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# Degradation and *de novo* synthesis of D1 protein and *psbA* transcript in *reinhardtii* during UV-B inactivation of photosynthesis

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UV-B induces intensity and time dependent inhibition of photosynthetic O<sub>2</sub> evolution and PS II electron transport  
*Chlamydomonas reinhardtii* chloroplast membranes are rapidly and

essential for the repair of damaged PS II as chloramphenicol accelerated UV-B inactivation of photosynthesis and  
*psb*

for the D1 protein. Cells showing 72% inhibition of PS II

protein. This shows that synthesis of D1 protein is not the only component involved in the recovery process. Our

events, which in turn may limit the repair of damaged PS II.

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## 1. Introduction

Excess of UV-B and visible light impairs the photosynthesis. UV-B is more potent in the inhibition of photosynthesis than the visible radiation (Jones and Kok 1966). As compared to vast literature available on visible light induced inhibition of photosynthesis, there are very few published studies on the mechanism of UV-B inhibition of photosynthesis *in vivo*. It is not clear whether visible light photoinhibition and UV-B inactivation of photosynthesis operate through similar mechanisms, though the primary site of damage in both the processes is PS II (Noorudeen and Kulandaivelu 1982; Renger *et al* 1989; Aro *et al* 1993). Degradation of D1 and D2 proteins of PS II reaction center occurs in visible as well as UV-B light (Greenberg *et al* 1989; Jansen *et al* 1996). A simultaneous repair process involving

the synthesis, removal and replacement of degraded D1 protein is reported to be operational during the course of photoinhibition of photosynthesis by visible light (Ohad *et al* 1984; Kyle and Ohad 1986). The observed photoinhibitory damage is the net result of a balance between the photodamage and the repair process (Samulesson *et al* 1985; Lidholm *et al* 1987; Shyam and Sane 1989). Whether similar repair phenomena are operational during the course of UV-B inactivation of photosynthesis in the presence of visible light is yet to be established. In the present communication, we have studied the D1 protein degradation and *psbA* transcript levels in the green alga *Chlamydomonas reinhardtii* in the course of UV-B inhibition of photosynthesis *in vivo*. We have investigated if the UV-B induced photosynthetic damage and its

**Keywords.** *Chlamydomonas reinhardtii*; D1 protein; photoreactivation; photosystem II; UV-B

Abbreviations used: CAP, Chloramphenicol; D1, 32 kDa photosystem II reaction center protein; D2, 34 kDa reaction center protein; DCPIP, Dichlorophenol indophenol; DPC, Diphenyl carbazide; MV, Methyl viologen; PBQ, p-benzoquinone; PPFD, photosynthetic photon flux density; TAP, Tris-acetate phosphate; UV-A, long wave ultraviolet radiation (320–400 nm); UV-B, medium wave ultraviolet radiation (290–320 nm); UV-C, short wave ultraviolet radiation.

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repair is a transcriptional, translational or post translational event.

## 2. Materials and methods

### 2.1 Strain and culture conditions

The cell wall deficient mutant of *C. reinhardtii* cw15, obtained from the Department of Plant Biochemistry, Ruhr University, Bochum, Germany, was grown in batch cultures in Tris-acetate phosphate (TAP) medium at  $25 \pm 1^\circ\text{C}$ . The photosynthetic photon flux density (PPFD) of  $250 \mu\text{mol m}^{-2} \text{s}^{-1}$  for 14 h daily was provided to the cultures by a combination of cool white fluorescent tubes and tungsten lamps (Singh *et al* 1996).

### 2.2 UV-B irradiation of algal cultures

Exponentially growing cells equivalent to  $60 \mu\text{g Chl ml}^{-1}$  were exposed to UV-B in a glass Petri dish embedded in a water jacketed metallic plate connected to thermostatic circulator (LKB, Sweden) to maintain a temperature of  $25 \pm 1^\circ\text{C}$ . The culture suspension occupying the depth of 1 cm in Petri dish was continuously stirred to provide equal exposure of UV-B to each cell. UV-B was provided by pre-burnt UVB-313 lamps (Q Panel, USA). Pre-solarized cellulose acetate filter (Courtauld Plastic, UK) was used to eliminate the UV-C emitted by these lamps. The filter transmits mainly in the UV-B region along with a little of UV-A. An equal amount of UV-A received by treatment cultures was also provided to the control by employing Mylar-D (DuPont, USA) which cuts both the UV-B and UV-C. The intensity of UV-C, UV-B and UV-A was measured by a radiometer (Vilber Lourmat, France) equipped with broad band sensors having half band width of 30, 26 and 31 nm with peaks at 254, 312 and 365 nm respectively. Cell suspensions were irradiated at UV-B intensities of 2.5 to  $10 \text{ W m}^{-2}$ . The high UV-B level was selected to allow studies on temporal changes in parameters related to the inhibition of photosynthesis in response to UV-B exposure within a reasonable time scale. Since the experimental material was a motile green alga, the actual response of UV-B could be achieved mainly at the UV-B dose of  $10 \text{ W m}^{-2}$ . The high dose requirement might be due to fast reactivation process operative under our set of experimental conditions. Antibiotics were added to the cell suspension 10 min before the start of UV-B treatment or the reactivation period. Chloramphenicol (CAP), cycloheximide and rifampicin were added at the concentration of 100, 10 and  $250 \mu\text{g ml}^{-1}$  respectively. Algal samples during UV-B treatment and in the course of reactivation were removed at different time intervals and inactivation of photosynthesis was assayed by measuring the photosynthetic  $\text{O}_2$  evolution or PS II electron transport activity. The rate of oxygen evolution or

consumption at the start of each experiment was used as control value for calculating per cent inhibition.

### 2.3 Monitoring of photosynthetic rate and electron transport activity

Photosynthetic ability of the intact algal cells and partial electron transport reactions of the thylakoid membranes were monitored by using a Clark type  $\text{O}_2$  electrode (Hansatech Ltd., Norfolk, UK). The measurement was carried out at  $25 \pm 1^\circ\text{C}$  under a saturating PPFD of  $250 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Photosynthesis was measured in terms of photosynthetic  $\text{O}_2$  evolved  $\text{mg}^{-1} \text{Chl h}^{-1}$  in the presence of 1 mM sodium bicarbonate. The chlorophyll was quantitated as in Porra *et al* (1989).

$\text{H}_2\text{O}$  to PBQ PS II activity *in vivo* was measured in the cells mildly disrupted under low pressure of 200 pounds/inch<sup>2</sup> in SLM Aminco French pressure cell as in Belknap and Togasaki (1981). The thylakoid membranes were prepared as in Shim *et al* (1990) and PS II and PS I activities were monitored as described by Singh *et al* (1990). In all experiments the data represent the mean  $\pm$  SE of five independent measurements.

### 2.4 Protein labelling, gel electrophoresis and immunoblotting

Cells were labelled with carrier-free  $\text{H}_2^{35}\text{SO}_4$  for 120 min following the method of Reismann and Ohad (1986) with minor modifications. The labelled cells were subjected to UV-B treatment ( $10 \text{ W m}^{-2}$  UV-B and  $150 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD) and chased at 30 min intervals for 90 min. In another set of experiments, cells were labelled with  $\text{H}_2^{35}\text{SO}_4$  immediately after the termination of UV-B treatment under suitable reactivating conditions (PPFD  $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ , temperature  $25 \pm 1^\circ\text{C}$ ) and simultaneously chased to follow the incorporation of label in the D1 and D2 proteins during recovery. The aliquots were homogenized for the preparation of thylakoid membranes and proteins were analysed by denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10–18% gradient of acrylamide in the presence of 4 M urea. The protein bands were visualized by fluorography. The disappearance of label in D1 and D2 was quantified by laser densitometry. Identity of the D1 protein was established by Western hybridization using alkaline-phosphate-conjugated secondary antibody as described by Harlow and Lane (1988). D1 protein was quantified by scanning the immunoblot with a laser densitometer.

### 2.5 RNA isolation and Northern blotting

Total RNA was isolated by using guanidine thiocyanate, following the method described by Malmberg *et al* (1985). A heterologous probe containing spinach *psbA* on an 800 bp

HindIII fragment was used to probe the *psbA* messenger RNA. Northern analysis was performed following the methods as described in Sambrook *et al* (1989).

### 3. Results

#### 3.1 UV-B inactivation of photosynthesis

The photosynthesis in *C. reinhardtii* growing at PPFD of  $150 \mu\text{mol m}^{-2} \text{s}^{-1}$  got saturated at  $250 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Exponentially growing cultures exposed to UV-B from 2.5 to  $10 \text{ W m}^{-2}$  for 60 min in the presence of a background PPFD of  $150 \mu\text{mol m}^{-2} \text{s}^{-1}$  showed UV-B intensity dependent inhibition of photosynthetic  $\text{O}_2$  evolution. The time course of UV-B inhibition showed a steep initial decline in the photosynthetic rate, followed by a slow phase, which usually occurred after 45 min of UV-B exposure (figure 1). The thylakoid membranes isolated from UV-B inactivated cells showed a time dependent loss of PS II activity, measured as electron flow from  $\text{H}_2\text{O}$  to DCPIP (table 1). When DPC was used as an artificial electron donor, a

marginal enhancement in the reduction of DCPIP was observed. PS I was, however, marginally affected.

Addition of cytoplasmic translation inhibitor cycloheximide did not change the time course of UV-B inactivation of photosynthesis (data not shown). However, the chloroplastic translation inhibitor, chloramphenicol (CAP) increased the susceptibility of the alga to UV-B inactivation. A faster decline in the rate of photosynthetic  $\text{O}_2$  evolution and PS II ( $\text{H}_2\text{O}$ -PBQ) electron transport activity occurred when CAP was added in the beginning of the UV-B treatment (figure 1, table 1).

#### 3.2 D1 and D2 protein degradation and steady state level of D1 protein

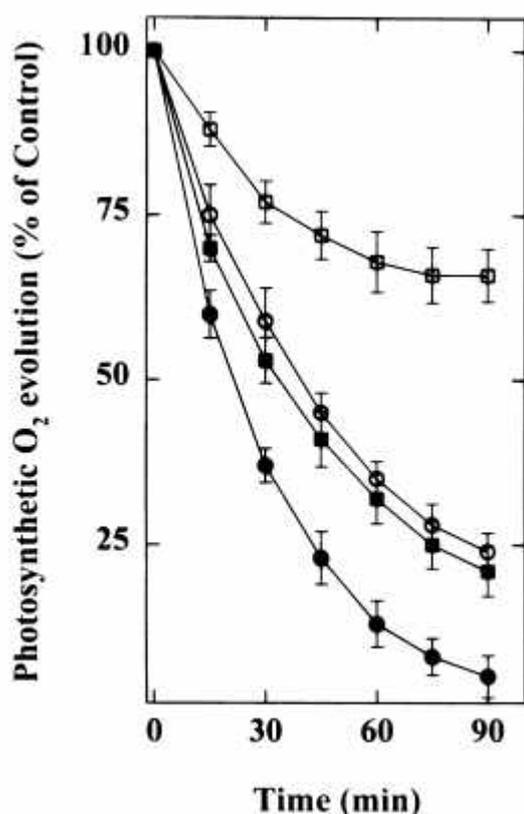
*In vivo* labelling with  $\text{H}_2^{35}\text{SO}_4$  for 2 h under a saturating PPFD in the presence of cycloheximide showed a high incorporation of radioactivity in 32 and 34 kDa protein bands, corresponding to the molecular mass of D1 and the D2 proteins respectively (figure 2). The 32 kDa band was also established, as the D1 protein by immunoblotting (figure 3). Following exposure to  $10 \text{ W m}^{-2}$  UV-B +  $150 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD, a time dependent decrease in the level of both the 32 and the 34 kDa proteins was noticed (figure 2). The decrease in the level of two proteins coincided well with loss of the PS II electron transport activity and photosynthetic  $\text{O}_2$  evolution. The rate of degradation of the D2 protein was marginally lower than the D1 protein following exposure to UV-B. Steady state level of the D1 protein in the thylakoid membranes, as determined by immunoblotting using D1 specific antibody, showed a reduction of 25% in its level in the course of 90 min of  $10 \text{ W m}^{-2}$  UV-B treatment while the loss of PS II activity amounted to 72% of the control. Steady state level of this protein reduced drastically when UV-B treatment was given in the presence of CAP (figure 3).

#### 3.3 Steady state levels of *psbA* transcript

Steady state levels of the transcripts of the *psbA* gene was analysed in the course of UV-B inactivation of photosynthesis. *C. reinhardtii* makes a single 1.4 kb *psbA* transcript. Steady state level of the *psbA* transcript showed a time dependent decrease during the course of UV-B inactivation. A maximum of 40% decrease in the transcript level was recorded 90 min after exposure of the algal cells to UV-B (figure 4).

#### 3.4 Photoreactivation of inhibited photosynthesis

Reactivation of the UV-B induced inhibition of photosynthesis was carried out by incubating UV-B treated cells (showing approximately 50% loss of photosynthetic activity) at different PPFDs ranging from 50 to

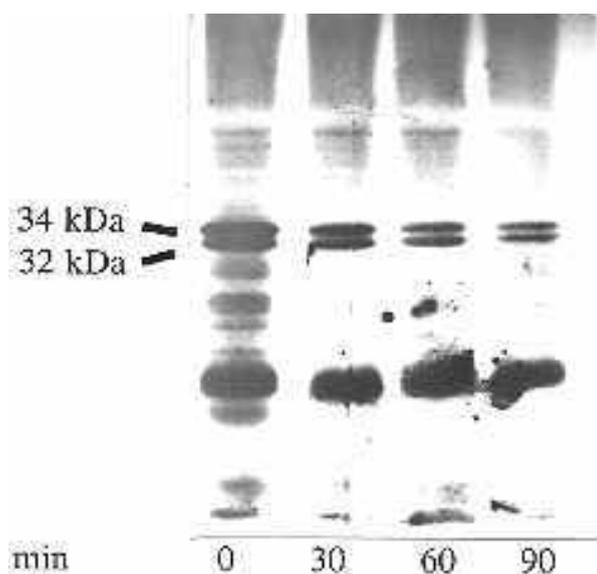


**Figure 1.** Time course of inhibition of photosynthetic  $\text{O}_2$  evolution. The cells were exposed to 5 (□, ■) and 10 (○, ●)  $\text{W m}^{-2}$  UV-B +  $150 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD in the presence (filled symbols) and absence (empty symbols) of  $100 \mu\text{g ml}^{-1}$  CAP. The initial photosynthetic rate taken as 100% was  $86 \pm 5 \mu\text{mol O}_2 \text{ evolved mg}^{-1} \text{ Chl h}^{-1}$ .

**Table 1.** Effect of UV-B on inactivation of electron transport activities in *C. reinhardtii*.

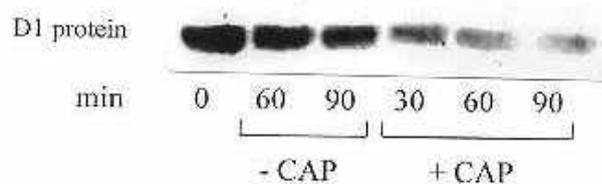
Electron transport reaction	Electron transport activity (% of control)			
	Time (min)			
	30	60	90	120
<b>PS II (<i>in vivo</i>)</b>				
H <sub>2</sub> O-PBQ	72 ± 6.5	48 ± 5.4	28 ± 3.5	24 ± 2.8
H <sub>2</sub> O-PBQ (cells exposed to UV-B in the presence of CAP)	53 ± 6.0	27 ± 4.0	14 ± 2.2	9 ± 1.4
<b>PS II (thylakoid membranes)</b>				
H <sub>2</sub> O-DCPIP	65 ± 3.2	40 ± 2.8	21 ± 2.0	17 ± 3.5
DPC-DCPIP	73 ± 4.5	50 ± 4.2	30 ± 4.0	26 ± 3.8
<b>PS I (thylakoid membranes)</b>				
Asc/DCPIP – MV; + DCMU	94 ± 3.0	90 ± 2.8	87 ± 4.8	85 ± 4.5

The cells were exposed to  $10 \text{ W m}^{-2}$  UV-B +  $150 \mu\text{mol m}^{-2} \text{ s}^{-1}$  PPF. The initial rates for H<sub>2</sub>O to PBQ and H<sub>2</sub>O to DCPIP reactions were  $154 \mu\text{mol O}_2 \text{ evolved mg}^{-1} \text{ Chl h}^{-1}$  and  $94 \mu\text{mol DCPIP reduced mg}^{-1} \text{ Chl h}^{-1}$  respectively. The PS II rates in the thylakoids of the control cells both with and without DPC were identical. The control rate for PS I reaction was  $380 \mu\text{mol O}_2 \text{ consumed mg}^{-1} \text{ Chl h}^{-1}$ .



**Figure 2.** UV-B induced degradation of the D1 and D2 proteins. Cells were pulse-labelled in the presence of  $10 \mu\text{g ml}^{-1}$  cycloheximide. Proteins visualized in aliquots drawn at 30 min interval were loaded in the lanes 1 to 4, following exposure to  $10 \text{ W m}^{-2}$  UV-B +  $150 \mu\text{mol m}^{-2} \text{ s}^{-1}$  PPF. The control samples chased under the condition of PPF ( $150 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ) alone did not show significant degradation of the two proteins (data not presented). The amount of radiolabel in D1 and D2 proteins at different time intervals was calculated relative to their initial level of '100%' at time zero.

$350 \mu\text{mol m}^{-2} \text{ s}^{-1}$ . The cells kept for reactivation in the dark did not show any recovery even after prolonged period of incubation (figure 5). Recovery under limiting and saturating PPFs showed divergent trends but inactivated photosynthesis was completely recovered in all the cases



**Figure 3.** Immunoblot showing steady state level of D1 protein in the thylakoid membranes isolated from the cells exposed to  $10 \text{ W m}^{-2}$  UV-B +  $150 \mu\text{mol m}^{-2} \text{ s}^{-1}$  PPF in the presence or absence of  $100 \mu\text{g ml}^{-1}$  CAP.

within one hour. Photorepair of UV-B damage of photosynthesis at PPFs higher than  $250 \mu\text{mol m}^{-2} \text{ s}^{-1}$  resulted into slow and incomplete reactivation (figure 5).

Since chloramphenicol increased susceptibility of the alga during UV-B inactivation, its effect was also studied on the photorepair of UV-B inactivated photosynthesis. Photoreactivation of UV-B inhibited photosynthesis was completely stopped by this drug (figure 5). Addition of cycloheximide or rifampicin, however, did not affect the process of photoreactivation (data not shown).

To identify the polypeptides synthesized during photorepair of UV-B damage of photosynthesis, 50% UV-B inactivated *Chlamydomonas* cells were simultaneously pulse labelled with  $\text{H}_2^{35}\text{SO}_4$  and chased in the course of photoreactivation. An indication of a time dependent increase in the incorporation of  $^{35}\text{S}$  in the D1 (32 kDa) and D2 (34 kDa) proteins was observed in the course of photoreactivation (figure 6). Increase in radiolabelling of the two PS II proteins was well correlated with the kinetics of restoration of the PS II activity as well as the

photosynthetic O<sub>2</sub> evolution. A trace amount of radiolabel was also detected in the D1 protein in the cells photo-reactivating in the presence of chloramphenicol (data not shown). Labelling of the D1 protein in the presence of cycloheximide was equal to or slightly more than that observed in its absence. Very low amount of radiolabelling of the D1 protein occurred when reactivation was carried out in the dark (data not shown).

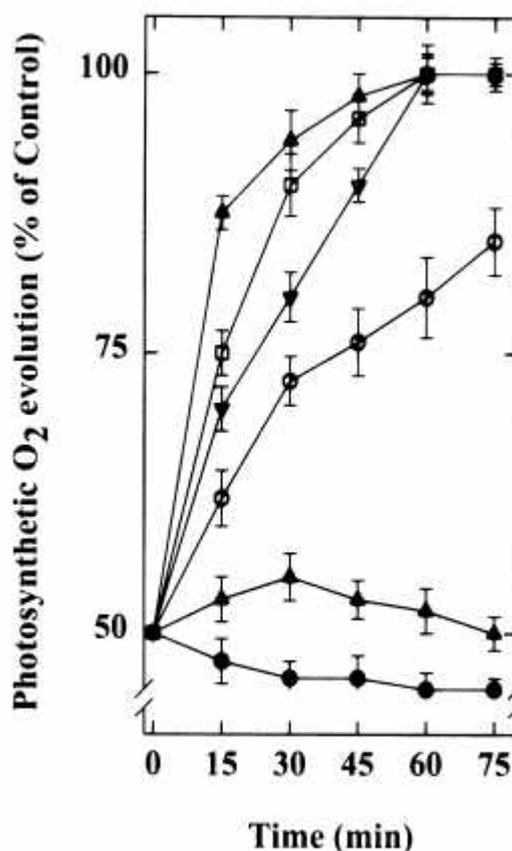
#### 4. Discussion

The time and intensity dependence of inactivation of photosynthesis in *C. reinhardtii* followed a pattern similar to photoinhibition of photosynthesis induced by strong visible light (Kyle and Ohad 1986). In both the cases structural and functional damages take place at the PS II (Aro *et al* 1993). UV-B damage of PS II has been shown to be associated with oxidizing as well as reducing side (Noorudeen and Kulandaivelu 1982; Renger *et al* 1989; Melis *et al* 1992). In the present study, UV-B mainly interfered with the electron transport from water to DCPIP. Since DPC which donates electrons to PS II by bypassing the water oxidation side restores the PS II activity partially in the thylakoid membranes isolated from the UV-B treated cells, it may be argued that UV-B predominantly affects the acceptor side of the PS II. A marginal effect of UV-B on the donor side of PS II preceding DPC donation, however, cannot be ruled out.

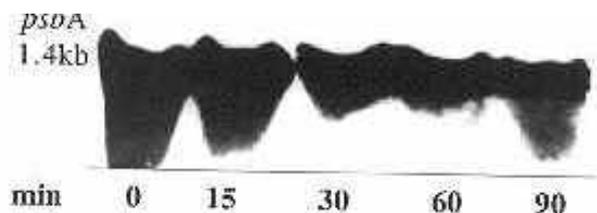
The kinetics of photoreactivation in *C. reinhardtii* exposed to UV-B in the present study is similar to that of the alga exposed to photoinhibitory light (Singh *et al* 1996). The reactivation of photoinhibition of photosynthesis induced by visible light mostly takes place in low light (Singh *et al* 1996) while photoreactivation of UV-B inactivated photosynthesis in the present study is accomplished even in the saturated light. Cycloheximide and rifampicin insensitivity of the recovery of UV-B damage suggests that synthesis of nuclear encoded proteins and chloroplast transcriptions are not involved in UV-B inactivation of photosynthesis and its photoreactivation. That the chloroplast protein synthesis is an essential and

regulatory component of the process of photoreactivation is apparent from the failure of recovery in the presence of chloramphenicol. The enhanced inactivation of photosynthesis in *C. reinhardtii* if chloramphenicol is added in the beginning of UV-B treatment suggests that the process of photoreactivation is functioning concurrently in the course of UV-B inactivation of photosynthesis.

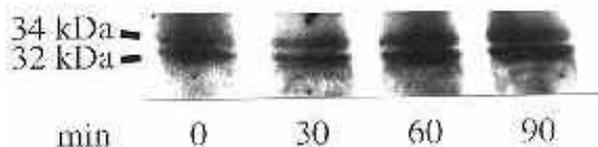
The present study revealed degradation of two bands of the thylakoid membrane proteins of molecular mass of 32 and 34 kDa. One of these proteins was identified as D1



**Figure 5.** Reactivation of photosynthesis in the cells exposed to 10 W m<sup>-2</sup> UV-B + 150 μmol m<sup>-2</sup> s<sup>-1</sup> PPFD. Reactivation of photosynthesis was carried out by incubating 50% UV-B inactivated cells under PPFDs of 50 μmol m<sup>-2</sup> s<sup>-1</sup> (▲), 150 μmol m<sup>-2</sup> s<sup>-1</sup> (□), 250 μmol m<sup>-2</sup> s<sup>-1</sup> (○), 350 μmol m<sup>-2</sup> s<sup>-1</sup> (○), 150 μmol m<sup>-2</sup> s<sup>-1</sup> + 100 μg ml<sup>-1</sup> CAP (▲), and dark (●).



**Figure 4.** Northern blots showing steady levels of *psbA* transcript in the cells exposed to 10 W m<sup>-2</sup> UV-B + 150 μmol m<sup>-2</sup> s<sup>-1</sup> PPFD for different durations of time.



**Figure 6.** Incorporation of radiolabel in the D1 and D2 proteins in the course of photo-reactivation of UV-B inactivated photosynthesis.

protein (32 kDa) of the PS II reaction center by immunoblotting. The 34 kDa protein band more seemingly represented the D2 protein. This, however, could not be confirmed in the present study due to non-availability of antibody to D2 protein. UV-B induced degradation of D1 and D2 proteins has recently been reported in certain studies (Greenberg *et al* 1989; Jansen *et al* 1996; Ihle 1997). The D2 protein during photoinhibition of photosynthesis is relatively stable and its degradation *in vivo* is quite low (Mattoo *et al* 1984; Greenberg *et al* 1989).

The increase in the level of incorporation of radioactivity in the 32 and 34 kDa proteins during photoreactivation indicate that the *de novo* synthesis of two reaction centre proteins was an essential step in the repair of UV-B induced damage of photosynthesis. Sass *et al* (1997) by using antibody to D1 and D2 proteins observed an increase in the levels of both the proteins in the course of recovery of UV-B inactivated photosynthesis in the cyanobacterium *Synechocystis*.

The present study showed that in spite of severe reduction in PS II activity, a relatively low reduction occurred in the amount of D1 protein. This observation along with the fast and almost complete loss of PS II activity accompanying concomitant disappearance of D1 protein in the presence of chloramphenicol further supports the simultaneous operation of degradation and synthesis involving the D1 protein. The steady state level of suppressed photosynthesis observed with moderate UV-B treatment in the present study therefore indicates a balance between degradation of D1 protein and its synthesis.

The reduction in the level of *psbA* transcript in the UV-B exposed *C. reinhardtii* is in contrast to that of the cyanobacterium *Synechocystis* where large accumulation of *psbA* mRNA occurs (Kanervo *et al* 1993; Constant *et al* 1995) during photoinhibition of photosynthesis by visible light. In higher plants, reduction in the level of *psbA* transcript is reported to occur in UV-B exposed pea (Jordan *et al* 1991), and wheat (Chaturvedi *et al* 1998). The role of transcription of genes encoding the D1 protein in the overall process of inhibition of photosynthesis and its reactivation is uncertain as photoreactivation proceeds even in the presence of transcription inhibitor rifampicin.

From the preceding discussion it is apparent that the basic mechanisms involved in the overall process of visible light and UV-B inactivation of photosynthesis and its reactivation are somewhat similar. It has, however, been suggested that different photosensitizers are involved in different spectral regions (Greenberg *et al* 1989; Jansen *et al* 1996). The molecular targets of UV-B damage in the thylakoid membranes are yet to be ascertained. UV-B, however, damages both the D1 and D2 proteins of PS II reaction center and the restoration of PS II activity requires *de novo* synthesis of the D1 and D2 proteins (Sass *et al* 1997). The degradation of other PS II core proteins including CP 47 and change in heterogeneity can not be ruled out. The apparent photosynthesis that observed in

UV-B treated *C. reinhardtii* is the net result of damage and repairs involves degradation and resynthesis of both the D1 and D2 proteins. The moderate reduction in the steady state level of D1 protein when PS II activity of UV-B exposed *C. reinhardtii* reduces considerably, suggests that UV-B does affect the synthesis of the D1 protein. This may also be true for the D2 protein. However, retardation in the synthesis of D1 and D2 proteins in the course of UV-B exposure may not be the sole limiting component for the simultaneous process of recovery induced by visible light. In fact the process of reactivation besides degradation and resynthesis of the D1 and D2 proteins also involves their insertion into PS II complex, religation of cofactors, assembly of subunits and finally activation of PS II. It appears that UV-B induces multiple lesions in the vicinity of the PS II complex and impairs these post translational processes which in turn become rate limiting factors for the repair of PS II.

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