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In vitro studies on light-induced inhibition of Photosystem II and D_1-protein degradation at low temperatures

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In order to get information on the molecular background behind the aggrevated photodamage to photosynthesis at low temperatures and to investigate the general mechanism of D_1-protein degradation, isolated spinach thylakoids were subjected to photoinhibitory treatment at various temperatures. The results reveal that: (i) the Photosystem II electron transport per se is less sensitive to high light at low temperatures in contrast to the overall photosynthetic process; (ii) the degradation of D_1-protein is severely retarded below 7°C; (iii) inhibition of Photosystem II electron transport and D_1-protein degradation are separate events since the two reactions could be completely separated in time; (iv) D_1-protein is degraded by enzymatic proteolysis and not by a direct photocleavage reaction; (v) degradation of the D_1-protein readily proceeds in the dark but its triggering for the proteolytic attack requires light; (vi) strong illumination at low temperature does not induce any lateral rearrangement in the location of Photosystem II; and (vii) D_1-protein fragments can be identified in vitro and be used to verify the specificity of D_1-protein degradation under various experimental conditions.

Introduction

Exposure of oxygenic photosynthetic organisms to strong light inhibits the overall photosynthetic process. It is generally accepted that the primary target for photoinhibition is the photochemistry of Photosystem II [1]. Light induced inhibition of photosynthesis in vivo is more severe at low temperature [2,3]. The molecular background for this synergistic light and cold stress is not known and can be sought at many different levels in the cell. Photosystem II as such could be destabilised in the cold and therefore more prone to light damage. A slowing down of the overall metabolic activity in the photosynthetic cell at low temperatures would reduce the amount of excitation energy dissipated through photosynthesis and thereby increase the probability for over-excitation of Photosystem II [4]. Moreover, mechanisms to protect Photosystem II against overexcitation, such as protein phosphorylation [5,6], become less efficient at low temperatures [7].

A subsequent event to the light-induced inhibition of electron transport is degradation of the D_1-protein [8,9], one of the two reaction centre subunits of Photosystem II [10–12]. Therefore, essential steps [9,13–15] in the repair process of Photosystem II after photodamage are degradation and removal of inactive D_1-protein, synthesis of new protein and its insertion into the stroma exposed thylakoids, subsequent migration of the newly synthesized protein to the appressed thylakoid regions where Photosystem II is reestablished through protein assembly and ligation of cofactors. The slowing down in the cold of any of these steps would result in reduced ability for repair and a decreased photosynthetic efficiency.

Studies on light stress on thylakoid membranes at low temperatures should therefore have physiological implications as well as provide further information about the molecular mechanism of Photosystem II inhibition and D_1-protein degradation. In the present study we have followed changes in the function and organization of Photosystem II in vitro after subjecting isolated spinach thylakoids to strong illumination in the cold. The thylakoid samples were subsequently incubated in the dark at 20°C and analyzed for changes in the

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properties of Photosystem II. The results reveal that photoinhibition of Photosystem II electron transport in vitro is less severe at low temperatures in contrast to known effects on the overall photosynthesis in vivo. Moreover, D_1-protein degradation is not operational below 7°C. Evidence is provided that D_1-protein degradation in itself does not require light but readily proceeds in the dark in an all enzymatic event.

Materials and Methods

Spinach (*Spinacea oleracea* L) was grown in a growth chamber at 25°C and 475 μmol photons · $m^{-2} \cdot s^{-1}$. Thylakoids were isolated according to [16] except that the last washing step and final suspension were made in 50 mM Tricine-KOH (pH 7.6), 100 mM sorbitol, 5 mM $MgCl_2$ and 20 mM NaCl (incubation buffer). For photoinhibitory illumination the freshly prepared thylakoids were suspended in the incubation buffer to a concentration of 0.2 mg chlorophyll/ml and equilibrated to the desired temperature in the dark. The thylakoid suspension, under constant slow stirring in a thermostatted vessel (2–20°C) was illuminated with heat-filtered white light from a 250 W projector lamp. This gave 6500 μmol photons $m^{-2} \cdot s^{-1}$ above the thylakoid suspension. At specified times, during the strong light exposure, 0.2 ml samples were withdrawn. These were either kept on ice in the dark or incubated at 20°C in the dark for a certain period prior to further analysis.

Grana and stroma thylakoid membranes were prepared by digitonin based fractionation essentially according to Ref. 17. Immediately after the light was turned off digitonin was added to the thylakoid suspension to a final concentration of 0.2% and incubated for 2 min at 20°C. The solubilization was stopped by adding 10 vol. of incubation buffer. The grana membranes were isolated by immediate centrifugation at 10000 × *g* for 5 min. Stroma thylakoids were isolated by further centrifugation of the supernatant at 40000 × *g* for 30 min and finally pelleted at 100000 × *g* for 30 min.

Photosystem II electron transport was measured at 20°C in a Hansatech oxygraph using saturating light. Phenyl-*p*-benzoquinone (0.5 mM) was used as an artificial electron acceptor. The assay medium consisted of 25 mM Hepes-NaOH (pH 7.5), 100 mM sucrose, 2.5 mM $MgCl_2$ and 10 mM NaCl. Thylakoids were added to a final concentration of 10 μg chlorophyll/ml.

SDS-polyacrylamide gel electrophoresis was carried out according to Ref. 18 using a 12 to 22.5% polyacrylamide gradient and 6 M urea in the separation gel. Samples were solubilized in Laemmli solubilization buffer immediately after being withdrawn from the illumination experiments. Prior to electrophoresis the samples were heated to 75°C and incubated for 5 min.

Western blotting, using antibodies against the D_1-, 22 kDa and 43 kDa proteins and the 9 kDa subunit of cytochrome *b*-559, was carried out essentially according to [19] using ^{125}I-labelled protein A for detection. For quantification autoradiograms were scanned with a laser densitometer.

Chlorophyll was determined in 80% acetone according to [20].

Results

Inhibition of Photosystem II electron transport

In the present study we have investigated the combined effects of low temperatures and high light intensities in vitro using isolated spinach thylakoids. Illumination (6500 μmol photons · $m^{-2} \cdot s^{-1}$) of the thylakoids at 20°C for 45 min resulted in 90% inhibition of oxygen evolution with an apparent inhibition half-time of approx. 10 min. (Fig. 1). The rate and degree of inhibition was highly influenced by the temperature during the illumination. Surprisingly, the lower the temperature, the less susceptible the Photosystem II activity was to inhibition. Thus, at 2°C the apparent half-time for the light induced inactivation was 25 min and after 45 min the inhibition was limited to 70%, as compared to 90% at 20°C. The illumination experiments at 7°C and 14°C gave intermediary inhibitions. Also, when the light level was decreased to 3000 μmol photons $m^{-2} \cdot s^{-1}$, the same temperature dependence of Photosystem II inactivation was observed with apparent half-times of

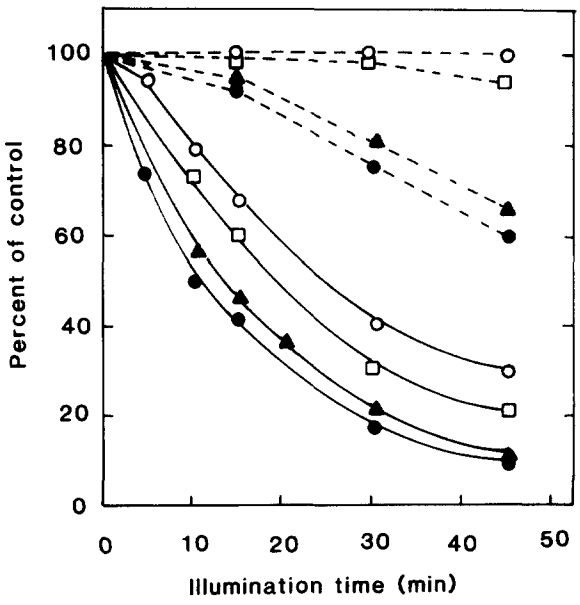

Fig. 1. The effect of strong illumination (6500 μmol photons · $m^{-2} \cdot s^{-1}$) on oxygen evolution (solid lines) and D_1-protein (broken lines) in thylakoid membranes incubated at different temperatures. D_1-protein content and oxygen evolution were measured immediately after termination of the photoinhibitory illumination which was performed at 20°C (●), 14°C (▲), 7°C (□) and 2°C (○). There was no D_1-protein degradation within 45 min in the dark.

approx. 19 and 45 min at 20°C and 2°C, respectively. It should be noted that within the time intervals used, the strong illumination did not induce any bleaching of chlorophyll.

In vitro D_1-protein degradation – light and temperature dependence

In Fig. 1 the inhibition pattern of Photosystem II is compared with changes in the relative D_1-protein content as determined by Western blotting (Fig. 2). At 14°C and 20°C the inhibition of the Photosystem II activity by strong light was accompanied by a loss of D_1-protein from the thylakoid membrane. Approx. 40% of the D_1-protein was lost after 45 min of illumination at 20°C compared to the 90% decrease in activity. When ^{35}S-labelled chloroplasts were illuminated the degradation of D_1-protein could be seen as a marked increase in the radioactivity in the supernatant (not shown). Illumination of isolated thylakoids does not result in a notable loss of other Photosystem II polypeptides [9] which is also indicated by a constant level of the 22 kDa polypeptide in Fig. 2. Occasionally, a limited degradation of D_2-protein was observed (not shown). The D_1-protein degradation was even more temperature dependent than the inhibition of the Photosystem II activity.

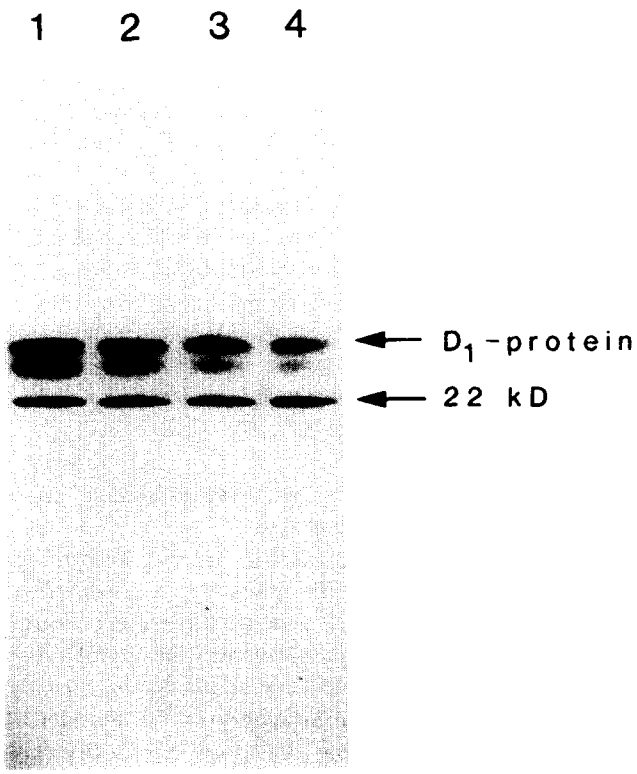

Fig. 2. Typical Western blot demonstrating the degradation of D_1-protein and its lower molecular weight conformer during strong illumination of thylakoid membranes at 20°C. The level of the 22 kDa polypeptide of Photosystem II is not affected by the illumination. Lane 1, control thylakoids; lanes 2–4, thylakoids illuminated for 15, 30 and 45 min, respectively.

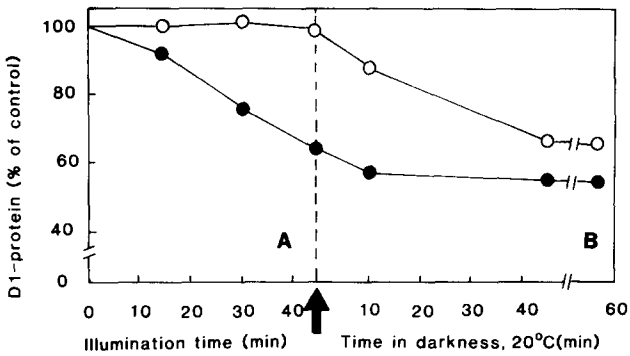

Fig. 3. (A) Loss of D_1-protein from the thylakoid membranes during strong illumination at 20°C (●) and 2°C (○). After 45 min the two thylakoid samples were transferred to darkness at 20°C (ARROW). (B) Degradation of D_1-protein in the dark at 20°C after the strong light illumination at 20°C (●) and 2°C (○). Incubation of control and photoinhibited thylakoid membranes in the dark at 20°C for 45 min did not effect the rate of O_2-evolution.

below 7°C virtually no reduction in the amount of D_1-protein could be seen. Thus, at 2°C, all D_1-protein remained in the thylakoid membranes despite a 70% inhibition of the Photosystem II activity (Fig. 1). These observations show that the D_1-protein degradation system is not operational under low temperatures and that strong illumination in the cold results in inhibited Photosystem II centres still containing D_1-protein.

The mechanism of D_1-protein degradation is poorly understood. Previous experimental approaches have been hampered by the problem of discriminating between direct light effects, inhibition of electron transport and proteolytic activities. It is still not verified to what extent D_1-protein degradation is simply a photocleavage event or simply a result of enzymatic proteolysis. The present photoinhibited but D_1-protein-containing Photosystem II complexes therefore offer a unique material for studies on the initiation and mechanism of D_1-protein degradation. Thus, after termination of the strong illumination at 2°C, the thylakoids were rapidly transferred to darkness and 20°C. They were then kept in the dark for various time intervals and analyzed for D_1-protein content in order to detect any degradation. Notably, the data in Fig. 3 reveal that, through this temperature rise in the dark, the degradation of D_1-protein gets started. Moreover, it can proceed in complete darkness, and, after 45 min approx. 30% of the D_1-protein becomes degraded. This dark degradation of D_1-protein was not accompanied by any further inactivation of the Photosystem II activity.

As can be seen in Fig. 4A the dark degradation of the D_1-protein at 20°C was dependent on the length of the preceding illumination at low temperatures in the same manner as upon illumination at 20°C. However, light treatments at 20°C resulted in a higher degree of D_1-protein degradation which is in accordance with a more severe inhibition of Photosystem II activity (Fig.

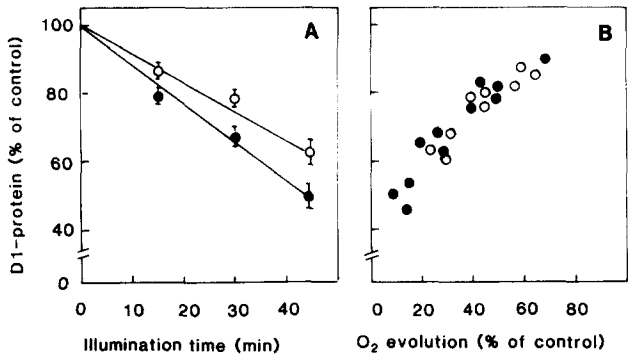

Fig. 4. (A) Final levels of D_1-protein in thylakoid membranes illuminated for various times at 20°C (●) and 2°C (○) and subsequently transferred to darkness and 20°C for 45 min. Less D_1-protein per light quanta was degraded after illumination at 2°C compared to 20°C. Bars indicate ± standard deviation. (B) Correlation between the remaining rate of oxygen evolution and the level of D_1-protein in thylakoid membranes photoinhibited for various times at 20°C (●) and at 2°C (○) and subsequently incubated in the dark at 20°C for 45 min.

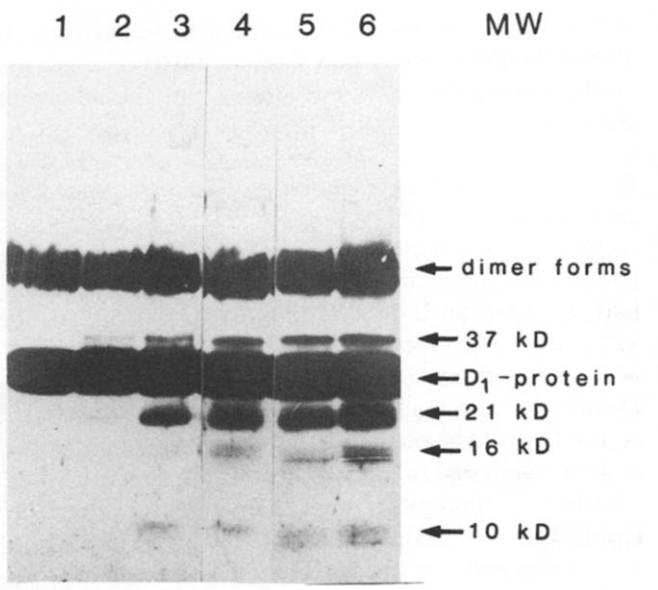

Fig. 5. Immunological detection of polypeptide fragments from degraded D_1-protein. In order to demonstrate the degradation fragments the gels had to be heavily overloaded which eliminated the possibilities for any quantitative analysis. Lane 1, control thylakoids before illumination; lane 2, thylakoids illuminated (6500 μmol photons·m^{-2}·s^{-1}) at 2°C for 30 min; lane 3, same thylakoids as in lane 2 but subsequently incubated in darkness at 20°C for 30 min; lane 4, thylakoids illuminated at 2°C for 45 min and then incubated in darkness and 20°C for 45 min; lane 5, thylakoids illuminated at 20°C for 30 min and lane 6 for 45 min. Incubation of the thylakoids in darkness at 20°C did not produce any fragments of D_1-protein.

1). Indeed, there is a pronounced correlation between the degree of inhibition of Photosystem II mediated O_2-evolution and the D_1-protein degradation within the two different sets of experiments (Fig. 4B). We therefore conclude that although degradation of the D_1-protein can occur in the dark it has to be sensitized in high light, possibly through the inhibition event itself.

Degradation fragments of the D_1-protein

The rapid turnover of the D_1-protein in high light is well established [21] but degradation fragments are not easily detected and have not been demonstrated in vitro. In pulse chase experiments in vivo only a 23.5 kDa polypeptide has been identified as a primary degradation product of the D_1-protein [22]. Fig. 5 shows a Western blot analysis of polyacrylamide gels heavily overloaded with thylakoid membranes. In control thylakoids there is a dominating band at 32 kDa indicative of the D_1-protein. In these overloaded gels a double band in the 65 kDa region becomes apparent. This double band probably represents D_1-protein homodimers [23,24] and/or D_1/D_2 protein heterodimers [25]. After photoinhibitory illumination at 20°C (Fig. 5, lanes 5 and 6) the D_1-antibodies recognize small amounts of polypeptides below the 32 kDa region. In particular a 21 kDa polypeptide becomes apparent but also 16 and 10 kDa fragments can be detected. In addition, there is an appearance of a 37 kDa polypeptide. Whether that is a fragment derived from dimerized D_1-protein or some aggregation product of the monomeric form cannot be judged at present. Photoinhibitory illumination at 2°C (Fig. 5, lane 2) does not produce any easily detectable polypeptide fragments, in agreement with the lack of D_1-protein degradation at low temperature. However, after the subsequent dark incubation at 20°C, polypeptide fragments appear in a pattern resembling that obtained after illumination at 20°C (Fig. 5, lanes 3 and 4).

The actual amount of fragments in both cases is very small, representing only some 1–2% of the total response obtained after the immunoblotting analysis. The loss of D_1-protein during illumination in vitro (Figs. 1 and 3) does not correlate with the amount of detectable degradation fragments (Fig. 5). This suggests a rapid further proteolysis of the D_1-fragments and/or their release from the membrane [22,26].

Temperature dependence of light induced changes in the lateral distribution of Photosystem II

In a previous communication it was shown that D_1-protein degradation at 20°C leads to a partial disassembly of Photosystem II and migration of subunits from the appressed to the nonappressed thylakoid regions [27]. In this study we have analyzed the temperature dependence of the disassembly of Photosystem II in grana membranes using digitonin based thylakoid subfractionation.

As revealed from Fig. 6A after illumination at 20°C and 14°C, as a consequence of D_1-protein degradation, there is a reduction in the amount of the 43 kDa chlorophyll *a* binding protein and the 9 kDa subunit of cytochrome *b*-559. This is accompanied by an increase

of these polypeptides in stroma thylakoids (Fig. 6B). Of all the Photosystem II polypeptides analyzed only the levels of the 22 kDa polypeptide remained constant in the grana thylakoids following strong illumination. Thus, the increased relative amounts of this polypeptide and the D_1-protein in the stroma thylakoids (Fig. 6B) suggest that there are not only lateral movements of Photosystem II subunits following photoinhibition but also a limited destacking. At 2°C the illumination did not induce any changes in the amounts of Photosystem II proteins in the appressed thylakoid regions suggesting the structural integrity of Photosystem II. This is consistent with the lack of D_1-protein degradation (Fig. 1) and the very low diffusion rate of proteins in the thylakoid membrane at low temperatures [7,28].

Discussion

The synergistic effects of low temperature and high light to induce stress in photosynthetic organisms in vivo is probably the result of modifications of many of the catalytic and regulatory reactions in photosynthesis as well as an influence on organization and turnover of components. The situation in isolated thylakoid membranes is less complex, thereby facilitating studies on individual factors contributing to the aggrevated light stress at low temperatures. The present study was aimed at investigating the effects of strong illumination of thylakoids in the cold with respect to Photosystem II activity and D_1-protein content.

Quite unexpectedly, we found that Photosystem II activity per se is less sensitive to photoinhibition at low temperatures (Fig. 1). The mechanism behind this reduced photoinhibition in thylakoids is not understood at present. However, the photoinhibited Photosystem II centres are efficient quenchers of exitation energy [29,30] and since the Photosystem II centres illuminated at low temperature remain in the appressed membranes and do not undergo any compositional changes (Figs. 1 and 6A), these may function as excitation quenchers and thereby protect the connected Photosystem II centres from light damage. Another interesting explanation

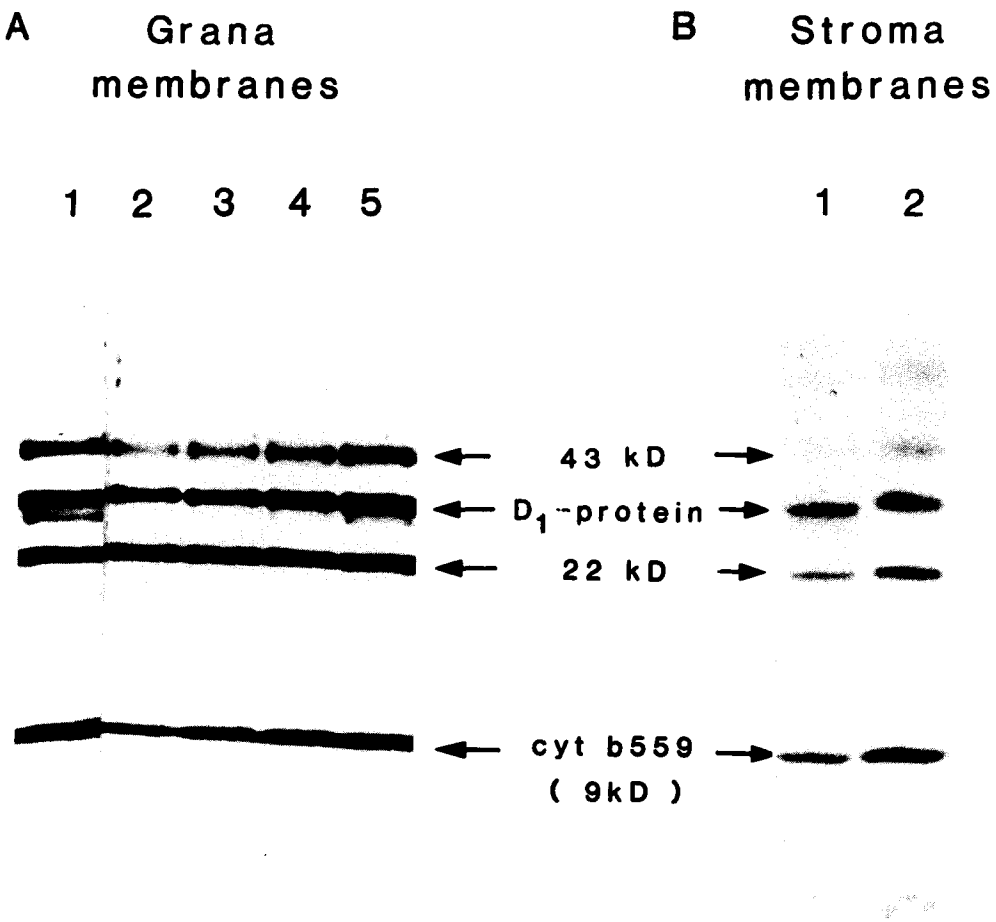

Fig. 6. Changes in the lateral distribution of Photosystem II polypeptides during strong illumination of the thylakoids at different temperatures. After 45 min of illumination the grana (A) and stroma (B) membranes were isolated and immunological detection of Photosystem II polypeptides was obtained by a mixture of antibodies against 43 kDa chlorophyll *a* binding protein, the D_1-protein, the 22 kDa polypeptide and the 9 kDa subunit of cytochrome *b*-559. Membranes containing 0.6 μg chlorophyll were loaded in each well. Lane 1, control; lane 2, thylakoids illuminated at 20°C; lane 3, at 14°C; lane 4, at 7°C and lane 5 at 2°C.

would be that in the cold, where the D_1-protein is not degraded, a spontaneous reactivation of the Photosystem II activity might be possible. At higher temperatures where D_1-protein degradation readily follows photoinhibition of photosystem II, such a reactivation would be limited and thereby lead to more severe net inactivation than at lower temperatures. The physiological significance and mechanism for such a reversibility of photoinhibition without replacement of D_1-protein is not clear and needs further experimentation. However, in light of a recent EPR-study on photoinhibited thylakoids [31] a spontaneous reoxidation of double reduced Q_A could be one possibility.

Moreover, the observation of less inhibition of electron transport at low temperature suggests that the increased photodamage seen at low temperatures in more intact photosynthetic systems cannot be directly attributed to the photochemistry of Photosystem II, but rather to a slowing down of other processes that indirectly influence Photosystem II function, organization and turnover.

At low temperatures, we show that there is virtually no loss of D_1-protein during illumination, despite a 70% inhibition of electron transport. This lack of D_1-protein degradation at low temperatures is consistent with in vivo pulse chase experiments in the green algae *Chlamydomonas reinhardtii* [32]. Most significantly, however, by increasing the temperature of such photoinhibited thylakoids in darkness we demonstrate that the degradation of the D_1-protein in itself does not require light. This observation excludes the possibility that D_1-protein degradation is the result of direct photocleavage of peptide bonds, which for example could occur through a modification of proline residues [33]. The results of Fig. 3 therefore give conclusive evidence that D_1-protein degradation is an all-enzymatic event.

Moreover, this experiment confirms previous observations in a very clear way [8,14] suggesting that light-induced inhibition of electron transport and D_1-protein degradation are separate events. Illumination of the thylakoid membranes in high light at 2°C inhibits Photosystem II electron transport without any loss of D_1-protein. On the contrary, during the subsequent dark incubation at 20°C, D_1-protein is degraded without any loss of activity. Although D_1-protein degradation proceeds readily in darkness the triggering requires light (Figs. 3 and 4). The mechanism behind this initiation could be a direct light effect on the D_1-protein or on a proteolytic enzyme. However, the results of Fig. 4 rather suggest that it is the Photosystem II inhibition as such that makes the D_1-protein susceptible to proteolysis. Loss of atrazine binding capacity of thylakoids subjected to strong illumination [34] under conditions where we would expect no D_1-protein degradation suggests changes in the D_1-protein prior to its digestion. Styring et al. [31] have suggested that during photoinhibition, Q_A becomes doubly reduced and may subsequently leave its site in the Photosystem II reaction centre which in turn could be associated with a conformational change in the D_1-protein. Also various reactive oxygen species produced in the light [19,35,36] have been suggested to trigger D_1-protein degradation. Our results do not appear to support any primary role of protein phosphorylation in the triggering of D_1-protein degradation [37] since the inhibition experiments were performed in the absence of added ATP.

The observed lack of D_1-protein degradation at low temperatures has several physiological implications since rapid degradation and removal of damaged D_1-protein are involved in the repair of Photosystem II [13,14]. The cold inhibition of D_1-protein degradation gives strong support for an inactive repair cycle of D_1-protein at low temperatures. This inactivation could involve both the actual degradation of the D_1-protein as well as an inability for lateral rearrangements of Photosystem II polypeptides in the membrane. The latter possibility is consistent with recent observations demonstrating that lateral migration of phosphorylated LHCII from appressed to non-appressed regions [6] is very slow below 10°C [7].

Our results give the first immunological in vitro identification of proteolytic fragments of the D_1-protein (Fig. 5). The fragment pattern mainly resembles that seen in in vivo studies [22]. Our results further strengthen the previous observations [9,27] that photoinhibition of isolated thylakoids does not lead to substantial protein aggregation [38] and that the D_1-protein degradation system is operational in an efficient way in vitro (Figs. 1 and 2). Some higher molecular weight bands appear in minor amounts after the high light exposure (Fig. 5) but these bands seem to reflect a specific increase in the relative levels of D_1-protein homodimers and D_1/D_2-protein heterodimers. Significantly, the digestion pattern seen after degradation of D_1-protein in the dark is the same as that obtained under high light at 20°C. This shows that D_1-protein degradation in the dark does not represent any unspecific proteolytic reaction but resembles that normally connected to turnover of D_1-protein and repair of Photosystem II.

Finally, photoinhibited but D_1-protein-containing thylakoid preparations should provide a unique material for studies on the mechanism of light-induced inhibition of Photosystem II electron transport without interference of protein degradation and structural rearrangements. Moreover, as shown in the present work, it will offer new possibilities for further characterization of the enzymes catalyzing D_1-protein degradation.

Studies are in progress to elucidate these problems in various Photosystem II preparations using different light and temperature conditions.

Acknowledgements

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