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Phosphorylation of PS II polypeptides inhibits D1 protein-degradation and increases PS II stability

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Abstract

To study the significance of Photosystem (PS) II phosphorylation for the turnover of the D1 protein, phosphorylation was compared with the synthesis and content of the D1 protein in intact chloroplasts. As shown by radioactive labelling with [$^{32}P_i$] phosphorylation of PS II polypeptides was saturated at light intensities of 125 mol m^{-2} s^{-1}. Under steady state conditions, in intact chloroplasts D1 protein, once it was phosphorylated, was neither dephosphorylated nor degraded in the light. D1 protein-synthesis was measured as incorporation of [^{14}C] leucine. As shown by non-denaturing gel-electrophoresis followed by SDS-PAGE newly synthesised D1 protein was assembled to intact PS II-centres and no free D1 protein could be detected. D1 protein-synthesis was saturated at light intensities of 500 mol m^{-2} s^{-1}. The content of D1 protein stayed stable even after illumination with 5000 μmol m^{-2} s^{-1} showing that D1 protein-degradation was saturated at the same light intensities. The difference in the light saturation points of phosphorylation and of D1 protein-turnover indicates a complex regulation of D1 protein-turnover by phosphorylation. Separation of the phosphorylated and dephosphorylated D1 protein by LiDS-gelelectrophoresis combined with radioactive pulse-labelling with [^{14}C] leucine and [$^{32}P_i$] revealed that D1 protein, synthesised under steady state conditions in the light, did not become phosphorylated but instead was rapidly degraded whereas the phosphorylated form of the D1 protein was not a good substrate for degradation. According to these observations phosphorylation of the D1 protein creates a pool of PS II centres which is not involved in D1 protein-turnover. Fractionation of thylakoid membranes confirms that the phosphorylated, non-turning over pool of PS II-centres was located in the central regions of the grana, whereas PS II-centres involved in D1 protein-turnover were found exclusively in the stroma-lamellae and in the grana-margins.

Abbreviations: chl – chlorophyll; F_v – yield of variable fluorescence, difference between F_m, the maximal fluorescence yield at saturating light, when all reaction-centres are closed, and F_o, the fluorescence yield in the dark, when all reaction-centres are open; LHC – light harvesting complex; PFD – photon flux density; PS – photosystem

Introduction

Protein-phosphorylation in eukaryotes has been shown to function as regulator of cellular processes. In plants the most extensively studied phosphorylation phenomenon is associated with proteins of the photosynthetic membrane especially those connected to Photosystem (PS) II (for recent reviews see Bennett 1991; Allen 1992). The main phosphorylated polypeptides belong to the light-harvesting complex (LHC) II protein family which forms the outer antenna of PS II. The other group of phosphoproteins is located in the inner antenna and in the reaction-centre core of PS II. The second group is represented by the 43 kDa protein, the 9 kDa *psbH* gene product, and the D1 and D2 protein, which form the PS II reaction-centre core containing all redox components (Nanba and Satoh 1987). All thylakoid polypeptides except the 27 kDa LHCII

polypeptide are phosphorylated at threonine residues facing the stroma side of the thylakoid membrane (Bennett 1991).

Most investigations regarding function and regulation of thylakoid protein-phosphorylation have been concentrated on the LHC II protein family (Bennett 1991; Allen 1992). Recently, phosphorylation of PS II proteins has attracted attention, but up to date, not much is known about its regulation and function. Like LHC II (Gal et al. 1988), phosphorylation of PS II polypeptides seems to be under the redox-control by the plastoquinone pool (Bennett 1991; Allen 1992; Elich et al. 1993). It has been proposed that phosphorylation of the D1 protein is induced by a photoinhibitory damage to PS II (Kettunen et al. 1991; Aro et al. 1993) and might regulate its rapid turnover (Elich et al. 1992; Anderson and Aro 1994) either by preventing (Aro et al. 1992) or stimulating its degradation (Callahan et al. 1990; Elich et al. 1992). The rapid turnover of the D1 protein is usually associated with the process of photoinhibition (Aro et al. 1993; Ohad et al. 1994) and is known to play an essential role in the repair of photoinactivated PS II-centres (Aro et al. 1993; Ohad et al. 1994). When degradation of the D1 protein becomes limiting, which is the case under high light conditions, inactivated PS II-centres accumulate leading to the phenomenon of photoinhibition (Kim et al. 1993; Aro et al. 1994). To repair inactivated PS II-centres the D1 protein has to be degraded and replaced by a newly synthesised one. This requires the partial disassembly of PS II. Furthermore, these partially disassembled PS II complexes have to migrate from the grana-lamellae, where D1 protein-degradation is initiated (Barbato et al. 1992), to the stroma regions, where new D1 protein is synthesised and inserted into the membrane (Mattoo and Edelman 1987). Both processes will lead to a decrease in PS II stability. It is therefore to assume, that phosphorylation, if changing D1 protein turnover and stability, would also alter the stability of the whole PS II complex.

To study the regulation of D1 protein-phosphorylation and its effects on the rapid turnover, we determined the steady state level of $^{32}P_i$ labelling of thylakoid proteins and compared it with PS II function and the synthesis and degradation of the D1 protein in intact chloroplasts with high activities of CO_2-fixation. The *in organello* system should posses similar regulatory mechanisms as intact leaves. However, it has the advantage that substrates can easily be applied in defined concentrations. Our results provide evidence that in intact chloroplasts phosphorylation of PS II polypeptides is saturated at low light intensities and is almost irreversible in light. It creates a pool of stable PS II-centres, which are located in the grana-lamellae and are not involved in D1 protein-turnover.

Materials and methods

Isolation of intact spinach chloroplasts

Spinach leaves from the greenhouse grown at an illumination with 250 μmol m^{-2} s^{-1} for 9 h were harvested and dark-adapted on ice overnight. Before isolating intact chloroplasts the leaves were preilluminated 30 min with white light of the same light intensity while floating on cold water. The leaves were then shaken dry and sliced to small stripes without midrib parts. Isolation of intact chloroplast was performed according to Walker (1987) with following modifications. 15 g spinach were blended in a domestic blender (Sorvall OMNI-MIXER 17106) for 5 s using maximum speed and 100 ml buffer containing 0.33 M sorbitol, 0.05 M Mes-KOH pH 6.5, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mm EDTA (consistency of melting snow). Then the mixture was kept on ice and immediately centrifuged for 90 s at 6200 rpm in a Hettich Roto Silenta II centrifuge. The supernatant was poured off and the pellet was washed twice using ice cold resuspension buffer containing 0.33 M sorbitol, 0.05 M HEPES-KOH pH 7.6, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA. Then the pellet was resuspended in 1 ml resuspension buffer and stored on ice in the dark. Only intact chloroplast preparation were used with a minimum rate of 50 μmol O_2 mg^{-1} chlorophyll h^{-1} oxygen evolution and an intactness above 80% determined by using FeCy as described in Ebbert and Godde (1994). The chlorophyll content was determined according to Arnon (1949).

Light-dependent phosphorylation of thylakoid proteins

Phosphorylation experiments were performed according to Ebbert and Godde (1994) by incubating a total amount of 50 μg chl equivalent per 1 ml assay buffer according to Walker (1987) containing 0.33 M sorbitol, 0.05 M Hepes-KOH (pH 7.6), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.05 mM KH_2PO_4 and 10 mM $NaHCO_3$ in presence of 40 μCi [^{32}P] orthophosphate. The incubation was carried out in glass tubes at 20 °C using a water bath. For illumination Hedler de Luxe 2000 white light sources were used. Phosphory-

lation assay reactions were terminated via freezing 250 μl aliquots of assay mixture in liquid nitrogen adding 10 mM NaF to prevent protein phosphatase reactions.

D1 protein-synthesis assay using [^{14}C]-leucine polypeptide labelling

Incubation of intact chloroplast was carried out in the same incubation medium as used for the phosphorylation assay with the modification that [^{14}C]-leucine (NEC-279E, NEN/ Du Pont) was used instead of [^{32}P] orthophosphate. For incubation total amount of 185 kBq [^{14}C]-leucine (11.5 GBq/mmol) per 1 ml assay mixture was added.

Thylakoid membranes were isolated according to Berthold et al. (1981) in the presence of 10 mM NaF except that BSA was omitted.

Subfractionation of thylakoid membranes in grana, stroma and intermediate lamellae

Intact chloroplasts according to 1.5 mg chl were adjusted to 0.4 mg chl/ml with a solution containing 0.1 M sorbitol, 7.5 mM NaCl, 10 mM MES (pH 6.5), 2.5 mM $MgCl_2$, 10 mM NaF and 0.5% Digitonin according to Leto et al. (1985) with slight modifications. After incubation for 30 min on ice with continuous vortexing, non fractionated membranes were centrifuged off for 10 min in a Sorvall SS 34 rotor at 1000 *g*. The pellet was discharged. The supernatant was centrifuged off in a Sorvall A841 for 30 min at 40000 *g*. The stroma lammellae in the supernatant were centrifuged off for 30 min at 120000 *g*. The pellet, which contained grana-lamellae and grana-margins, was resuspended in solution b containing 150 μl 15 mM NaCl, 20 mM MES (pH 6.5), 5 mM $MgCl_2$, 10 mM NaF and 0.2 M sorbitol. The chl content was adjusted to 1.5 mg/ml and the sample was diluted with Triton X-100 (1.5 mg/ml) in a ratio of 1:15. After stirring for 1 min on ice, non-fractionated particles were centrifuged off for 3 min at 1000 *g*. The supernatant was centrifuged for 30 min at 40000 *g*. The pellet contained grana-margins. The intermediate membranes in the supernatant were centrifuged off at 120000 *g*. All pellets were resuspended in solution a. All fractions were stored in liquid N_2.

Sodium dodecyl sulfate-PAGE, immunoblotting and autoradiography

Sodium dodecyl sulfate-Page and Western-blotting were performed according to (Ebbert and Godde 1994). Immunodetection of D1, D2 and LHC II was carried out according to Godde et al. (1992). After Western-blotting the nitrocellulose was air dried for 2 h. Radiolabelled polypeptides were visualised by phosphoimaging of western blots using the Bioimaging System TINA 2.07 d (Raytest Isotopenmeßgeräte GmbH, Germany). Western-blots were exposed on a FUJI Imaging plate Typ BAS-IIIs, which were scanned with a FUJI Bio-imaging analyser BAS 1000 and analysed with the BAS reader for windows.

LiDS-gelectrophoresis was performed at 4 °C on a gel-system containing 0.1% LiDS (w/v), 0.375 M Tris pH 8.8, 14,4% acrylamide, 0.84 mM TEMED and 0.084% APS. The stacking gel contained 0.1% LiDS, 0.125 M Tris pH 6.5, 4.1% acrylamide, 1.4 mM TEMED and 0.14% APS. As separating buffer 0.024 M Tris containing 0.192 M glycine and 0.1%(w/v) LiDS.

2-Dimensional gel-electrophoresis in a non-denaturing/denaturing system

For the first dimension a non-denaturing gel system according to Peter and Thornber (1991a,b) with modification according to Heinze (1992) was used. Intact chloroplasts according to 1 mg chl were solubilised with 1% Dodecylmaltoside. Chlorophyll containing protein complexes were separated on a 2 mm deriphate-gel (1216 cm) in the dark at 4 °C. After separation, gel strips were cut and solubilised for 45 min in SDS containing solubilisation mixture before they were set on a SDS gel (Schägger et al. 1985) described above.

Pulse modulated chlorophyll fluorescence induction

F_o and F_m were determined in intact chloroplasts by pulse modulated fluorescence induction according to Schreiber et al. (1986) using a PAM fluorometer (Walz, Effeltrich, Germany). The intensity of the actinic light was 60 or 1000 μmol m^{-2} s^{-1}, respectively, and the 500 ms saturating flashes had an intensity of 4000 μmol m^{-2} s^{-1}. Before measuring the chloroplasts were dark-adapted for 5 min. The intrinsic fluorescence F_o, when all reaction-centres are open, was determined by applying only measuring light (peak wavelength 710 nm). F_m was estimated from the fluorescence yield achieved on the addition of a saturating 500 ms pulse.

Results

To test whether phosphorylation of the PS II D1 reaction-centre polypeptide depends on a photoin-

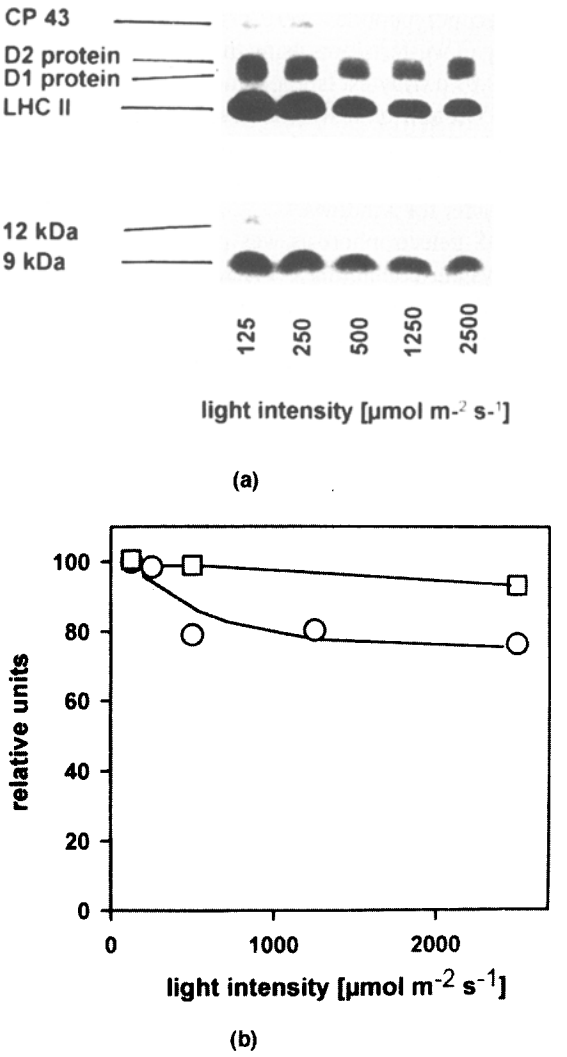


Figure 1. **Phosphorylation of PS II polypeptides is already maximal** at low light intentisites. Intact chloroplast were illuminated with increasing light intensities in the presence of [$^{32}P_i$]. No NaF was added. For this experiment PFDs of 125 (a), 250 (b), 500 (c), 1250 (d) and 2500 (e) mol m^{-2} s^{-1} were used. Intact chloroplast were solubilised and protein according to 2.5 g chl was separated on a 15% SDS-gel. Proteins were transferred in the electrical field onto a nitrocellulose membrane, which was exposed to an X-ray film. (a) shows the autoradiogram. (b) compares the light dependency of the phosphorylation level of the D1 protein (○) as determined by phosphoimaging of the Western-blots and the functionality of PS II measured by the chlorophyll fluorescence parameter F_v/F_m (□) after 10 min illumination. Both parameters are indicated as relative units of the value measured at 125 mol m^{-2} s^{-1}. At this light intensity, phosphorylation was maximal (1a), F_v/F_m of the intact chloroplasts was 0.78±0.015.

hibitory damage to PS II as has been proposed (Kettunen et al. 1991; Aro et al. 1993; Ghiardi 1993), the incorporation of $^{32}P_i$ into the D1 protein was determined after 10 min illumination at increasing light intensities from 125 to 2500 µmol m^{-2} s^{-1}. The phosphorylation level was compared with the chlorophyll fluorescence parameter F_v/F_m which indicates the functionality of PS II (Krause and Weis 1991). As shown in Figure 1a, the D1 protein in intact chloroplasts was already maximally phosphorylated at light intensities as low as 125 µmol m^{-2} s^{-1}. With increasing light intensities up to 2500 µmol m^{-2} s^{-1}, the level of D1 protein-phosphorylation decreased slightly about 20%. This decrease was probably caused by an inhibition of the phosphorylation reaction occurring at higher light intensities (Ebbert and Godde 1994). Illumination with light intensities of 125 µmol m^{-2} s^{-1} for 10 min did not result in any loss of PS II functionality as determined by F_v/F_m (Figure 1b). An accumulation of inactivated PS II-centres started to occur only after a 15 min illumination with light intensities around 800 µmol m^{-2} s^{-1}. Under these conditions photosynthetic oxygen evolution was still at its maximum (Ebbert and Godde 1994). This indicates that the phosphorylation level of PS II polypeptides and especially of the D1 protein is not strictly coupled to accumulation of inactivated PS II-centres.

To determine the turnover of the phosphate groups under steady-state conditions in the light, intact chloroplasts were preilluminated for 0, 20, 40 and 60 min in the presence of cold P_i before [$^{32P}]_i$ was added. The samples were then further illuminated for another 10 min in the presence of radioactive phosphate. From the autoradiogram (Figure 2), it can be seen that LHC II could be phosphorylated at any time during the experiment indicating a high turnover of phosphate groups bound to LHC II. The PS II polypeptides, instead, were only phosphorylated during the first 10 min of the illumination. Preillumination with non-radioactive P_i prevented any further radioactive labelling. This shows that the phosphate groups, once they were bound to the PS II polypeptides, were not removed. Obviously, PS II specific phosphatase activity in intact chloroplasts was very low in the light. Since the content of the D1 protein stayed stable even at higher light intensities (Figure 3b), the lack of dephosphorylation of PS II shows that phosphorylated D1 protein is only a poor substrate for degradation.

To investigate the effect of protein-phosphorylation on D1 protein-turnover, D1 protein-synthesis was measured as light-dependent incorporation of ^{14}C leucine

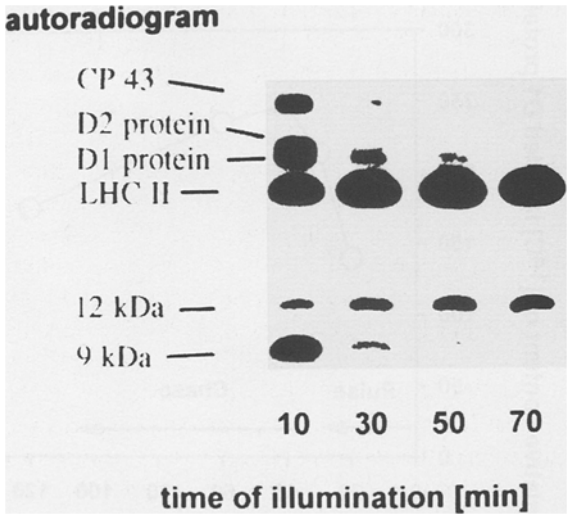


Figure 2. Possibility of thylakoid protein-phosphorylation in intact chloroplasts during increasing times of preillumination (autoradiogram). Intact chloroplasts were illuminated for 0, 20, 40 and 60 min in the presence of 0.0 5 mM KH_2PO_4 without NaF at 500 μmol m^{-2} s^{-1}. After this time [^{32} P]-orthophosphate and NaF were added and the samples were illuminated for another 10 min to get total illumination times as indicated in the figure.

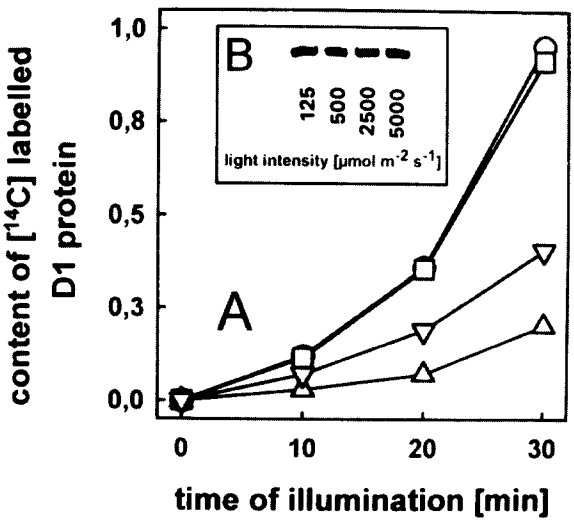


Figure 3. D1 protein-synthesis is saturated at light intensities of 500 mol m^{-2} s^{-1}. Intact chloroplast were illuminated in the presence of [^{14}C] leucine up to 30 min at increasing PFDs from 125 (△), 500 (▽), 2500 (□), to 5000 (○) mol m^{-2} s^{-1}. Solubilised chloroplast proteins were separated by SDS gel-electrophosresis, the D1 protein was located by Western-blotting and the incorporation of radioactivity into the D1 protein was evaluated by lasercanning of the autoradiogram shown in (A). The content of D1 protein is not changed even by 30 min illumination with 5000 μmol m^{-2} s^{-1} as shown by Western-blotting (B).

into the D1 protein in intact chloroplasts and compared with the actual D1 protein content determined by Western-blotting (Figure 3a,b). As shown in Figure 3a, D1 protein was already synthesised at light intensities as low as 125 μmol m^{-2} s^{-1}. D1 protein-synthesis reached its maximum at 500 μmol m^{-2} s^{-1}. No decrease in D1 protein content was observed during the 30 min light treatment even when light intensities were increased further to 5000 μmol m^{-2} s^{-1} (Figure 3b). Thus, also D1 protein-degradation was maximal at 500 μmol m^{-2} s^{-1}. The higher light saturation point of D1 protein-turnover indicates a complex regulation of D1 protein-turnover by phosphorylation.

We therefore separated the D1 protein into its two populations, in the phosphorylated and the non-phosphorylated one using a special LiDS-gelsystem (Figure 4a). By differential labelling of intact, light adapted chloroplasts with either $^{32}P_i$ or ^{14}C leucine, it could be shown that the upper form of the D1 protein was phosphorylated (Figure 4a), but not labelled with ^{14}C leucine. This clearly demonstrates that this form was not identical with precursor of D1 protein, which should be highly labelled with [^{14}C] leucine. The radioactive aminoacid, instead, was exclusively found in the lower form of the D1 protein (Figure 4b). This form did not become phosphorylated in the light. Obviously, no exchange between the phosphorylated and the dephosphorylated form of the D1 protein was possible in the light under steady state conditions. Only when phosphorylation of PS II polypeptides was not complete and phosphatase was still active, as it was in dark adapted chloroplasts during the first ten minutes of illumination, an incorporation of ^{14}C leucine into the phosphorylated upper form of the D1 protein was observed (Figure 4c). Later on, when the maximum of D1 phosphorylation was reached, phosphorylation of ^{14}C leucine labelled D1 protein stopped. Thus, D1 protein newly synthesised in the light under steady-state conditions, does not become phosphorylated. This might be due to the missing dephosphorylation of the PS II polypeptides found in intact chloroplast in the light.

The same result was obtained studying the distribution of [^{14}C] labelled D1 protein between its two forms during a 1.5 h chase period. During the 20 min pulse, [^{14}C] leucine was again only incorporated into the non-phosphorylated form of the D1 protein. After 1.5 h chase with cold leucine, 30% of the radioactively labelled D1 protein was degraded (Figure 5a), however during the whole chase period [^{14}C] leucine labelled D1 protein never appeared in the phosphorylated band

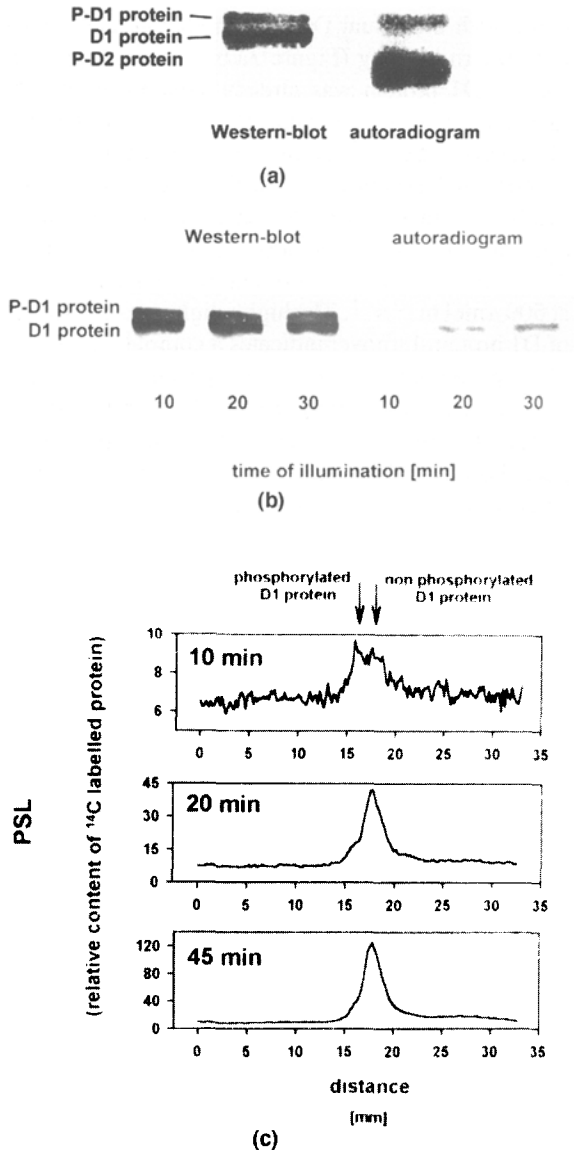


Figure 4. Newly synthesised D1 protein is not phosphorylated in intact chloroplast under steady state illumination. To identify the phosphorylated form of the D1-protein, intact chloroplasts isolated from light-adapted leaves were either illuminated for 30 min at 500 μmol m^{-2} s^{-1}in the presence of [$^{32}P_i$]. Solubised chloroplast proteins were sperated by LiDS-gel-ectrophoresis. On this gel-system, D1 protein was separated into two forms as indicated by the Westernblot (a). As shown by the autoradiogram, the upper band of D1 protein was phosphorylated (P-D1) in the light. No phosphorylation was found in the lower band. The heavily labelled band with a lower molecular weight was identical to LHC II. To test whether newly synthesised D1 protein could become phoshorylated intact chloroplasts isolated from light-adapted leaves were illuminated for 30 min with [^{14}C] leucine at 500 μmol m^{-2} s^{-1}. (b) D1 protein labelled with [^{14}C] leucine was only found in the non-phosphorylated band. No newly synthesised D1 protein was located in the upper, phosphorylated band of the D1 protein. (c) Only in chloroplast isolated from dark-adapted plant material newly synthesised D1 protein labelled with [^{14}C] leucine appeared in the phosphorylated band in the first 10 min of illumination as is shown in the evalution of the phosphoimager.

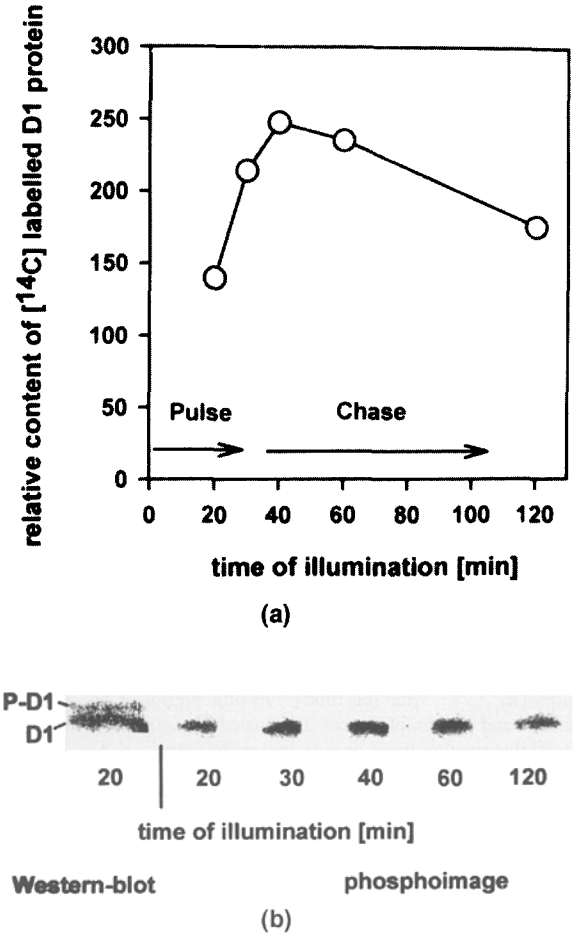


Figure 5. D1 protein is mainly degraded in its dephosphorylated form. Intact chloroplasts were labelled with [^{14}C] leucine for 30 min at 500 mol m^{-2} s^{-1}. To start the chase period the radioactive aminoacide was diluted by adding 1.55 mM cold leucine. After further illumination at the same light intensity sample were taken off, chloroplast proteins were solubilised and seperated on the LiDS-gelsystem. (a) After a 90 min chase period about 30% of the D1 protein labelled with [^{14}C] leucine was degraded. (b) The content of the two forms of D1 protein did not change during the experiment (Western-blot). As shown by the phosphoimage, no ^{14}C labelled D1 protein occured in the upper, phosphorylated band.

(Figure 5b). Thus, only the non-phosphorylated form was degraded in the light. This result confirms again that probably due to the absence of PS II phosphatase activity phosphorylation in intact chloroplasts creates a stable pool of PS II-centres which is not involved in D1 protein-turnover.

The lack of dephosphorylation of PS II polypeptides in intact chloroplasts also influences the lateral distribution of PS II centres with phosphorylated and newly synthesised D1 protein in the thylakoid membrane. It has already been shown that phosphorylated PS II centres are exclusively located in the grana parts of the thylakoid membrane. To study the distri-

bution of the different PS II centres under conditions when PS II phosphatase is inhibited, we determined the distribution of [$^{32}P_i$] or [^{14}C] leucine labelled PS II-centres in the stroma-lamellae, the central parts of the grana-lamellae and an additional fraction, which can be washed of the grana-lamellae with triton and presumably represents grana-margins as indicated by the Coomassie stain of the membrane fractions (Figure 6a). When intact chloroplasts were illuminated at light intensities of 500 μmol m^{-2} s^{-1}, phosphorylated PS II polypeptides like D1 and D2 protein, CP 43 and the *psbH* gene product were almost exclusively found in the grana-cores lamellae as has been already been known (Figure 6b,c). The grana-cores had also the highest content of D1 protein (Elich et al. 1992; Stefansson et al. 1994). Phosphorylated D1 protein in the grana-margins and stroma-lamellae was hardly detectable.

However, D1 protein, which was involved in the rapid turnover and was labelled with [^{14}C] leucine, was only located in the stroma-lamellae and in the grana-margins (Figure 6d). No newly synthesised D1 protein was detected in the grana-lamellae, even after 30 min labelling. D1 protein located in the stroma-lamellae had the highest specific labelling, since here hardly any D1 protein could be detected by Western-blotting. This confirms results of Mattoo and Edelman (1987) that newly synthesised D1 protein is integrated into the stroma-lamellae. It then could diffuse to the grana-margins but was not able to reach the central part of the grana-membranes. Obviously, due to the lack of PS II dephosphorylation an exchange between newly synthesised and phosphorylated PS II-centres even on the level of the thylakoid membrane was not possible.

To test whether newly synthesised D1 protein was integrated into intact PS II complexes, we separated chlorophyll containing protein complexes according to their molecular size on a non-denaturing deriphate-gel in the first dimension (Figure 7a). On this gel-system eight different chl containing bands were identified which were identical to the ones identified by Peter and Thornber (1991a,b). The polypeptides of the chl containing complexes were further separated on a SDS-gel in the second dimension. To localise PS II complexes, D1 (Figure 7b) and D2 protein (data not shown) were identified by Western-blotting as well as LHC II (Figure 7b). Most of the D1 protein and of the D2 protein was found in the PS II complex with the highest molecular weight named PS II A corresponding to band 3 on the non-denaturing gel. A second complex, called PS II B, was located near band 4 of the non-denaturing gel which contains trimeric LHC II (Peter and Thornber 1991b). These two band were also detected by Adir et al. (1990) using a comparable gel-system. Another third spot 7* contained minor amounts of D1 and D2 protein. This spot was named PS II C. It did not contain chl and was located below band 7 which is assumed to contain monomeric LHC II (Peter and Thornber 1991b). This spot was not detected by Adir et al. (1990) presumably because of its low protein content. The highest amount of [^{14}C] leucine integrated into the D1 protein was detected in PS II A, however some radioactivity was also found in PS II B and in PS II C (Figure d). Thus, [^{14}C] labelled D1 protein in thylakoids was integrated into intact PS II complexes. The highest phosphorylation level was shown by the high molecular PS II A complex (Figure 6c) showing that PS II centres with newly synthesised or phosphorylated D1 protein do not differ in their molecular structure.

A high phosphorylation level was also shown by band 8 of the non-denaturing deriphate-gel. This band is assumed to contain 'free pigments'. However, its separation on the second dimensional SDS-gel reveales, that band 8 contains phosphorylated proteolytic breakdown products. We assume that they derive from phosphorylated LHC II, since its content is unusually low after non-denaturing gel-electrophoresis. No such phosphorylated polypeptides have ever been observed after SDS-electrophoresis in the first dimension.

Discussion

The aim of the studies presented in this paper was to clarify the role of phosphorylation for the rapid turnover of the D1 protein. It has been proposed by several authors (Kettunen et al. 1991; Aro et al. 1993; Ghiardi 1993) that phosphorylation of D1 protein is induced by a photoinhibitory damage to PS II. As a consequence, the phosphorylation level of the D1 protein should increase with the degree of photoinhibition. However, in intact chloroplasts, maximal PS II phosphorylation is reached at low light intensities of 125 μmol m^{-2} s^{-1}, whereas a substantial loss of photosynthetic activity was observed only after 15 min illumination with light intensities higher than 800 μmol m^{-2} s^{-1}. This indicates that accumulation of phosphorylated PS II polypeptides is saturated before a substantial accumulation of inactivated PS II-centres occurs as has already been reported by Rintamäki et al. (1995, 1996).

In intact chloroplasts, phosphorylation of PS II polypeptides in the light is stable and no exchange of phosphate groups was observed. This effect was

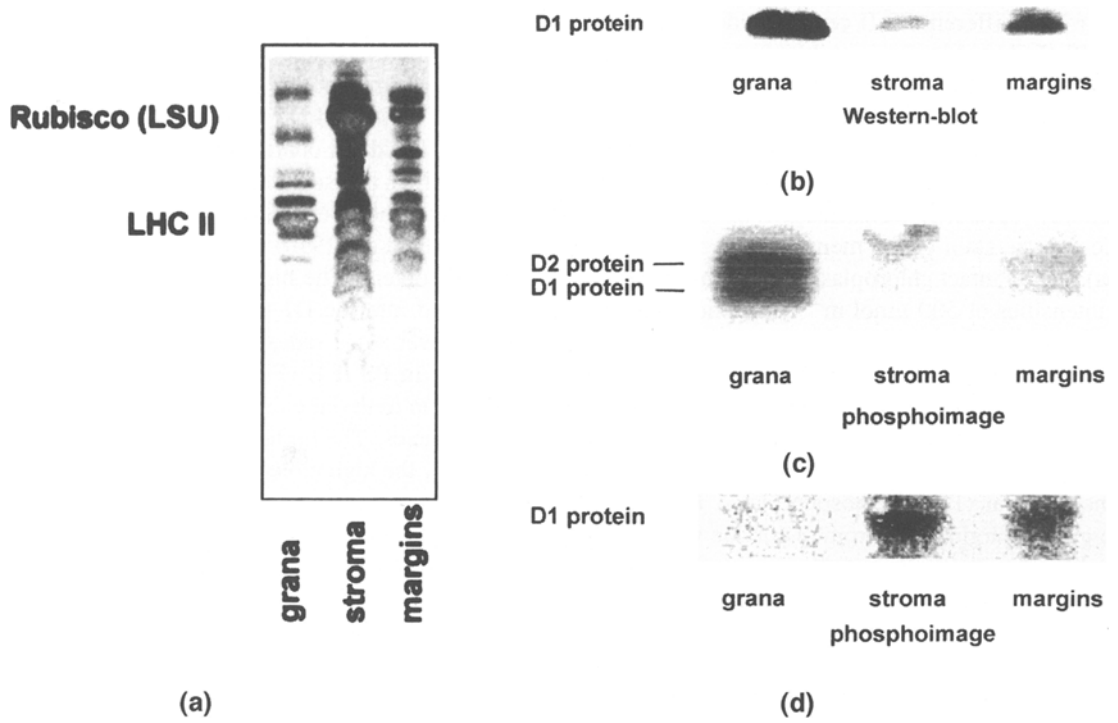
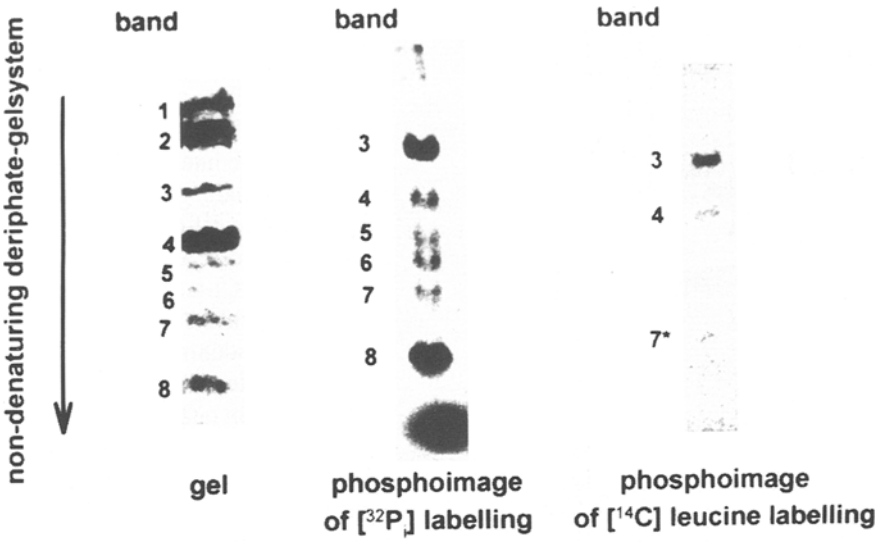


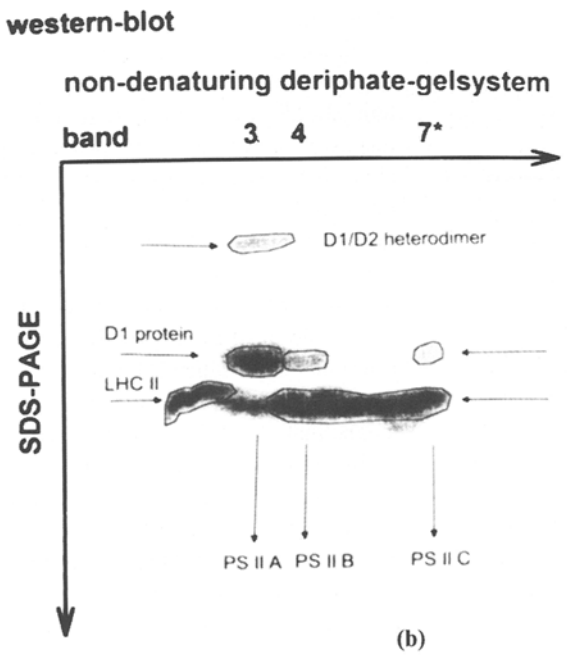
Figure 6. Phosphorylated PS II-centres show a different lateral distribution in the thylakoid membrane than PS II-centres involved in the rapid turnover of the D1 protein. Intact chloroplasts were incubated for 30 min at 500 mol m^{-2} s^{-1} with either [$^{32}P_i$] or [^{14}C] leucine. The thylakoid membrane isolated from the intact chloroplasts were fractionated into grana-cores lamellae (G), stroma-lamellae (S) and a third fraction containing grana-margins (M) indicated by the Coomassie stain of the protein pattern (a). (b) Western-blotting was used to determine the D1 protein content as indicator for the PS II content in the three different membranes. (c) As revealed by phosphoimaging, phosphorylated PS II-centres were only found in the grana-cores lamellae, (d) wheras PS II-centres with newly synthesised D1 protein, labelled with [^{14}C] leucine were only found in stroma-lamellae and grana-margins.

specific for PS II polypeptides. Polypeptides of the LHC II family, instead, were rapidly phosphorylated and dephosphorylated in the light. Thus, lack of PS II protein-dephosphorylation is not caused by any preparation artefact, but is due to the regulation of the PS II specific phosphatase, itself. Recently, Koivuniemi et al. (1995) have presented evidence that dephosphorylation of D1 protein is inhibited by high levels of ATP. Such high ATP-levels are likely to occur in intact chloroplasts, where due to limitations in CO_2-fixation membrane-energetisation is already high at moderate light intensities (Ebbert and Godde 1994). In plants or intact leaves, where membrane-energetisation does not reach such high levels, dephosphorylation of D1 protein is possible at low to moderate light intensities (Elich et al. 1993; Ebbert and Godde, unpublished results). As has been already pointed out by Koivuniemi et al. (1995) PS II specific phosphatase should be inhibited at photoinhibitory light intensities, which is in agreement to our first observations in pea plants (Ebbert and Godde, unpublished). Thus, the lack of D1 protein-dephosphorylation in intact chloroplasts offers the possibility to study exclusively the effect of protein-phosphorylation on D1 protein-turnover when dephosphorylation is inhibited. Recently, it has been reported that dephosphorylation of photoinactivated PS II centres requires light and does not proceed in the dark, whereas non-inactivated PS II centres can be dephosphorylated under both conditions (Rintamäki et al. 1996). Such a difference could not be observed in our intact chloroplast system where dephosphorylation of PS II polypeptides did occur in the dark (Ebbert and Godde 1994).

As indicated by the constant content of D1 protein even at extremely high light intensities, D1 protein-synthesis and degradation are strongly coupled in intact chloroplasts as it is in other systems (Komenda and Barber 1995). In combination with the constant protein content, the stability of D1 protein-phosphorylation in the light therefore indicates that phosphorylated D1



(a)



(b)

Figure 7. To test whether newly synthesised D1 protein was integrated into PS II-centres non-denatured, chl-containung protein complexes of thylakoid membranes isolated of intact chloroplasts labelled for 30 min at 500 μmol m^{-2} s^{-1} with either [$^{32}P_i$] or [^{14}C] leucine were separated on a non-denaturing deriphate-gelsystem. At this gel-system 8 chl containing bands could be identified (a). Of these bands, band 3, 4, 5, 6, 7 and 8 were labelled with [$^{32}P_i$], band 3, 4, 7* and 8 were labelled with [^{14}C] leucine. (b) Additional separation of the green complexes on a SDS-gel in the second dimension followed by Western-blotting against D1 protein, D2 protein (data not shown) and LHC II revealed, that D1 and D2 protein were located in band 3 (PS II A), 4 (PS II B) and 7* (PS II C). (c) Phosphorylated D1 and D2 protein were located in PS IIA, (d) wheras [^{14}C] leucine labelled D1 protein was located in PS II A, PS II B and PS II C.

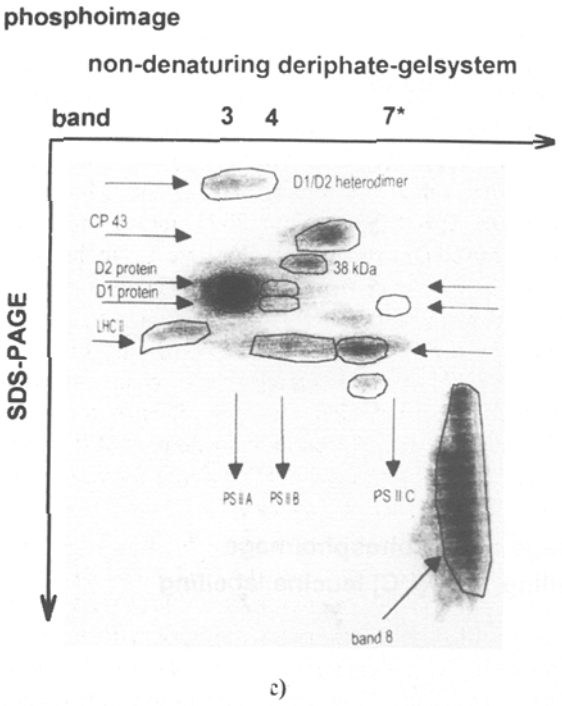


Figure 7. Continued.

protein was neither dephosphorylated nor degraded, confirming the results of Aro and coworkers (Aro et al. 1992; Koivuniemi et al. 1995).

To get more information about D1 protein-turnover, we measured synthesis the of D1 protein as light-dependent incorporation of [^{14}C] leucine and compared it with its actual content determined by immunoblotting (see also Ebbert and Godde 1994). To test whether newly-synthesised D1 protein was integrated into PS II-centres in the intact chloroplast system, we separated the chl-containing thylakoid protein complexes by non-denaturing gel-electrophoresis followed by SDS-gelelectrophoresis as has been described by Adir et al. (1990). [^{14}C]-labbelled D1 protein was found to be integrated into three PS II complexes differing in size but all containing D1 and D2 protein. No free D1 protein was detected in thylakoids of intact chloroplasts. Obviously, in intact chloroplasts newly synthesised D1 protein is as rapidly integrated into complete PS II-centres as it is in other systems (Adir et al. 1990). In the two-dimensional gelsystem no difference in the location of phosphorylated and [^{14}C] leucine labelled PS II-centres was found indicating that phosphorylated and newly-synthesised PS II-centres were structurally identical.

Since phosphorylated D1 protein could not be degraded, an accumulation of phosphorylated D1 protein should result in a retardation of D1 protein-turnover as has also been proposed by Rintamäki et al. (1995). Therefore, we determined the light saturation points of both phosphorylation and D1 protein-turnover. D1 protein-synthesis in intact chloroplasts reached its maximum at light intensities of 500 μmol m^{-2} s^{-1}, which was substantially higher than the saturation point of PS II protein-phosphorylation at 125 μmol m^{-2} s^{-1}. Since D1 protein-synthesis and degradation were in equilibrium in intact chloroplasts, this means that also D1 protein-degradation was saturated at this light intensity. Thus, D1 protein-turnover, both synthesis and degradation, were accelerated under conditions where phosphorylation was already maximal and an inhibition or at least a retardation of D1 protein-turnover was to be expected. We therefore assume that phosphorylation creates a population of PS II-centres which is not involved in D1 protein-turnover.

To test this hypothesis, we separated the phosphorylated and non-phosphorylated forms of the D1 protein by LiDS gel-electrophoresis. By this way, we could demonstrate that newly synthesised and [^{14}C] leucine labelled D1 protein did not become phosphorylated in the light in intact chloroplasts under steady-state conditions. Only during the first few minutes of illuminating dark adapted chloroplasts, phosphorylation of newly synthesised D1 protein could be

observed. At this time, phosphorylation of the PS II polypeptides was not complete and phosphatase was still active. However, when the maximum phosphorylation level was reached and phosphatase was inhibited, no further phosphorylation of PS II centres with newly synthesised D1 protein was possible. Furthermore pulse-chase experiments with [^{14}C] leucine revealed that only newly synthesised, but not phosphorylated D1 protein was rapidly degraded in intact chloroplasts. Obviously, phosphorylation in intact chloroplasts creates a stable pool of PS II-centres which are not involved in the rapid turnover of the D1 protein. However, it does not control the rate of D1 protein-turnover in the non-phosphorylated pool. Thus, in addition to the heterogeneity concerning antennae size (Melis 1991) and ability of Q_B-reduction, PS II-centres also differ in their involvement in the D1 protein-turnover. Recently, Park et al. (1996) have proposed a functional heterogeneity of PS II-centres regarding their stability against photoinactivation. These authors have observed that a small part of PS II-centres easily loose their ability to evolve oxygen when the D1 repair cycle is inhibited by the addition of chloramphenicol. The other population of PS II is relatively stable and their rate of inactivation is not influenced by the addition of chloramphenicol. The difference in the susceptibility against chloramphenicol might be ascribed to differences in the rate of D1 protein-turnover. We assume that the instable PS II-centres belong to the rapidly turning over population whereas the stable PS II-centres are members of the phosphorylated pool.

The authors further propose that the stable PS II-centres are dimers located in the grana-lamellae whereas the instable population should be identical with momomers located in the stroma-lamellae of the thylakoid membrane. It is known that phosphorylation affects the lateral distribution of PS II-centres in the thylakoid membrane with phosphorylated PS II-centres exclusively located in the grana-lamellae of the thylakoid membrane (Elich et al. 1992; Stefansson et al. 1994). We also found the highest specific phosphorylation of PS II in the central grana-lamellae. Some phosphorylated PS II-centres were located in the grana-margins, but their specific phosphorylation was low. No phosphorylated PS II centres could be detected in the stroma-lamellae. The almost exclusive location of phosphorylated PS II-centres in the grana parts of the thylakoid membrane has to be expected for PS II-centres not involved in D1 protein-turnover (Anderson and Aro 1994). According to Rögner et al. (1996) it is likely that these centres have a dimeric structure. The PS II-centres involved in the rapid turnover of the D1 protein with the highest specific labelling with [^{14}C] leucine were found to be located in the stroma-lamellae, where D1 protein is synthesised and integrated into the membrane (Mattoo and Edelman 1987). Some of the PS II-centres with newly synthesised D1 protein were also detected in the grana-margins. However, they never reached the central parts of the grana-membranes. This observation substantiates the results of Park et al. (1996) who locate the instable PS II-centres, susceptible to chloramphenicol, in the stroma lamellae. However, it is in contrast to the observations of Mattoo and Edelman (1987), who found newly synthesised D1 protein moving to the grana-membranes of *Spirodela*.

The difference between the two results might be ascribed to the fact that in intact chloroplasts D1 protein-phosphorylation is irreversible in the light and neither phosphorylation nor dephosphorylation of PS II polypeptides occur in intact chloroplasts under steady state conditions. Thus, PS II-centres assembled under these conditions cannot be stabilised by phosphorylation and do not move into the grana-coress. Instead, their D1 protein is rapidly degraded. In contrast, *in vivo* systems like low light grown *Spirodela* seem to posses an active phosphatase. Here, D1 protein is constantly phosphorylated and dephosphorylated. Under these conditions, newly assembled PS II-centres can be stabilised by phosphorylation and are able to migrate to the grana-lamellae of the thylakoid system (Elich et al. 1992). Thus, it seems to be the rate of dephosphorylation that regulates the exchange of PS II-centres from the rapidly turning over pool in the stroma-lamellae into the phosphorylated stable pool in the central parts of the grana and vice versa.

By this way, the activity of the phosphatase might also regulate the degree of photoinhibition. As has been shown by Koivuniemi et al. (1995), PS II specific phosphatase is inhibited by high levels of ATP which are likely to occur under photoinhibitory conditions. Due to this fact, inactivated phosphorylated PS II-centres cannot be degraded and accumulate at high light intensities. At lower light intensities, when PS II specific phosphatase is active, inactivated PS II-centres can be dephosphorylated and can be repaired by D1 protein-turnover. This also explains why recovery from photoinhibition is optimal at lower light intensities. Whether the stabilisation of PS II is only caused by the phosphorylation of the D1 protein, cannot be answered. Since phosphorylation of D1 protein always

comes together with the phosphorylation of the other PS II polypeptides like the D2 protein, CP 43 and the 9 kDa *psbH* gene product (Ebbert and Godde 1994), it might as well be the phosphorylation of the other PS II polypeptides that is responsible for protection of the D1 protein against degradation and the stabilisation of PS II.

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References

Adir N, Shochat S and Ohad I (1990) Light-dependent D1 protein-synthesis and translocationis regulated by reaction-centre II. J Biol Chem 265: 12563–12568

Allen JF (1992) Protein-phosphorylation in regulation of photosynthesis. Biochim BiophysActa 1098: 275–335

Anderson JM and Aro EM (1994) Grana stacking and protection of Photosystem II in thylakoid membranes of higher plants leaves under sustained high irradiance: Hypothesis. Photosynth Res 41: 315–326

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. Plant Physiol 24: 1–5

Aro EM, Kettunen R and Tyystjärvi E (1992) ATP and light regulate D1 protein modification and turnover. Role of D1* in photoinhibition. FEBS Lett 297: 29–33

Aro EM, Virgin I and Andersson B (1993) Photoinhibition of Photosystem II - inactivation, protein damage and turnover. Biochim Biophys Acta 1143: 113–134

Aro EM, McCaffery S and Anderson JM (1994) Recovery from photoinhibition in peas acclimated to varying growth irradiances: Role of D1 protein-turnover. Plant Physiol 104: 1033–1041

Barbato R, Friso G, Rigoni F, Dalla Vecchia F and Giacometti G (1992) Structural changes and lateral redistribution of Photosystem II during donor side photoinhibition of thylakoids. J Cell Biol 119: 325–335

Bennett J (1991) Protein-phosphorylation in green plant chloroplasts. Annu Rev Plant Physiol Plant Mol Biol 42: 281–311

Berthold DA, Babcock GT and Yoccum CF (1981) A highly resolved, oxygen-evolving Photosystem II preparation from spinach thylakoid membranes. FEBS Lett 134: 231–234

Callahan FE, Ghirardi ML, Sopory SK, Metha AM, Edelman M and Mattoo AK (1990) A novel metabolic form of the 32 kDa-D1 protein in the grana-localized reaction center of Photosystem II. J Biol Chem 265: 15357–15360

Ebbert V and Godde D (1994) Regulation of thylakoid protein-phosphorylation in intact chloroplasts by the activity of kinases and phosphatases. Biochim Biophys Acta 1187: 335–346

Elich TD, Edelman M and Mattoo AK (1992) Identification, characterization, and resolution of the in vivo phosphorylated form of the D1 Photosystem II reaction center protein. J Biol Chem 167: 3523–3529

Elich TD, Edelman M and Mattoo AK (1993) Dephosphorylation of Photosystem II core is light-regulated in vivo. EMBO J 12: 4857–4862

Gal A, Schuster G, Frid D, Canaani O, Schwieger HG and Ohad I (1988) Role of cytochrome *b6/f* complex in the redox-controlled activity of the Acetabularia thylakoid protein kinase. J Biol Chem 263: 7785–7791

Ghirardi MT (1993) Phosphorylation and dissambly of the Photosystem II core as an early stage of photoinhibition. Planta 90: 107–113

Godde D, Buchhold J, Ebbert V and Oettmeier W (1992) Photoinhibition in intact spinach plants: effect of high light intensities on the function of the two photosystems and on the content of the D1 protein under nitrogen. Biochim Biophys Acta 1140: 69–77

Heinze J (1992) Inaktivierung und Abbau von Proteinen in der Photoinhibierung von isolierten Thylakoidmembranen. Dissertation, Ruhr-Universität, Germany, Bochum

Kettunen R, Tyystjärvi E and Aro EM (1991) D1 protein-degradation during photoinhibition of intact leaves. A modification of the D1 protein precedes degradation. FEBS Lett 290: 153–156

Kim JH, Nemson JA and Melis A (1993) Photosystem II reaction center damage and repair in Dunaliella salina (green alga). Plant Physiol 103: 181–189

Koivuniemi A, Aro E-A and Andersson B (1995) Degradation of the D1- and D2 proteins of Photosystem II in higher plants is regulated by reversible phosphorylation. Biochemistry 34: 16022–16029

Komenda J and Barber J (1995) Comparison of *psbO* and *psbH* deletion mutants of *Synechocystis* PCC 6803 indicates that degradation of D1 protein is regulated by the Q_B site and dependent on protein synthesis. Biochemistry 34: 9625–9631

Krause GH and Weis E (1991) Chlorophyll fluorescence and photosynthesis: The basics. Annu Rev Plant Physiol Plant Mol Biol 42: 313–349

Leto KJ, Bell E and McIntosh L (1985) Nuclear mutation leads to an accelerated turnover of chloroplast-encoded 48 kDa polypeptides in thylakoids lacking Photosystem II. EMBO J 4: 1645–1653

Mattoo AK and Edelman M (1987) Intramembrane translocation and posttranslational palmitoylation of the chloroplast 32-kDa herbicide binding protein. Proc Natl Acad Sci USA 84: 1497–1501

Melis A (1991) Dynamics of photosynthetic membrane composition and function. Biochim Biophys Acta 1058: 87–106

Nanba O and Satoh K (1987) Isolation of a Photosystem II reaction center consisting of D1 and D2 polypeptides and cytochrome *b*559 . Proc Natl Acad Sci USA 84: 109–112

Ohad I, Keren N, Zer H, Gong H, Mor TS, Gal A, Tal S and Domovich Y (1994) Light-induced degradation of the Photosystem II reaction-centre D1 protein. In: Baker NR and Bowyer JR (eds) Photoinhibition of Photosynthesis: From Molecular Mechanisms to the Field, pp 161–178. Bios Scientific Publishers, Oxford

Park Y-I, Anderson JM and Chow WS (1996) Photoinactivation of functional Photosystem II and D1-protein-synthesis in vivo are independent of the modulation of the photosynthetic apparatus by growth irradiance. Planta 198: 300–309

Peter GF and Thornber JP (1991a) Biochemical composition and organization of higher plant Photosystem II light-harvesting pigment-proteins. J Biol Chem 266: 16745–16754

Peter GF and Thornber JP (1991b) Biochemical evidence that higher plant Photosystem II core complex is a organized as a dimer. Plant Cell Physiol 32: 1237–1250

Rintamäki E, Kettunen R, Tyystjärvi E and Aro EM (1995) Light-dependent phosphorylation of D1 reaction-centre protein of Photosystem II: hypothesis for the functional role *in vivo*. Physiol Plant 93: 191–193

Rögner M, Boekema EJ and Barber J (1996) How does Photosystem 2 split water? the structural basis of efficient energy conversion. TIBS 2: 44–49

Schägger H, Borchart U, Aquila H, Link TA and von Jagow G (1985) Isolation and aminoacid composition of the smallest subunit of beef heart *b/c*1 complex. FEBS Lett 190: 89–94

Schreiber U, Schliwa U and Bilger W (1986) Continuous recording of photochemical and non-photochemical fluorescence quenching with a new type of modulation fluorometer. Photosynth Res 10: 51–62

Stefansson H, Wollenberger L and Albertsson PA (1994) Fragmentation and separation of the thylakoid membrane. Effect of light-induced protein-phosphorylation on domain composition. J Chromatogr 668: 191–200

Walker D (1987) The Use of the Oxygen Electrode and Fluorescence Probes in Simple Measurements of Photosynthesis. Oxygraphic Limited, Sheffield, UK