# Light is required for efficient translation elongation and subsequent integration of the D1-protein into Photosystem II

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**Abstract The light dependence of translation and successive assembly of the D1 reaction center protein into Photosystem II subcomplexes was followed in fully developed chloroplasts isolated from the dark phase of diurnally grown spinach. The incorporation of synthesized D1 protein into Photosystem II (PSII) was analyzed by fractionation of radiolabeled unassembled protein and PSII (sub)complexes on sucrose density gradients. The ribosomes with attached nascent chains were recovered as pellets in the same gradients, and nascent chains of the D1 protein were immunoprecipitated. The analysis showed that absence of light during translation leads to an increased accumulation of polysome-bound D1 translation intermediates, indicating that light is required for efficient elongation of the D1 protein. The accumulation of the D1 protein and CP43 decreased three-fold in darkness, whereas accumulation of the D2 reaction center protein was not affected by light. In addition, light was also required for efficient incorporation of the D1 protein into the PSII core complex. In darkness, the newly synthesized D1 protein accumulated predominantly as unassembled protein or in PSII subcomplexes smaller than 100 kDa.**

*Key words:* Assembly; D1 reaction center protein; Light regulation; Photosystem II; Photoinhibition

## 1. Introduction

The D1 protein forms, together with the D2 protein, the α and β subunits of cyt$b_{559}$, the *psbI* gene product and possibly the *psbW* gene product, the reaction center of Photosystem II (PSII) located in the thylakoid membrane [1]. The turnover rate of the chloroplast-encoded D1 protein is much higher than of any other PSII protein and is a consequence of a light-induced modification of the PSII reaction center, followed by proteolysis of the D1 protein (reviews [2,3]). To compensate for the relatively short life-time of the D1 protein, the chloroplasts synthesize the D1 protein at a much higher rate than the other PSII proteins. Newly synthesized D1 protein is reassembled into existing PSII complexes. In two recent studies it has been shown that such replacement of the D1 protein can be achieved both in isolated chloroplasts and in isolated thylakoids [4,5]. The sequence of synthesis and assembly was partially resolved by pulse-chase experiments in these in vitro systems [5].

The D1 and the D2 proteins are homologous proteins, interacting strongly with each other and sharing 1–2 reaction center chlorophyll a molecule(s) (P680) within the PSII reaction center (e.g. see [6,7]). The fate of the reaction center chlorophyll a molecules upon damage and degradation of the D1 protein is unknown. Possibly, new chlorophylls have to be synthesized in order to properly assemble the PSII reaction center. Alternatively, these chlorophylls can be recycled and rebound after de novo synthesis of the D1 protein. A redistribution of chlorophylls between different chlorophyll-binding proteins (CP1, LHCII, CP43) has been proposed to occur when greening tissue was transferred to darkness [8,9].

The D1 and the D2 proteins are assumed to be inserted into the membrane during their translation on membrane-bound ribosomes [10,11]. It is not clear whether the reaction center chlorophylls bind to these proteins during translation elongation (co-translationally) or after termination of translation (post-translationally). If efficient elongation depends on the presence of chlorophylls, elongation might be halted and pausing translation intermediates could accumulate if no chlorophylls are located in the proximity of the nascent chain. Pausing intermediates of the D1 protein have been observed in chloroplasts and etioplasts and were suggested to facilitate such co-translational chlorophyll binding (e.g. [12,13]). However, in later studies, no effect of de novo chlorophyll synthesis on the elongation rate in run-off experiments could be detected in barley etioplasts [14]. During translation in darkness in mature, fully greened chloroplasts, in the presence of exogenous ATP but in absence of the reductant dithiothreitol, accumulation of at least two D1 translation intermediates (of 17.5 and 22 kDa) has been observed [15,16]. However, the reason for this accumulation was not further investigated.

The stability of most nuclear- and chloroplast-encoded chlorophyll-binding proteins is strongly increased after binding of chlorophyll and carotenoids (e.g. [14,17,18]). Since chlorophyll biosynthesis is a strictly light-controlled process in higher plants, light indirectly controls the accumulation of most chlorophyll-binding proteins by providing an increased protein stability.

Several other post-transcriptional mechanisms for light-controlled accumulation of chloroplast proteins have been discussed [19–22]. It has been shown that translation initiation of *psbA* mRNA, encoding the D1 protein, is a light-induced process via redox-controlled binding of nuclear-encoded proteins to the 5′ untranslated region (UTR) of *psbA* mRNA [23–26]. Modulation of the phosphorylation state of the mRNA-binding protein complex by ATP/ADP in the chloroplast further controls the initiation process [26].

In this paper, the light requirement of the replacement of the D1 protein into existing PSII complexes in mature, fully developed chloroplasts has been addressed. The reductant dithiothreitol and ATP were present during all translation assays to stimulate translation, especially in the dark. We have dissected the effect of light on D1 synthesis and assembly

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into different steps: (i) elongation and release of the nascent chain from the ribosomes, (ii) accumulation of the unassembled D1 protein in the membrane and (iii) assembly of the D1 protein into the PSII complex. It will be shown that the accumulation rate of the D1 protein in the dark is threefold lower than in the light and that in the dark a strong accumulation of polysome-bound N-terminal translation intermediates occurs. We show that in the absence of light, the newly synthesized D1 protein is only poorly incorporated into the PSII core complex. Interestingly, absence of light decreases accumulation of D1 and CP43 to an equal extent, while accumulation of the D2 protein is not affected.

## 2. Material and methods

Spinach was grown hydroponically at 23°C in a light/dark cycle of 12/12 h. For all experiments, mature leaves were harvested at the end of the dark period and kept in darkness. Isolation of intact chloroplasts was carried out in the dark as described in [4].

In vitro translation in isolated chloroplasts was carried out in the dark or in the light (approximately 50 μmol photons $m^{-2}$ $s^{-1}$) at 23°C, as described in [4]. The chloroplasts (0.5 μg chlorophyll $\mu l^{-1}$) were incubated for 5 min at 23°C in a translation mixture consisting of 330 mM sorbitol, 50 mM HEPES-KOH (pH 8.0), 10 mM dithiothreitol, 10 mM MgATP and 40 μM of each amino acid except methionine. After this preincubation time, carrier-free $^{35}S$-labeled methionine was added to a final concentration of 0.5 μCi $\mu l^{-1}$. After 10 min pulse labeling, the labeled methionine was chased for 30 min by the addition of 10 mM cold methionine to the translation mixture. Translation was stopped by dilution with a 10-fold volume of ice-cold medium containing 330 mM sorbitol, 50 mM HEPES-KOH (pH 8.0). To separate the thylakoid-bound translation products from the stromal products, the chloroplasts were lysed in RNase-free lysis buffer, containing 46 mM HEPES-KOH (pH 7.6), 118 mM potassium acetate, 7 mM magnesium acetate, 5 mM dithiothreitol, 10 μg $ml^{-1}$ heparin, and the thylakoid membranes were collected by 3 min centrifugation at 5000×*g*. Subsequently, the thylakoids were washed twice in the same buffer.

To separate the native PSII (sub)complexes, unassembled proteins and ribosomes, thylakoids were solubilized for 50 min on ice with *n*-dodecyl-β-D-maltoside (1% w/v) at a chlorophyll concentration of 0.5 mg $ml^{-1}$ in a medium containing 200 mM Tris-HCl (pH 8.0), 25 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mg $ml^{-1}$ heparin and 0.2 mM of the protease inhibitor phenylmethylsulfonyl fluoride. The suspension was subsequently centrifuged (20 h, 3°C, 180000×*g*) on sucrose gradients containing 0.1–1 M sucrose in 25 mM Tris-HCl (pH 8.0), 25 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mg $ml^{-1}$ heparin and 0.03% *n*-dodecyl-β-D-maltoside and 19 fractions of equal volume were collected from bottom to top. The pellets, containing the ribosomes, were resuspended in 600 μl $H_2O$.

The distribution of PSII proteins and PSII (sub)complexes in the sucrose gradient fractions was determined by several biophysical and biochemical techniques as described in [4].

Proteins in the sucrose gradient fractions were precipitated in 10% ice-cold trichloroacetic acid (> 30 min on ice), collected by centrifugation, and finally resuspended in an SDS-solubilization buffer. PSII proteins in the ribosome pellets were immunoprecipitated with specific antisera kindly provided by Dr. J.E. Mullet (Texas A & M). SDS-PAGE was performed using 14% linear gels containing 6 M urea. Western blotting and immunodetection were done using chemiluminescence (goat anti-rabbit IgG horseradish peroxidase conjugate; BioRad). In the case of $^{35}S$-labeled samples, gels were stained by Coomassie brilliant blue or silver nitrate, followed by incubation for 15–20 min in Amplify (Amersham) and drying. Quantification of proteins was performed by scanning the autoradiograms with a laser densitometer, using the software package Image Quant (Molecular Dynamics).

## 3. Results

Synthesis, accumulation and assembly of D1, D2 and CP43 were followed in intact chloroplasts in darkness or in the light. Since the main goal of this study was to investigate the effect of light on synthesis and assembly of the D1 protein, chloroplasts were isolated in the dark at the end of the dark period from diurnally grown, fully mature leaves. This prevented possible accumulation of light-induced factors and accumulation of chlorophyll precursors or pools of chlorophyll. Prior to the addition of the radiolabeled methionine, the chloroplasts were incubated for 5 min under translation conditions (in light or dark) to preadapt the chloroplasts to the translation conditions and to decrease the amount of radiolabel in apoprotein breakdown products [13].

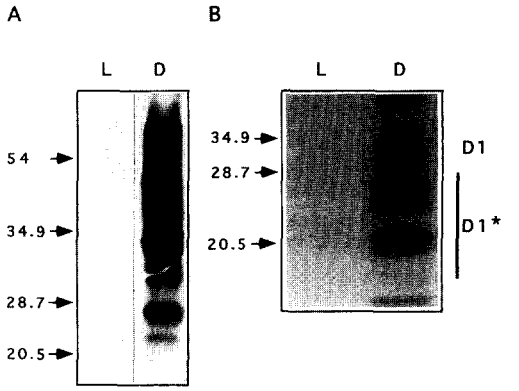



Fig. 1. Ribosome-bound radiolabeled translation products after translation in intact chloroplasts in the light and in the dark. The intact chloroplasts were pulse-labeled for 10 min and chased for 30 min with an excess of unlabeled methionine. After translation, the chloroplasts were lysed and the thylakoid-bound polysomes were collected as pellets after centrifugation on sucrose density gradients of the solubilized thylakoid membranes. A: Autoradiograms of 1% of the polysome pellets after translation in the light (L) and the dark (D). B: Autoradiogram of immunoprecipitated radiolabeled N-terminal translation intermediates of the D1 protein. Immunoprecipitation was carried out using 8% of the polysome pellet. The position of molecular weight markers is indicated.

After translation (pulse 10 min, followed by a 30 min chase with cold methionine) in the light and in the dark, the chloroplasts were lysed and thylakoid membranes were collected. To separate the radiolabeled polysome-bound nascent chains from unassembled radiolabeled membrane proteins and from radiolabeled PSII (sub)complexes, the membranes were solubilized and subjected to a sucrose density gradient centrifugation. The distribution and characterization of the different PSII (sub)complexes and unassembled proteins in such gradients were described in [4]. A combined stabilization of polysomal structures and PSII complexes was achieved through a replacement of the Tricine buffer employed in the earlier work [4] by Tris and addition of $MgCl_2$, KCl, dithiothreitol and RNase inhibitor [27] to all solutions used during the fractionation. This allowed the complete recovery of polysomes and monosomes with attached nascent chains as pellets on the bottom of the gradients. It should be noted that changing of the buffer system did not alter the distribution of the PSII polypeptides in the sucrose density gradient (van Wijk, unpublished results).

### *3.1. Accumulation of polysome-bound D1 translation intermediates*

After fractionation of the sucrose gradients into equal (volume) fractions, the polysomal pellets were resuspended in a

small volume and incorporated radiolabel was measured by scintillation counting. The polysomal pellets of the translation in the dark contained five times more radiolabel than after translation in the light. SDS-PAGE of the solubilized polysomal pellets showed a large number of radiolabeled polypeptides in case of the translation in the dark but hardly any radiolabel after translation in the light (Fig. 1A). Immunoprecipitation of the polysomal pellets with N-terminal D1 antiserum revealed that pellets from the dark translation contained D1 translation intermediates of about 18, 20, 24, 26 and 30 kDa and full-length precursor protein (Fig. 1B). Interestingly, hardly any radiolabeled D1 translation intermediates or full-length apoprotein could be detected on polysomes from the illuminated chloroplasts (Fig. 1B). In a recent study [5] it was shown that in the light, elongation and termination were completed within 10 min in isolated chloroplasts. This explains why no translation intermediates were observed in the ribosomal pellets after a chase time of 30 min in the light. Thus, translation in darkness must have resulted in an increased pausing of the ribosomes and/or in a strongly decreased elongation rate.

### 3.2. *Accumulation and assembly of radiolabeled D1, D2 and CP43*

To investigate the role of light on the accumulation and assembly of the D1 protein, the sucrose gradient fractions from translation in light and darkness were precipitated and analyzed on SDS-PAGE (Fig. 2). Autoradiograms were scanned, and the quantitative distribution of radiolabeled D1, D2 and CP43 in the sucrose gradient fractions is shown in Fig. 3. The total amount of radiolabeled D1, D2 and CP43 after translation in light and in darkness is summarized in Table 1.

As can be observed from the autoradiogram (Fig. 2A) and Table 1, translation in the light resulted primarily in synthesis and accumulation of the D1 protein. Accumulation of radiolabeled D1 protein was about three-fold higher than of the D2 protein and of CP43 (Table 1). However, in the dark, accumulation of radiolabeled D1 and CP43 was approximately three-fold lower than in the light (Table 1). Surprisingly, accumulation of the D2 protein was nearly unaffected by the light condition.

In the light, a large proportion (45%) of the radiolabeled D1 protein could be assembled into PSII core complexes, which were recovered in sucrose gradient fraction 9 and 10 (Figs. 2A and 3A). These core complexes were found to have a molecular weight of 190–240 kDa and contained at least the PSII reaction center proteins (D1, D2, cyt$b_{559}$, *psbI* gene product), CP43 and CP47 [4]. The residual proportion of the radiolabeled D1 protein was found in PSII reaction center particles (in fraction 12), in smaller complexes or as unassembled protein in fractions 13–16. Such assembly pattern of radiolabeled D1 protein is in agreement with a recent assembly study in which chloroplasts isolated during the light period of diurnally grown plants were employed [4]. In contrast, during translation in darkness, the amount of synthesized D1 protein was not only three-fold lower than in the light, but the D1 protein was also poorly incorporated into PSII core complexes (about 10%). The D1 protein was mainly found in small PSII (sub)complexes and as unassembled protein (Fig. 2B, Fig. 3A). Thus, it can be concluded that light is not only required for efficient elongation and accumulation of

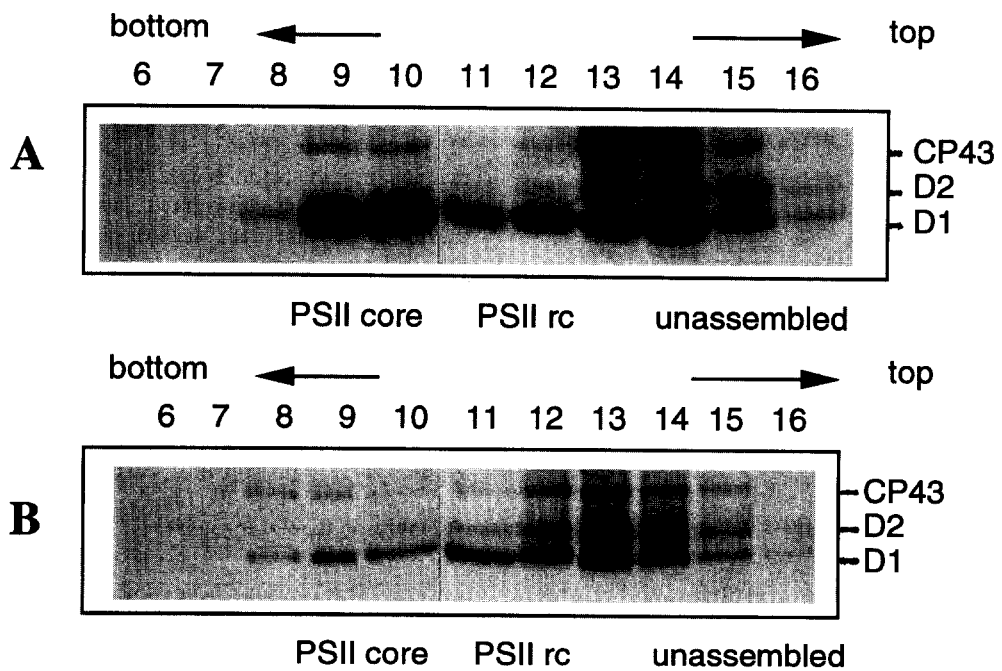
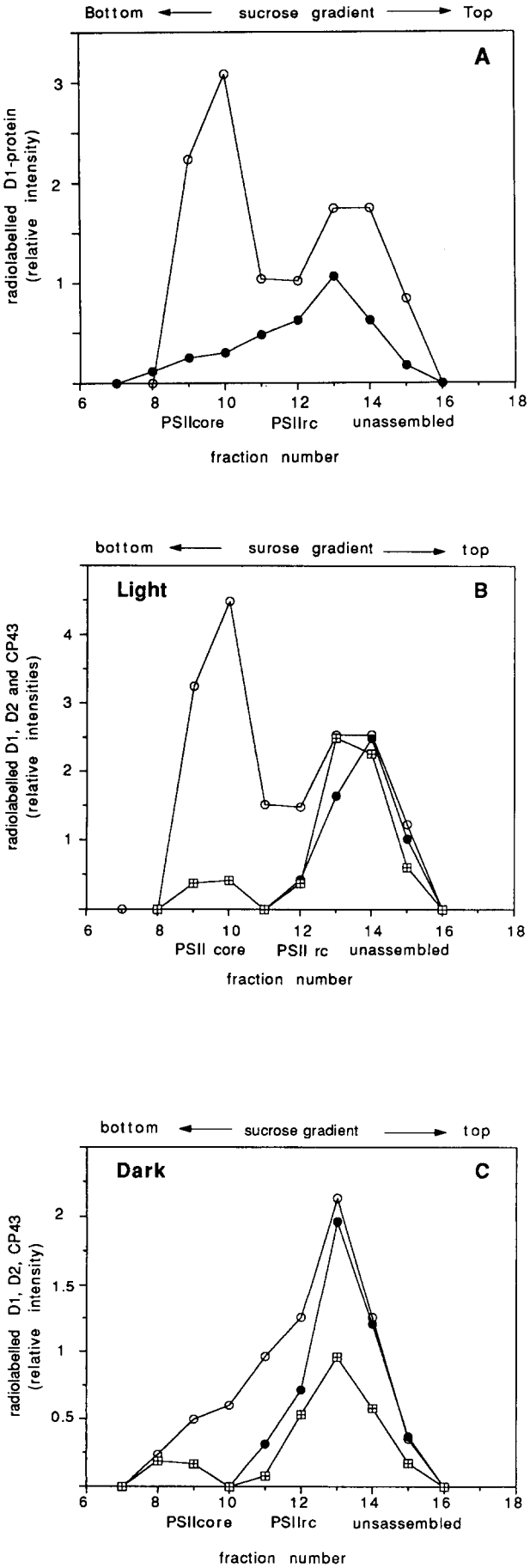



Fig. 2. Autoradiogram of sucrose density gradient fractions of solubilized thylakoid membranes after translation in intact chloroplast in the light (A) and in the dark (B). The intact chloroplasts were pulse-labeled for 10 min and chased with excess unlabeled methionine. The sucrose gradient fractions are numbered from bottom to top. The different PSII (sub)complexes are indicated below the autoradiograms. The identification and characterization of the different PSII proteins and PSII complexes is described in [4] (PSII rc is the PSII reaction center, containing D1, D2, cytb559, and the *psbI* gene product).




the D1 protein but seems also required for efficient assembly into PSII core complexes.

Radiolabeled CP43 and D2 were only poorly incorporated into the PSII (sub)complexes both in light (Figs. 2A and 3B) as observed earlier [4] and in darkness (Figs. 2B and 3C). CP43 accumulated nearly completely as unassembled protein in sucrose fractions 12–14, whereas the D2 protein accumulated in a small PSII subcomplex of less than 100 kDa or as unassembled protein (in fractions 13–15).

## 4. Discussion

It is important to emphasize that the experiments in this paper concern leaves which were fully developed; many earlier studies on light-regulated protein synthesis concerned etiolated tissue in which no photosynthetic complexes had assembled nor chlorophyll had accumulated. In mature leaves, synthesis of PSII proteins is predominantly required to replace damaged subunits, and not to assemble completely new complexes as is the case in etioplasts (see [4,5]). Thus in mature leaves, de novo synthesis and accumulation of PSII proteins is inversely correlated with the lifetime of each protein and is therefore dominated by accumulation of the D1 protein.

In this study, we have demonstrated a strong regulatory effect of light on elongation and accumulation of the D1 protein and CP43. Thus a light-induced stromal factor or de novo chlorophyll biosynthesis seems to be required for the elongation process. Synthesis and accumulation of CP43 and D1 were equally stimulated by light, possibly indicating that they are regulated by a similar light-controlled mechanism or light-induced factor.

In contrast, elongation and accumulation of the D2 protein were nearly unaffected by light, in agreement with earlier observations in etioplasts [28]. Thus translation elongation and accumulation of the D2 protein might be independent of de novo chlorophyll biosynthesis. This suggests that the synthesis of the D1 protein is more tightly controlled than the synthesis of the D2 protein. This supports the notion that the D2 protein plays an important role in the early stabilization of the D1 protein during biogenesis of PSII, as suggested from PSII deletion mutants studies (see [21]). It is likely that the D2 protein can bind chlorophyll post-translationally, possibly during association with the D1 protein in the formation of the PSII reaction center.

The sucrose gradient analysis demonstrated that in the light, de novo synthesized D1 protein was effectively incorporated into the PSII core complex. Apparently, the chloroplasts isolated from leaves at the end of the night cycle contained damaged PSII complexes from the previous light period, requiring replacement of the D1 protein. Assembly of newly synthesized D1 protein into PSII (sub)complexes was inhibited in darkness (Fig. 2B, Fig. 3), demonstrating that also the assembly of the D1 protein had a strong light requirement.

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Fig. 3. Quantification of radiolabeled D1, D2 and CP43 in the autoradiograms as shown in Fig. 2. A: Distribution of radiolabeled D1 protein after translation in the light (open circles) or in the dark (filled circles). B, C: Distribution of radiolabeled D1 protein (open circles), D2 protein (closed circles) and CP43 (squares) after translation in the light (B) or in the dark (C). To allow direct comparison of the three proteins, the intensity was corrected for the number of methionine residues in each of the proteins.

Table 1
Total amount of synthesized D1, D2 and CP43 during translation in the dark or in the light (50 μmol photons $m^{-2}$ $s^{-1}$)

| | Light | Darkness |
|---|---|---|
| D1 | 1 | 0.31 |
| D2 | 0.32 | 0.27 |
| CP43 | 0.38 | 0.16 |

The amount of each protein was calculated from the autoradiograms as shown in Fig. 2. A correction for the number of methionine residues in each of the proteins was carried out (spinach D1, D2 and CP43 contain 11, 8 and 8 methionine residues, respectively). The amounts were normalized to the amount of D1 protein synthesized in the light.

This might be an indication for the need of newly synthesized chlorophylls in order to replace damaged reaction center chlorophylls. A limited extent of recycling of chlorophylls could, however, account for the small but significant amount of integration of the D1 protein into the PSII core complex in darkness.

Although in vitro (in isolated thylakoids) degradation of the damaged D1 protein and disassembly of the PSII complexes does occur in darkness [2,3], it is possible that in vivo degradation and disassembly are influenced by light through phosphorylation and dephosphorylation of the D1 protein [29]. We can therefore not exclude that the light dependence of D1 assembly, as observed in our experiments, resulted partially from a light control of degradation and disassembly of the PSII reaction center.

The sucrose gradient analysis as used in this paper allowed us to follow elongation rates, accumulation of pausing intermediates as well as the assembly of the newly synthesized proteins into PSII complexes. These tools will now be further applied to identify the light-induced regulatory factors required for translation elongation and assembly of the chlorophyll-binding PSII reaction center proteins.

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