-terminal extension (Lers et al., 1992; Nixon et al., 1992), it might still have a controlling role in PSII assembly, especially under conditions of rapid D1-protein turnover.

The enzyme involved in the carboxyl-terminal processing of the pD1 protein has been isolated and partially purified from higher plants (Inagaki et al., 1989; Bowyer et al., 1992; Taguchi et al., 1993; Fujita et al., 1995) and from an alga, *Scenedesmus obliquus* (Taylor et al., 1988; Bowyer et al., 1992). Similarly, a novel gene (*ctpA*) recently isolated in *Synechocystis* sp. PCC 6803 has been shown to encode the carboxyl-terminal processing protease of the pD1 protein (Anbudurai et al., 1994; Shestakov et al., 1994). Considering the impaired pD1 processing of the fatty acid mutant in the present study, either the function of the processing protease may be impaired, or the processing site in the pD1 protein may remain hidden or unrecognizable to the processing protease. Given the former case, the protease, located on the lumenal surface of the thylakoid membrane (Bowyer et al., 1992; Fujita et al., 1995; Oelmuller et al., 1996), may become distorted because of lowered membrane fluidity. This could result in insufficient interaction between the protease and the substrate. It should be noted that the processing protease was active even in the presence of a protein-synthesis inhibitor, provided the thylakoid membranes were sufficiently fluid (Fig. 3), indicating that there is no need for de novo protein synthesis under those conditions. The fluidity of the membranes is modulated by the level of lipid unsaturation, the importance of which in D1-protein processing is particularly evident at low temperatures. A significant sequence similarity has been observed between the *CtpA* protease in *Synechocystis* sp. PCC 6803 and the interphotoreceptor retinoid-binding proteins in human, bovine, and insect eye systems (Anbudurai et al., 1994), which bind hydrophobic molecules such as fatty acids and cholesterol. The sequence similarities found between these two proteins may suggest that the *CtpA* protease binds to thylakoid membrane fatty acids, or that the recognition of the substrate pD1 by this protease involves interactions with fatty acids. Also, the reduced fluidity of the thylakoid membranes may affect the conformation of the pD1 protein. Consequently, an aberrant conformation and/or orientation of the carboxyl terminus of pD1 may debilitate pD1 processing, especially under low-temperature stress.

In conclusion, this study offers, to our knowledge, the first direct evidence at the molecular level of the importance of thylakoid membrane polyunsaturation in the maintenance of proper D1-protein synthesis and posttranslational carboxyl-terminal processing at low temperatures. A low level of thylakoid fatty acid unsaturation drastically enhances the detrimental effects of low temperature on PSII by debilitating the processing of pD1, thus impairing reparation of photodamaged PSII centers. Whether the effect of membrane lipid unsaturation on the posttranslational processing of membrane proteins at low temperature is a phenomenon specific to the D1 protein alone, or if it may affect other membrane proteins as well, remains to be elucidated.

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