INVOLVEMENT OF PROTEASES, CYCLIC NUCLEOTIDES AND SMALL HEAT SHOCK PROTEINS IN PSII REPAIR IN *SYNECHOCYSTIS* sp. PCC6803

PhD Thesis

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CONTENTS

ABBREVIATIONS

APS- ammonium persulphate

ATP- adenosine triphosphate

A_{650}, A_{665}- chlorophyll absorption at 650 and 665 nm

BCIP- 5-bromo-4-chloro-3-indolyl phosphate

BME- *β*-mercapto-ethanol

cAMP- 3′, 5′-cyclic adenosine monophosphate

cGMP- 3′, 5′-cyclic guanosine monophosphate

CAT- catalase

Chl- chlorophyll

D1, D2- proteins of the PSII reaction center

DCBQ- 2,5-dichloro-*p*-benzoquinone

DCMU- 3(3,4- dichlorophenyl)-1,1-dimethylurea

Deg- degradation of periplasmic proteins

DM- *n*-dodecyl-*β*,D-maltoside

DMBQ- 2,5-dimethyl-*p*-benzoquinone

DMSO- dimethyl-sulfoxide

FtsH- filamentation temperature sensitive

GM- grinding medium (for thylakoid membranes isolation)

GSH- glutathione

HSP- heat shock proteins

sHSP- small heat shock proteins

Hsp17- 16.6 kDa heat shock protein

MES- 2-(N-morpholino) ethansulfonic acid

MGDG- monogalactosyldiacylglycerol

Na_2EDTA- ethylene-diamine-tetraacetate (disodium salt)

NBT- nitro-blue-tetrazolium

NO- nitric oxide

OD- optical density

OE33- lumenal 33 kDa protein of the oxygen-evolving complex

OEC- oxygen evolving complex

P_{680}- photosystem II reaction center chlorophyll

P_{700}- photosystem I reaction center chlorophyll

PAGE- polyacrylamide gel electrophoresis

p-BQ- *p*-benzoquinone

PG- phosphatidylglycerol

PMSF- phenyl-methyl-sulfonyl-fluorid

PSII- Photosystem II

PSI- Photosystem I

SDS- sodium dodecyl sulphate

SOD- superoxide dismutase

SQDG- sulfoquinovosyldiacylglycerol

TEMED- N,N,N',N'-tetramethyl-ethylenediamine

Tris- Tris(hydroymethyl)aminomethane

TyrD, Y_D- tyrosine-161 of the D2 protein, a slow electron donor to P_{680}

TyrZ, Y_Z- tyrosine-161 of the D1 protein, the immediate donor to P_{680}

UV-A,B,C- ultraviolet radiation emitted between 320-400 nm(A), 280-320 nm(B) and 200-280 nm (C)

Q_A, Q_B - primary and secondary quinone electron acceptors in PSII

1. INTRODUCTION

1.1. Structure of photosynthetic apparatus

The photosynthetic apparatus is located in the thylakoid membrane, the internal membranes of chloroplast and cyanobacteria. In algae and higher plants the thylakoid membrane is organized in grana and stroma regions which represent folded and outstretched regions of the membrane, respectively. In prokaryotes like cyanobacteria and prochlorophytes the thylakoid membrane is a closed membrane system located in the soluble cytosol which encloses an interior aqueous phase, the thylakoid lumen.

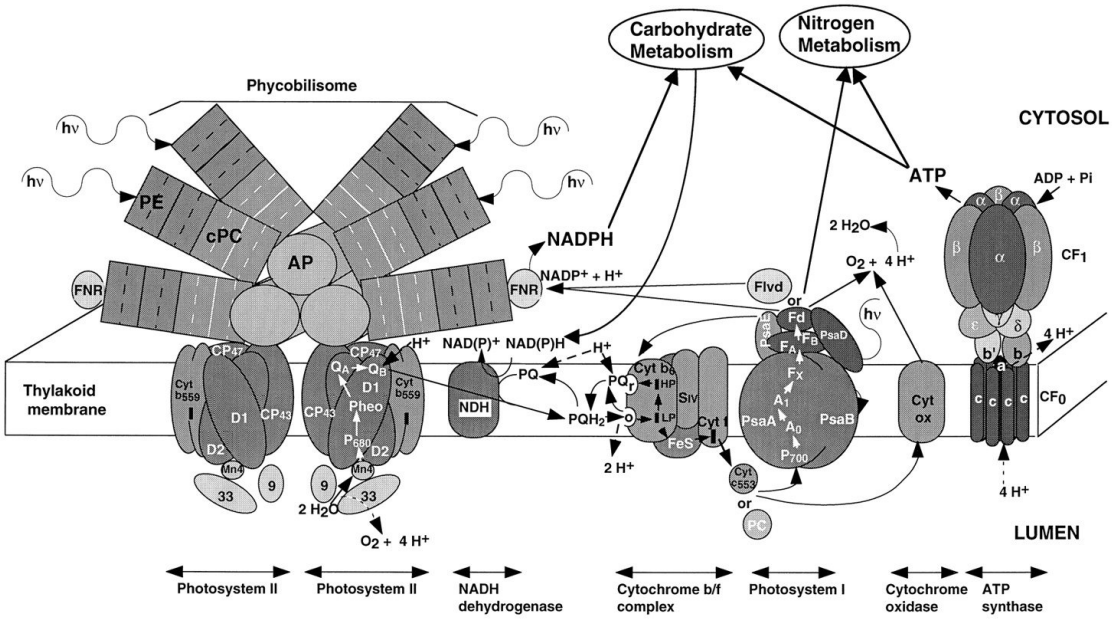

Fig. 1.1. Photosynthetic apparatus in cyanobacteria (Donald A Bryant 1994)

The thylakoid membrane - a unique assembly of protein, pigment and lipid molecules - accommodates the energy trapping and energy transduction functions. Four enzymatic complexes are involved in energy conversion (Fig.1.1): Photosystem II (PSII), cytochrome b_6f complex (cyt b_6f), Photosystem I (PSI) and ATP synthase.

Both Photosystems I and II consist of a reaction center (RC) carrying redox cofactors of the electron transfer chain and surrounded by the light harvesting complexes. In the prokaryotic cyanobacteria and eukaryotic red algae, light harvesting is carried out primarily by a group of pigmented proteins, called phycobiliproteins, the constituents of a macromolecular complex called the phycobilisome (PBS). Although a PBS is composed of

hundreds of biliproteins and linker polypeptides, light energy absorbed anywhere within the particle is efficiently transferred towards a specific biliprotein, which functions as a terminal-energy emitter and transfers the energy to a RC. Reaction centers acting as "energy sinks" consist of several molecules of Chl *a* associated with a protein heterodimer, which bind most of the redox cofactors involved in the electron transport chain. Chlorophyll molecules at the heart of reaction centers absorb light at different wavelengths, 680 and 700 nm and are referred to as P_{680} and P_{700}, respectively.

In photosynthesis light is converted to chemical energy and this chemical energy is further used for the synthesis of glucose. These two phases are separated in time and in space: the conversion of light energy into redox energy takes place in the thylakoid membrane during the "light phase ", whereas the synthesis of glucose takes place in the stroma or in the cytosol, during the "dark reaction".

Once light hits the PSII, its energy is transferred to the pair of special chlorophyll molecules P_{680}, which become excited. As a result, an electron is translocated from P_{680} through an accessory chlorophyll and a pheophytin molecule to the tightly bound quinone electron acceptor, Q_A; this is followed by the reduction of a mobile quinone electron acceptor, Q_B. The oxidized P_{680} is reduced by an electron from water via the redox active tyrosine, Tyr-Z. Water oxidation is catalyzed by a cluster of four Mn ions, which undergo light-induced changes in their oxidation states, called S-states. The complex cycles through five S-states denoted as $S_0 \ldots S_4$ and oxygen is released during the $S_3 \rightarrow S_4 \rightarrow S_0$ transition. After two photochemical cycles, the doubly reduced Q_B (Q_B^{2-}) takes up two protons from the stroma to form Q_BH_2 and then it is released into the bilayer lipid to be replaced by an oxidized quinone (PQ) from the membrane quinone pool. PQH_2 passes the electrons to the cyt b_6f complex and then to plastocyanin (PC). PC transports the electron to PSI and reduces oxidized P_{700}. PSI, in turn, reduces $NADP^+$ to NADPH via the action of ferredoxin and ferredoxin-NADP reductase. During the electron transfer reactions protons are transported from the stromal side of the membrane toward the lumenal side. At the same time, the process of water splitting also releases protons into the lumen. This creates a pH-gradient across the thylakoid membrane, which drives the synthesis of ATP via ATP-synthase.

Through these processes, the light reactions of photosynthesis have trapped solar energy and used it to synthesize the highly energetic compounds NADPH and ATP. These are then transported to other parts of the cell, where they are used in the dark reactions of photosynthesis to reduce CO_2 to carbohydrates.

While light is essential for photosynthesis, when absorbed in excess of the photosynthetic capacity it is harmful, creating high light (HL) stress that can lead to photodamage of the function and structure of PSII. The main mechanisms of PSII photoinhibition are the acceptor- and donor-side mechanisms; visible light can lead to both, but the main consequence of UV-B radiation is the donor-side inhibition.

1.2. PSII damage

1.2.1. Photodamage by UV-B radiation

UV-B light is an important contributor to the irradiation budget (8-9% of total solar radiation) but does not drive efficiently the photosynthetic process. However, due to its high energy content it has many negative effects on terrestrial and aquatic biosphere. Research on the effects of UV-B radiation is boosted by the increasing concern of the diminishing ozone layer and the consecutive increase in the UV flux at the Earth surface. The increase in the solar flux of UV-B combined with climatic changes due to the global warming are affecting terrestrial ecosystems of the temperate (Caldwell et al. 2007) and polar regions (Rozema et al. 2005) and also the ecosystems of aquatic organisms (Häder et al. 2007). UV-B stress is a main issue for agriculture influencing the growth, yield and biomass of main crop species: wheat, rice, soybean (Teramura 1983; Teramura et al. 1994) and maize (Gao et al. 2004).

UV radiation covers the 200-400 nm region of the spectrum and is divided into three spectral regions: UV-C, UV-B and UV-A. The UV-C band is defined between 200 and 280 nm and has no biological relevance since is very efficiently filtered by the atmosphere. The UV-B band comprises the 280-320 nm regions and has been attributed to a large range of detrimental effects on biological systems. UV-A, with wavelengths between 320 and 400 nm, reaches the Earth surface without being absorbed by the ozone layer. The effects of UV-A irradiation are less damaging than those of UV-B, at the same energy dose, but new results point to an ameliorating effect of UV-A radiation over the UV-B induced damage through the activation of xanthophyl cycle and/or maintaining a constant level of β- carotene in the chloroplasts of irradiated plants (Joshi et al. 2007).

UV-B radiation is absorbed by the majority of essential biological compounds: nucleic acids, proteins, pigments and lipids (Stapleton 1992). High intensity UV-B radiation damages almost all components of the photosynthetic apparatus (reviewed by Vass et al. 2001). Inhibition of photosynthetic activity following UV-B irradiation might be the result of the destruction of chloroplast ultrastructure (reviewed by Holzinger et al. 2006) or of direct

damage of key components of PSII such as D1 and D2 proteins (Vass 1996). Other effects of UV-B radiation include: loss of the photosynthetic pigments (Lutz et al. 2005), damage of the Rubisco enzyme (Bischof et al. 2002) and a general decrease of mRNA transcripts for photosynthetic complexes (Mackerness et al. 1999).

The most UV-B susceptible parts of PSII are donor side components like the CaMn cluster of water oxidation (Vass et al. 1999), Tyr_Z and Tyr_D (Vass et al. 1996), but also quinones Q_A and Q_B from the acceptor side (Melis et al. 1992; Hideg et al. 1993). UV-B radiation damages the D1 and D2 proteins almost to the same extent and the repair process includes de novo synthesis of both subunits (Greenberg et al. 1989; Melis et al. 1992; Sass et al. 1997). In isolated preparations UV-B treatment promotes the degradation of D1 and D2 proteins via non-enzymatic reactions (Friso et al. 1994a; Friso et al. 1994b; Friso et al. 1995).

1.2.2. Photodamage by visible light

Inactivation of electron transport by visible light can be located at the acceptor- or at the donor side of PSII and results in increased D1 protein turnover (Aro et al. 1993). In acceptor-side photoinhibition Q_B and the plastoquinone pool become fully reduced and it can produce relatively stable double-reduced Q_A^{2-} molecules. The presence of reduced Q_A species facilitates the formation of Chl triplets, which in the presence of oxygen readily react to produce singlet oxygen (Vass et al. 1992; Hideg et al. 1994a) that can damage the D1 protein. Triplet P680 can also be formed through recombination of Q_A^- or Q_B^- with the positively charged S_2 or S_3 states of the water oxidizing complex leading to singlet oxygen mediated damage of PSII (Keren et al. 1997; Szilárd et al. 2007). However, double reduction of Q_A has not been seen under aerobic conditions (Vass et al. 1993). Vass et al. (2007) have recently proposed a new form of acceptor-side hypothesis, which is based on the fact that singlet oxygen is produced during photoinhibition (Hideg et al. 1994a; Hideg et al. 1998; Hideg et al. 2001; Vass et al. 2007). It is suggested that in the presence of oxygen, Q_A is stably reduced producing singlet oxygen via charge recombination reactions between $Pheo^-$ and $P680^+$, which leads to the damage of PSII (Vass et al. 2007).

In the donor-side type of photoinhibition impairment of the electron pathway between the CaMn cluster and P680 leads to stabilized $P680^+$ and $TyrZ^+$ cations, which in turn can oxidize the surrounding environment (Andersson et al. 2001). This type of inactivation does not lead to the production of singlet oxygen but hydroxyl radicals are formed (Hideg et al. 1994b). Donor-side photoinhibition has been directly observed after chemical inactivation of

oxygen evolving complex (OEC) (Chen et al. 1995). Under natural conditions, where both visible and UV-B light occur, inhibition of PSII donor-side by UV-B radiation may trigger donor-side induced photoinhibition by visible light (Sicora et al. 2003). Recently it has also been proposed that the blue component of visible light can directly damage the CaMn cluster and induce donor-side type photoinhibition (Hakala et al. 2005; Ohnishi et al. 2005).

As mentioned earlier, singlet oxygen is the main damaging species generated during different types of stresses and it targets especially the D1 protein. The damaged protein has to be removed and replaced by a newly synthesized copy. The removal of the D1 polypeptide is most likely triggered by a conformational change within PSII (Andersson et al. 2001).

1.3. The D1 and D2 proteins

The protein backbone of the PSII reaction center is consisted of a heterodimer of the homologous D1 and D2 subunits. D1 and D2 bind all the essential redox components of PSII required to transfer the electrons from manganese cluster of the water-oxidizing complex to the plastoquinone pool: P_{680} (the primary electron donor), pheophytin (the primary electron acceptor), the Q_A and Q_B quinone electron acceptors, as well as the redox-active Tyr-Z and Tyr-D amino-acid residues. It also harbors the CaMn cluster (Mn_4Ca) and its cofactors (Ca^{2+}, Cl^-).

D1 and D2 are integral membrane spanning proteins with five transmembrane helices. Their C-termini are oriented towards the lumen of the thylakoid membrane and the N-termini oriented toward the cytosol (Fig.1.2). The molecular mass of the D1 and D2 proteins, estimated from their mobility in SDS-polyacrylamide gels (Marder et al. 1987), is 32 and 34 kDa, respectively.

In cyanobacteria the D1 and D2 proteins are encoded by small multigene families of the *psbA* (1-5) and *psbD* (1-2) genes, respectively (Golden 1995). By contrast, in plants and eukaryotic algae, *psbA* exists as a single copy in the chloroplast genome. In *Synechocystis* PCC6803 there are 3 *psbA* genes (Williams 1988). While *psbA1* is not expressed in this strain, *psbA2* and *psbA3* encode identical proteins but are differentially expressed in response to external stimuli like UV-B (Máté et al. 1998): the transcript from *psbA2* accounts for 90% of the total *psbA* pool transcript under normal conditions (Mohamed et al. 1993) but UV-B exposure determines a 20-30-fold increase in the transcript pool of *psbA3*.

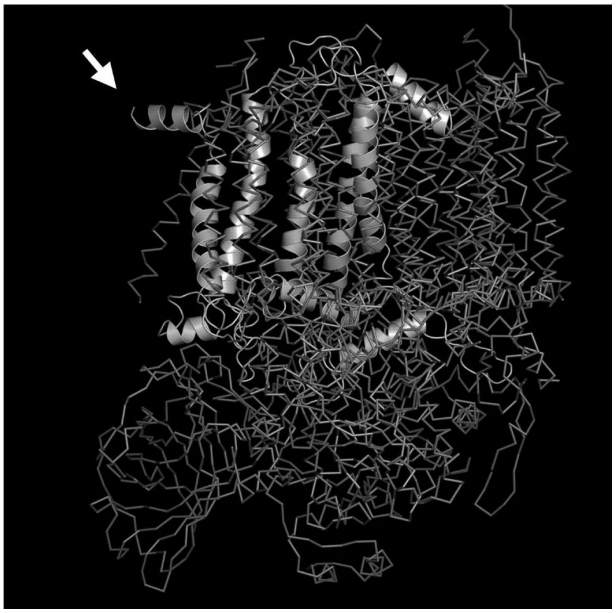

Figure 1.2. Crystal structure of PSII from Thermosynechococcus elongatus showing the N-terminal region of the D1 subunit exposed at the periphery of the complex. The D1 polypeptide chain is shown only in one of the PSII monomers in the dimer. The image is modeled from the coordinates determined by Ferreira et al. (2004). The first nine residues of D1 could not be resolved in the structure. Amino acid residues 11 to 20, forming the parallel helix protruding from the structure, are indicated by the arrow (Komenda et al. 2007).

In *Synechococcus* sp. PCC7942 the three *psbA* genes encode two distinct D1 isoforms (Golden et al. 1986; Clarke et al. 1993a; Clarke et al. 1993b; Campbell et al. 1995). Under environmental stress conditions such as high light (Bustos et al. 1990; Clarke et al. 1993b), blue light (Tsinoremas et al. 1994), low temperature (Campbell et al. 1995; Sippola et al. 1998), UVB (Campbell et al. 1998), or oxygen depletion (Campbell et al. 1999) *psbA* expression is altered to selectively exchange the D1:1 isoform encoded by *psbA1* with the D1:2 isoform, encoded by *psbA2* and *psbA3*. Mutant strains of *Synechococcus* PCC7942 in which the exchange of D1:1 to D1:2 is blocked suffer enhanced inhibition under UV-B (Campbell et al. 1998) showing that the two isoforms are functionally distinct.

Further examples for light and UV dependent differential *psbA* regulation have been observed in *Anabaena* sp. PCC7120 (Sicora et al. 2006) and *Gloeobacter violaceus* PCC7421 (Sicora et al. 2007), both having five *psbA* genes, which encode 3 different D1 protein isoforms, as well as in *Thermosynechoccus elongatus*, which has 3 *psbA* genes and 2 D1 protein isoforms (Kós et al. 2006; Kós et al. 2008).

There is a significant difference between cyanobacteria and higher plants concerning the regulation of the *psbA* genes. In plants, the D1 protein is encoded by the single copy plastid *psbA* gene and translated on thylakoid membrane-bound polysomes. Regulation of *psbA* gene is post-transcriptional at the level of translational elongation. Light, optimal electron transfer and availability of assembly partners are limiting factors in the translational elongation step of D1 protein synthesis (Zhang et al. 2002). By contrast, in cyanobacteria, the main regulation of D1 synthesis is at the transcriptional level, and the exchange of D1 protein isoforms is induced by environmental factors. In *Synechococcus* PCC7942, the involvement of regulatory levels other than transcription was suggested by data showing that almost no D1:1 proteins accumulated in thylakoid membranes after long high-light treatments or exposure of cells to UV-light (Campbell et al. 1998; Sippola et al. 2000), despite the presence of high amounts of *psbA1* mRNA.

Translation of *psbA* mRNAs proceeds similarly for both plants and cyanobacteria and begins on cytosolic ribosomes, followed by targeting of the ribosome-nascent D1 polypeptide chain complexes to the thylakoid membranes, where the D1 polypeptide is co-translationally inserted into the membrane and assembled into the PSII complex (Zhang et al. 1999). Recent results suggest that targeting, membrane export, and assembly of the D1 reaction center protein of photosystem II (PSII) might be performed by components of the cpSRP and cpSec pathways: cpSRP54, Alb3p, and cpSecY (components of import pathways of nuclear encoded proteins across the thylakoid membrane) (Nilsson et al. 1999; Zhang et al. 2001b; Ossenbuhl et al. 2004). Specifically, cpSRP54 was found to interact early with the nascent D1 protein (D1 fragments smaller than 17 kD) (Nilsson et al. 1999), whereas nascent D1 fragments between 17 and 25 kD were found in interaction with the translocase cpSecY (Zhang et al. 2001a). In *Synechocystis* 6803 the Oxa1/Alb3p homolog is essential for membrane integration of the D1 precursor protein pD1 (Ossenbuhl et al. 2006).

Although cyanobacteria usually contain only two different *psbD* genes, which encode identical D2 polypeptides their expression is also differentially regulated by light conditions. This has been demonstrated for *Synechococcus* PCC7942 as well as *Synechocystis* PCC6803. In both organisms the relative contribution of *psbD1* represents the dominating transcript under low light conditions, which is decreased on the expense of *psbD2* when the cells are exposed to high light (Bustos et al. 1992) or UV-B radiation (Viczián et al. 1999). The *psbD* operon of higher plant plastids is regulated transcriptionally through the activity of an upstream light promoter (Allison et al. 1995). In cyanobacteria the *psbD* gene is regulated, as

the *psbA* gene, mainly at the transcriptional level. In addition, it appears that D1 is co-translationally assembled with other PSII polypeptides like D2 or CP47 (Zhang et al. 1999).

In most oxygenic photoautotrophs the D1 polypeptide is synthesized with an 8 to 10 amino acid C-terminal extension that is removed after the insertion of D1 into the PSII complex in order to form the mature D1 (Nixon et al. 1992). For cyanobacteria and red algae the extension is 16 amino acids long and is processed to the mature protein by CtpA protease (Anbudurai et al. 1994).

One property unique to PSII, apart from water oxidation, is the rapid, light-induced turnover of D1 protein which takes place even under non-stress-light conditions with a half-life of 20-60 minutes and speeds up with increasing light intensity. During active photosynthesis the D1 and, to a lesser extent, the D2 proteins are degraded and replaced by newly synthesized polypeptides in the PSII repair cycle.

1.4. PSII repair

Although PSII is damaged by visible and UV-B light, damaged PSII complexes do not usually accumulate due to a rapid and efficient repair mechanism that operates in the thylakoid membrane. Crucial steps of the repair process are (Fig.1.3.):

- monomerization and partial disassembly of the PSII complex to allow access to the damaged subunits;
- degradation of the damaged D1 and D2 proteins with the involvement of proteases;
- signaling events leading to induction of the genes encoding the D1 and D2 proteins, and de novo synthesis of the proteins;
- religation of various extrinsic proteins and the photoactivation of CaMn cluster;
- reassembly and dimerisation of PSII complexes.

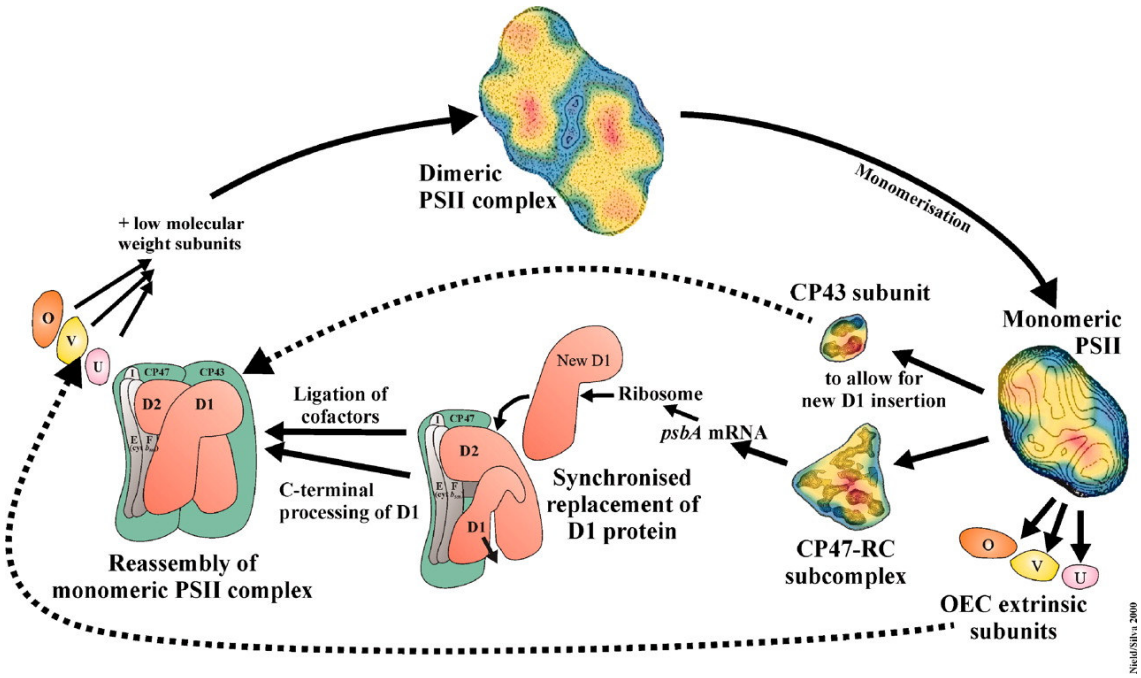

Fig.1.3. Model of the PSII repair cycle in Synechocystis PCC6803. A functional dimeric PSII complex undergoes a series of disassembly steps to allow the synchronized replacement of a damaged D1 subunit by a newly synthesized copy. The PSII complex is then reassembled and the water-oxidizing CaMn cluster photoactivated (Nixon et al. 2005).

In the following section we provide a brief description of the factors involved in the above-mentioned steps of PSII repair.

1.4.1. Proteolysis of D1 and D2 proteins

Regardless of the exact mechanism involved in photodamage of PSII, the process of D1 degradation is mediated by the action of specific or non-specific proteases. Identification of the proteases responsible for primary cleavage and secondary degradation of the D1 protein is currently a main topic of research on photoinhibition and repair of PSII.

Based on studies conducted *in vitro*, a model has been proposed in which damaged D1 is removed through the action of two proteases (Adam et al. 2002). DegP2, a serine protease, is proposed to perform the primary cleavage within the Q_B-binding pocket (Haussuhl et al. 2001) in a GTP-dependent process (Spetea et al. 1999). After this primary cleavage, the breakdown products are removed by one or more members of the FtsH (*F*ilamentation *t*emperature-*s*ensitive) protease family (Lindahl et al. 2000). However, *in vivo*

analysis of *var2-2 Arabidopsis thaliana* mutants which lack FtsH2 (a member of the FtsH family) has suggested that FtsH2 might be responsible for the primary cleavage of the D1 protein under high-light treatment and that FtsH2 is required for the efficient turnover of the D1 protein and protection against photoinhibition (Bailey et al. 2002). As chloroplasts have a prokaryotic origin, the proteases found in higher plants have their homologues in cyanobacteria. In *Synechocystis* PCC6803, the inactivation of one FtsH2 homologue (Slr0228) increased the sensitivity to high-light treatment. Furthermore, FtsH/Slr0228 was shown to bind to PSII and to be involved in the early steps of D1 degradation (Silva et al. 2003). FtsH2 is also involved in the heat-induced primary cleavage of the D1 protein of plants and cyanobacteria and the production of its corresponding fragments (Kamata et al. 2005; Yoshioka et al. 2006). In *Synechocystis* PCC6803, the documented role of the FtsH2 protease is not restricted to the selective turnover of only the D1 protein, but is also involved in the removal of unassembled PSII subunits and non-functional, partially assembled PSII complexes (Komenda et al. 2006).

FtsH proteases are ATP- and zinc-dependent metallo-type peptidases. Most avalaible information on this protease comes from the *E. coli* enzyme. Based on the X-ray crystallographic analysis, FtsH forms a homo-oligomeric hexameric ring (Krzywda et al. 2002) and substrate proteins are translocated through a central cavity in an ATP-dependent manner. FtsH has two transmembrane domains towards the N-terminus that anchor it in the plasma membrane, while the protease domain and the C-terminus face to the cytoplasm. FtsH proteases can interact with both membrane and soluble substrates, and their activity can be divided into two main categories: protein quality control by degradation of unassembled, unfolded and damaged proteins and regulatory function by degradation of short-lived regulatory proteins (like σ^{32}).

All prokaryotic genomes contain a single *ftsH* gene except photosynthetic cyanobacteria that contain 4 such genes (Mann et al. 2000). This number is further multiplied to different extent in higher plants: 12 *ftsH* genes in *Arabidopsis* (Sokolenko et al. 2002), 9 in rice (Yu et al. 2005), 18 in *Populus* (Garcia-Lorenzo et al. 2006). In *Synechocystis* PCC6803 inactivation of 2 *ftsH* genes proved to be lethal (*slr1390* and *slr1604*), one had no obvious phenotype (*slr1463*) (Mann et al. 2000) and the mutation of *slr0228* showed light-sensitive growth, impaired PSII repair and a slower rate of D1 degradation *in vivo* (Silva et al. 2003).

Nine of the 12 Arabidopsis *ftsH* genes reside in chloroplast (*ftsH*1, 2,5,6,7,8,9,11,12) and the remaining three in mitochondria (*ftsH*3, 4, 10) (Sakamoto et al. 2003). Of these, *ftsH*2 is by far the most abundant species, followed by *ftsH*5, *ftsH*8 and *ftsH*1 (Sinvany-Villalobo et

al. 2004). In terms of phylogenetic relations, *ftsH*1 and *ftsH*5 are duplicated genes, and so are *ftsH*2 and *ftsH*8. Each pair of duplicated genes constitutes a separate type of subunit and a functional FtsH complex is composed of subunits of type A (FtsH1 and FtsH5) and type B (FtsH2 and FtsH8). Within each type the subunits are interchangeable but in the absence of either type no active complex accumulates (Zaltsman et al. 2005b).

The variegated phenotype of *ftsH* mutants suggests that the FtsH protease is essential for chloroplast biogenesis. A knock-out strain of one of the four *ftsH* genes (*slr0228*) in *Synechocystis* PCC6803 resulted in impaired PSI activity and up to 60% reduction in abundance of functional PSI subunits, without affecting the cellular level of PSII or phycobilisomes (Mann et al. 2000). Based on genetic, biochemical and physiological analyses, the proposed functions of FtsH proteases in photosynthetic organisms are as follows: (i) major proteases involved in PSII repair (Bailey et al. 2002; Silva et al. 2003; Komenda et al. 2006); (ii) involved in thylakoid formation at an early step of chloroplast development (Zaltsman et al. 2005a).

Recent results of Komenda et al. (2007) suggest a new model of D1 degradation with emphasis on the interaction between N-terminus of the protein and the FtsH protease (Komenda et al. 2007). In *E. coli*, membrane protein degradation by FtsH protease is highly processive and it starts from either the N-or the C-terminus of the target molecule (Chiba et al. 2002). For N-terminal proteolysis, there is a structural requirement that the tail should be longer than 20 amino acid residues (Chiba 2000). In the case of D1 protein, the N-terminus is oriented toward the stromal side of the thylakoid membrane, on the same side with the proteolytic domain of FtsH protease (Fig.1.4). In recent crystal structures (Ferreira et al. 2004; Loll et al. 2005) the N-terminus of D1 protrudes from the cyanobacterial PSII complex (Fig.1.2). Its length and localization are ideal to engage in a proteolytic process with FtsH protease. In *Synechocystis* PCC6803, removal of 5 or 10 residues from the N-terminus resulted in blocked D1-synthesis while removal of 20 residues inhibited PSII repair and selective D1 degradation (Komenda et al. 2007). In the case of chloroplast, it has been suggested that D1 degradation by FtsH might be facilitated by cleavage of D1 by Deg proteases on the opposite lumenal side of the membrane (Kapri-Pardes et al. 2007; Sun et al. 2007).

Mutagenesis experiments have so far demonstrated the role of the FtsH/Slr0228 protease in PSII repair in cyanobacteria at an early stage in D1 degradation: the primary cleavage. However, it remains unclear to what extent the Deg proteases are important for D1 degradation *in vivo*. This is a crucial question to address since recent biochemical experiments

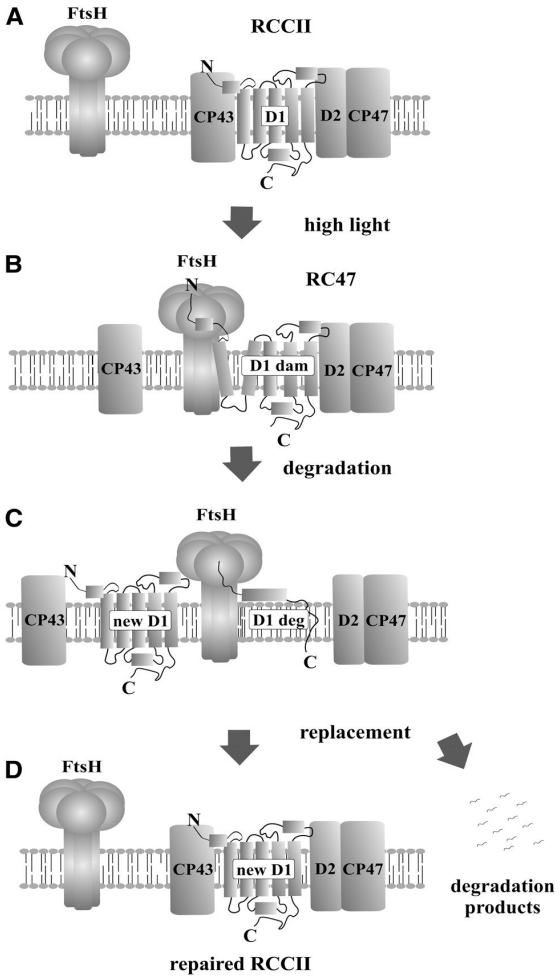

Fig.1.4. Selective replacement of D1 protein during PSII repair following photoinhibition. For clarity, just one of the monomers in the PSII dimer is shown, and the extrinsic and small transmembrane subunits of PSII are omitted. (A) Intact PSII core complex with the functional and correctly folded D1 protein. (B) High light–induced inactivation of PSII is followed by the release of CP43 and extrinsic proteins. In the resulting core complex lacking CP43 (RC47), the structure of damaged D1 protein (D1 dam) is destabilized, the protein is recognized by FtsH, and its released N terminus is caught by the protease. (C) The damaged D1 subunit is degraded (D1 deg) by FtsH processively from the N to the C terminus, releasing short oligopeptides but no distinct larger fragments. (D) Insertion of the new D1 molecule and reassembly of the active dimeric PSII core complex (RCCII) (Komenda et al. 2007).

have suggested that a homologue of Deg2 in *Synechocystis* PCC6803 extracts could be involved in cleaving D1 during PSII repair (Kanervo et al. 2003; Huesgen et al. 2005).

The DEG/HTR family proteases are ATP-independent Ser endopeptidases, which are present in both prokaryotes and eukaryotes (Adam et al. 2002; Huesgen et al. 2005). DEG/HTR proteases were initially identified in *E. coli* and named DegP (for *deg*radation of *p*eriplasmic proteins) or HtrA (for *h*igh *t*emperature *r*equirement), DegQ (HhoA) and DegS (HhoB) (Clausen et al. 2002). PDZ-like domains at their C-termini are interesting structural features of all these proteins (Ponting 1997). PDZ domains mediate protein-protein interactions and are important for substrate recognition and/or for the regulation of proteolytic activity (Wilken et al. 2004). Determination of its three-dimensional structure has revealed that it forms a hexamer made of two staggered trimers.

In *Synechocystis* PCC6803 there are three homologues of the Deg peptidase family: HtrA (DegP), HhoA (DegQ) and HhoB (DegS) (Sokolenko et al. 2002). However, the number of *deg* genes can vary between two and five in various cyanobacterial species (Huesgen et al. 2005).

HhoA has been found in the periplasm of *Synechocystis* PCC6803 and HtrA in the outer membrane (Huang et al. 2004). Analysis of the double or the triple Deg mutants has proven that the Deg proteases do not play an essential role in D1 turnover and repair *in vivo*, although they are required for photoprotection during heat and light stress (Barker et al. 2006).

Like other chloroplast proteases, Deg in *Arabidopsis* are encoded by multiple genes (16 genes) of which 4 are targeted to chloroplast (Peltier et al. 2002). DEG1, DEG5 and DEG8 were found in the thylakoid lumen, and DEG2 was peripherally attached to the stromal side of the thylakoid membrane. The Deg1 protease from *Arabidopsis* has been expressed in *E. coli*; this *in vitro* assay demonstrated the proteolytic activity of Deg1 against the non-physiological substrate β-casein and against thylakoid lumen proteins such as *in vitro* translated OE33 and plastocyanin. The proteolytic activity of recombinant Deg1 increased with temperature and had an optimum around pH~6 (Chassin et al. 2002). In a recent study, a mutant with reduced levels of Deg1 proved to be more sensitive to photoinhibition than the WT, accumulated higher levels of D1 protein and less of its C-terminal degradation products than in the WT (Kapri-Pardes et al. 2007). Moreover, it seems that the accumulation of Deg1 and FtsH proteases might be coordinated: the mutant containing less Deg1 also contained less FtsH protease and FtsH mutants contained less Deg1 (Kapri-Pardes et al. 2007).

Incubation of recombinant Deg2 with isolated plant thylakoid membranes pretreated with heat or high light intensity showed a selective degradation of the D1 protein (Haussuhl et al. 2001). The results of this *in vitro* study were not confirmed *in vivo*, since mutants lacking Deg2 protease showed the same rate of D1 degradation under the conditions of high light stress like the WT (Huesgen et al. 2007). The other two Deg proteases, Deg5 and Deg8, form together a protein complex which is not associated with PSII but it is localized in the thylakoid lumen. Individual inactivation of *deg5* and *deg8* genes resulted in increased sensitivity to photoinhibition. The double mutants *deg5deg8* showed the same sensitive phenotype and also impaired turnover of newly synthesized D1 protein (Sun et al. 2007). It seems reasonable to speculate that DEG could cooperate with FtsH in efficiently cleaving the multiple transmembrane D1 proteins from both sides of the thylakoid membrane (Sun et al. 2007).

1.4.2. *De novo* protein synthesis

Although the effects and consequences of UV-B radiation are known in details the mechanisms for sensing and responding to UV-B radiation are largely unknown. In higher plants recent results point to the role of *Arabidopsis thaliana* UV Resistance Locus8 (UVR8) protein as a UV-B–specific signaling component (Brown et al. 2005; Kaiserli et al. 2007). The signaling molecule NO has been shown recently to alleviate oxidative damage produced by UV-B irradiation by increasing the activity of SOD, CAT, peroxidase, the accumulation of GSH and elimination of O_2^- (Xue et al. 2007).

Another category of signaling molecules, cyclic nucleotides, govern the adaptation of cell to its surroundings in primitive organisms like bacteria and fungi but also in algae, plants and animals. The discovery by Earl Sutherland of cyclic nucleotides as the intracellular receptors of extracellular hormones and hence named “second messengers” awarded him with a Nobel Prize. Accumulating evidence of research carried out over the last three decades proved that cyclic nucleotides AMP (3′, 5′-cyclic adenosine monophosphate) and GMP (3′, 5′-cyclic guanosine monophosphate) represent the classic set of second messengers, effectors of extracellular signaling.

Cyclic nucleotides are derivatives of nucleic acids with three functional groups: an aromatic base (adenine or guanine), a sugar (ribose) and a phosphate. Cyclic nucleotides differ from other nucleotides in that the phosphate group is linked to 3′ and 5′ groups of the

ribose sugar and hence forms a cyclic ring. This cyclic conformation allows cAMP and cGMP to bind to proteins to which other nucleotides cannot.

cAMP signalling is very diverse: in *E.coli* cAMP binds to a dimer of the catabolite receptor protein (CRP, also known as catabolite activator protein) which requires the allosteric effector cAMP in order to bind efficiently to DNA (Kolb et al. 1993). In *E. coli* CRP activates transcription at more than 100 promoters, by binding to a well-conserved palindromic binding motif (TGTGAN6TCACA). In *Synechocystis* PCC6803 inactivation of the adenylyl cyclase or of its receptor protein Sycrp1 (*sll1371*) resulted in an apparently nonmotile phenotype (Yoshimura et al. 2002). Also, a blue light-cAMP signal cascade stimulates the motility of *Synechocystis* PCC6803 (Terauchi et al. 2004). In *Anabaena cylindrica* the intracellular cAMP concentration depends on the light quality: red light determines a rapid decrease in cAMP content and far-red light causes a rapid increase in its content (Yoshimura et al. 2002; Ohmori et al. 2002). It is worth mentioning that in cyanobacteria light signals are mediated by cAMP whereas in vertebrate visual cells it is the cGMP that transduces the photosignals. The role of cAMP as a second messenger is not restricted to light initiated cascades: low pH-high pH, oxic-anoxic and nitrogen depleted-repleted conditions change its cellular level. In marine diatoms the regulation of the cytosolic level of cAMP is a general mechanism that operates in CO_2 sensing and regulation of CCM (carbon concentrating mechanism) (Harada et al. 2006).

The role of cGMP is well established in the literature: together with Ca^{2+} it is involved in the phytochrome mediated induction of chalcone synthase gene and the development of chloroplast (Bowler et al. 1994), it is a second messenger for NO signaling in animals and plants by inducing defence-related genes (Durner et al. 1999).

Cyclic AMP and cyclic GMP are both present in eukaryotes, but prokaryotes possess only one class; the only exception is cyanobacteria. The cellular level of cyclic nucleotides is determined by the opposing activities of cyclases and phosphodiesterases :

-Adenylyl cyclases (catalyse synthesis of cAMP from ATP).

-Guanylyl cyclases (catalyse synthesis of cGMP from GTP).

-cNMP phosphodiesterases (catalyse breakdown of cyclic nucleotides)

In *Synechocystis* PCC6803, the components of the cGMP and cAMP signaling pathways identified are as follows: the adenylyl and guanylyl cyclase (Terauchi et al. 1999; de Alda et al. 2000a), cAMP-phosphodiesterase (Sakamoto et al. 1991), 2 hypothetical cNMP phosphodiesterases (de Alda et al. 2000b) and 5 proteins- possible receptors of cyclic nucleotides (de Alda et al. 2000b).

Since light of different qualities: red, far-red and blue light affects the level of cNMP, other wavelengths could be transmitted through the same mechanism. The documented role of cNMP as transducers of light with various wavelengths makes them a potential candidate for transducing UV-B signals. When light with a damaging potential is perceived it is very important that the defense mechanisms are rapidly induced through gene induction and protein synthesis. In the case of photosynthetic process affected by UV-B, the defense mechanisms require the induction of the genes for the proteolysis of damaged proteins and of the genes coding for the replacing of damaged subunits.

1.4.3. PSII reassembly

The striking features of the PSII complex are its susceptibility to damage and the consecutive repair and photoreactivation. Maintaining of PSII function requires the selective replacement of damaged subunits through degradation and resynthesis while the rest of the subunits in the complex are recycled. At the level of resynthesis and integration into the membrane of D1 protein there are few candidates: the D1 protein is synthesized as a precursor (pD1) with a 16 aminoacids carboxyl-terminal extension that is cleaved in two separate steps. The first cleavage is after Ala-352, resulting in formation of a processing intermediate termed iD1, which in *Synechocystis* PCC6803 is mainly associated with RC complexes (Komenda et al. 2004). The role of small subunits PsbI (Dobakova et al. 2007) and PsbH (Komenda et al. 2005) in D1 processing and integration into the membrane cannot be neglected. The Sec translocon (Zhang et al. 2001a) and the chaperone Hsp70 (Yokthongwattana et al. 2001) seem to be important factors during PSII reassembly. Under normal conditions of light intensity, temperature and solutes concentration PSII repair is a coordinated series of these intermediary steps. Different stress conditions like cold, heat and salt stress affect the photosynthetic activity by impairing the PSII repair (reviewed by Murata et al. 2006). Lower (Gombos et al. 1994b; Nishida et al. 1996) and higher temperatures (Gombos et al. 1994a) than the physiological ones modify the fluidity of the thylakoid membrane with direct consequences on the PSII repair cycle (Allakhverdiev et al. 2004). Against the heat inactivation of PSII function photosynthetic organisms protect themselves by rapidly synthesizing heat shock proteins (HSP) (Heckathorn et al. 1998; Heckathorn et al. 2002). Among these, Hsp17 small heat shock proteins (sHSP) have dual role: stabilize heat-stressed membranes and bind denatured proteins in the cytosol for subsequent chaperone-mediated refolding (Török et al. 2001).

The cyanobacterium *Synechocystis* PCC6803 has only one sHSP: Hsp17 (also known as Hsp16.6). Subtle changes in membrane physical order lead to the induction of *hsp17* gene to the same extent as the heat treatment, suggesting a role of the Hsp17 protein in membrane quality control. In the same experiment it was demonstrated that the newly synthesized Hsp17s are associated with the thylakoid membrane (Horvath et al. 1998). In terms of oxygen evolution rates and viability, inactivation of this gene rendered the mutant more sensitive to heat stress compared with the wild type (Lee et al. 2000b). Constitutive expression of a small heat shock protein from *Synechococcus vulcanus* in *Synechococcus* sp. PCC 7942 increased the thermal resistance of PSII and protected light-harvesting phycocyanin from heat-light induced photobleaching (Nakamoto et al. 2000; Nakamoto et al. 2006).

The Hsp17 protein in *Synechocystis* PCC6803 can antagonize the heat-induced hyperfluidization of membrane domains and thereby preserves the structural and functional integrity of biomembranes (Török et al. 2001).

The chaperone function of sHSP is temperature dependent: the oligomers dissociate into dimers, bind the heat denatured proteins and form the sHSP-denatured protein complex, preventing protein aggregation and insolubilization (Van et al. 2001). The sHSP-bound proteins can be refolded into their native state by the cascade action of the ATP-dependent chaperones DnaK/DnaJ/GrpE (the DnaK system) or GroEL/GroES (Lee et al. 1997; Lee et al. 2000a). Besides their documented role during heat stress, sHSPs can be induced by other stresses and can confer cross-tolerance, indicating a broader role for sHSPs (Fulda et al. 1999).

The structure of sHSPs is defined by a conserved α-crystallin domain with high similarities to the α-crystallin of the vertebrate eye lens, flanked by an N-terminal region of variable length and sequence and by a short C-terminal extension (Kappe et al. 2002). sHSPs, with molecular masses of the monomers ranging from 16-42 kDa, are usually found in the cells as large oligomers of 12 to 32 subunits, depending on the type of sHSP (Stamler et al. 2005). sHSPs are ubiquitous in terms of cellular localization; they can be found in the cytoplasm, nucleus, chloroplast, mitochondria and endoplasmic reticulum in higher plant cells (Boston et al. 1996).

2. AIMS OF THE STUDY

In our experiments we tried to identify factors and their contribution to the repair of UV-B damaged PSII reaction center in the cyanobacterium *Synechocystis* PCC6803. The aims of our work were:

1. To identify which protease is involved in the degradation of the UV-B damaged D1 and D2 proteins of the PSII reaction center complex. Therefore, we took advantage of a series of mutants for two different families of proteases: Deg and FtsH.
2. The open reading frame *slr2100* is a proposed cNMP phosphodiesterase because it carries a HD domain. The questions we addressed were: what is the *in vivo* function of this gene? Do cyclic nucleotides play a role in the signaling pathways of PSII repair?
3. Due to the preferential and selective association of the mutated Q16R-Hsp17 protein with the thylakoid membrane after heat shock our goal was to verify if this event confers increased resistance to PSII damage or facilitates the recovery/repair from UV-B damage.

3. MATERIALS AND METHODS

3.1. *Synechocystis* PCC6803 growth conditions

The cyanobacterium *Synechocystis* PCC6803 was obtained from the Pasteur Culture Collection of axenic cyanobacterial strains. Cells were grown in liquid culture in an illuminated orbital shaking incubator (120 rpm) in BG11 medium (Rippka 1988) at 30º C, under a 5% CO_2-enriched atmosphere and 40 $\mu E \cdot m^{-2} \cdot s^{-1}$ white light intensity. Cyanobacterial growth was followed by recording the optical density at 580 nm. Cell preservation was done at -80 ºC, in 5% methanol or 8% DMSO.

The glucose tolerant strain of *Synechocystis* PCC6803 was used to construct the studied mutants (Williams 1988), in the laboratory of Prof. Peter Nixon (Imperial College London). The ΔFtsH/*slr0228* mutant was constructed by interrupting the *slr0228* gene with a chloramphenicol-resistance cassette (Silva et al. 2003). The three *deg* genes were inactivated stepwise using the plasmids described by Silva et al. (2002): first *hhoA*, then *hhoB* to generate the *hhoAhhoB* double mutant and finally the *htrA* to give the triple Δ*deg* mutant (Barker et al. 2006). The genes were interrupted by chloramphenicol, erythromycin and kanamycin-resistance cassettes, respectively.

3.2. Thylakoid isolation

Thylakoid membranes were prepared by breakage of the cells with glass beads (150-212 μm in diameter, Sigma) at 4 ºC followed by differential centrifugations according to (Komenda et al. 2004). Usually, 10 ml of cells were spinned down at 7000x*g* at room temperature for 10 minutes. The resulting pellet was resuspended in 1 ml of grinding medium (GM), pH 6.5, containing 50 mM MES, 5 mM Na_2EDTA, 1 mM benzamidine and 2 mM amino-caproic acid. The resuspended pellet was transferred to dark and centrifuged at 6500x*g*, at 4 ºC for 5 minutes followed by resuspension in 0.5 ml GM and transfer to Eppendorf with 0.5 ml glass beads. The mixture of cells and glass beads was beaten in a bead beater (Biospec Products, USA) at 4 ºC, 3x 90 sec with 1 minute interruption for cooling on ice. Beads were washed three times with 0.5 ml GM, aliquots were pooled and centrifuged at 6500x*g* at 4 ºC, 20 seconds, just to spin down the remaining glass beads and cell debris. Membranes were collected from the supernatant following 15 min centrifugation at 13000x*g*

at 4 ºC. The final sediment was resuspended in preparation medium, pH 7.5, containing 50 mM Tris and 1 M sucrose and stored at -80 ºC.

3.3. Chlorophyll content determination

Chlorophyll content of the isolated thylakoid membranes was determined in methanol. Various volumes of thylakoids were diluted in 100% methanol. Absorption of each sample was determined at 650 nm and 665 nm. Chl (a) concentration was calculated as:
Chl (a) = ($16.5 \times A_{665} - 8.3 \times A_{650}$) x dilution factor. The concentration was given in µg/ml units.

3.4. Ultraviolet and visible light treatment

UV-B irradiation experiments were carried out using a VL-215M (Vilbert-Lourmat, France) lamp with maximal emission at 312 nm in combination with 0.1 mm cellulose acetate filter (Clarfoil, Courtalouds Chemicals, UK) to exclude radiation shorter than 290 nm (UV-C). The UV-B intensity measured with a UV-B radiometer (9750300, Cole-Palmer) at the surface of the sample was ~4.5 $W \cdot m^{-2}$, corresponding to 12 $\mu E \cdot m^{-2} \cdot s^{-1}$. UV-B irradiation was performed in open-, square-shaped, glass-containers in which 100 ml cell culture of 6.5 µg Chla/ml formed 1 cm layer height, maintained in suspension by magnetic stirring. The temperature during the illumination was kept constant at 30 ºC. In some cases, a protein synthesis inhibitor, either lincomycin (300µg/ml) or spectinomycin (200µg/ml) was added to the cell culture.

Visible light illumination was performed during the recovery period after the UV-B irradiation and was produced by an array of halogen spot lamps in the 40-50 $\mu E \cdot m^{-2} \cdot s^{-1}$ intensity ranges.

3.5. Measurement of photosystem II electron transport activity

PSII electron transport activity was assessed by measuring the light-saturated rates of oxygen evolution from whole cells, in the presence of 0.5 mM 2,5-dimethyl-p-benzoquinone as electron acceptor, using a Hansatech DW2 O2 electrode. In each measurement, 1 ml of cells at 6.5 µg Chla /ml were used. The standard oxygen evolution of the WT *Synechocystis* PCC6803 measured in the presence of 0.5 mM 2,5 DMBQ at 40 $\mu E \cdot m^{-2} \cdot s^{-1}$ light intensity and 30˚C was ~200 µmol O_2/mg Chl/h.

Flash-induced increase and subsequent decay of chlorophyll fluorescence yield was measured by a double-modulation fluorometer (Photon System Instruments, Brno, Czech Republic) (Trtilek et al. 1997) in the 150 μs to 100 s time range, in samples which were dark adapted for 3 minutes prior to measurements, as described in (Vass et al. 1999). Multicomponent deconvolution of the measured curves was done by using a fitting function with three components, two exponentials and one hyperbolic as described earlier (Vass et al. 1999):

$F(t)- F_0 = A_1\exp(- t/T_1)+A_2\exp(-t/T_2)+ A_3/(1+t/T_3)$,

where $F(t)$ is the variable fluorescence yield, F_0 is the basic fluorescence level before the flash, A_1- A_3 are the amplitudes, T_1- T_3 are the time constants. The non-linear correlation between the fluorescence yield and the redox state of Q_A was corrected for by using the Joliot model with a value of 0.5 for the energy-transfer parameter between PSII subunits (Joliot et al. 1964).

3.6. Gel electrophoresis and immunoblotting

Thylakoid membranes were isolated from the samples irradiated for various periods of time and with various treatments, as described above. The isolated thylakoid membranes were solubilized in 0.313 M Tris-HCl buffer (pH 6.8) containing 3% (w/v) SDS, 6% (w/v) glycerol, 10% (v/v) BME and bromophenol blue (~0.001% w/v) for 15 minutes at 45ºC.

Protein composition of the solubilized thylakoids was assessed by SDS-PAGE (SDS-polyacrylamide gel electrophoresis) in a Tris-glycine buffer system of discontinous pH (LAEMMLI 1970). Gels containing 6 % (stacking gel) and 12-17 % linear gradient (separation gel) acrylamide were prepared from a stock solution of 60 % (w/v) acrylamide and 1.6 % (w/v) bis-acrylamide. The buffers used for the separation and stacking gels are: 0.8 M Tris-HCl (pH 8.83) containing 6 M urea and 0.1 M Tris-HCl (pH 6.8), respectively (Barbato et al. 1991). Chemical polymerization of the acrylamide/bis-acrylamide in the separation and stacking gels was achieved by the addition of 0.5 and 1 μl/ml TEMED, respectively, and 0.25 and 0.75 μl/ml APS, respectively. For electrophoresis, 0.02 M Tris, 0.2 M glycine buffer (pH 8.3) containing 0.1% (w/v) SDS was used. Electrophoresis on 1x16x14 cm slabs was performed with a constant current of 10 mA in the cold room for about 12-14 hours, until the bromophenol blue marker reached the bottom of the separation gel.

The thylakoid extracts, adjusted to 0.7-1 μg Chla per lane, were loaded and the gel was runned in the above described conditions. For the purpose of individual protein

recognition with the immunoblotting technique, the gels were soaked for 30 minutes in transfer buffer consisting of 3 mM Na_2CO_3, 10 mM $NaHCO_3$ and 10% (v/v) methanol (Dunn 1986). The resolved proteins were electroblotted onto nitrocellulose membranes (0.45 μm, Schleicher& Schuell, Germany) at a constant voltage of 25 V for 2 hours. For the blocking of the membrane we used 10% (w/v) skimmed milk in 0.01 M Tris-HCl buffer (pH 7.6) containing 0.15 M NaCl (TBS buffer) for 30 minutes at room temperature. To detect the D1 and D2 proteins, the membranes were incubated with the corresponding antibodies: D1 polyclonal antibody (from Agrisera) and D2 polyclonal antibody (from Peter Nixon) for 120 minutes at room temperature. The antibody dilution was 1:4000 for anti-D1 and 1:7500 for anti-D2. Immunoreacted bands were further immunodecorated with secondary antibody-alkaline phosphatase conjugate at a dilution ratio of 1:5000 in TBS buffer, for 60 minutes at 37ºC. The antigen-antibody complexes were visualized by colorimetric reaction using the BCIP-NBT system -0.165 mg/ml BCIP and 0.3 mg/ml NBT in 0.1 M Tris-HCl buffer (pH 9.8) containing 0.15 M NaCl and 0.5 M $MgCl_2$. The enzymatic reaction was stopped by washing the membrane with distillated water. The linearity of the immuno-response was checked by loading dilution series. The bands from the scanned blotts were quantified using a NIH program, ImageJ. Data are averages of at least three independent experiments.

4. RESULTS AND DISCUSSION

4.1. The role of FtsH and Deg proteases in the degradation of UV-B damaged D1 and D2 proteins

4.1.1. Effects of inactivating the *deg* and *ftsH* genes on PSII activity in UV-irradiated cells

The consequences of UV-B induced damage to the function and structure of PSII are known in details. In what concerns the protein damage, the D1 and D2 proteins of the PSII reaction centre are the most sensitive components. The PSII repair cycle proceeds in a stepwise fashion to remove the damaged protein components and ensure their replacement with newly synthesized, functional copies. Proteolytic removal of the damaged D1 and D2 subunits is the first step of the repair process and the details are under careful scrutiny in many laboratories working on the topic. FtsH and Deg proteases are the main candidates for this role, due to their conserved role in nature in the degradation of damaged or unassembled proteins.

The role of proteases in protein turnover can be examined *in vivo* through the analysis of defined knockout mutants. In this work we used a ΔFtsH/*slr0228* mutant (Mann et al. 2000) and a triple Δ*deg* mutant (Barker et al. 2006), with inactivated *slr1204*, *sll1679* and *sll1427* genes. To determine whether the loss of FtsH or Deg proteases impaired the ability of cells to repair damaged PSII, light-saturated rates of oxygen evolution were monitored in cells during and following exposure to UV-B light either in the absence or in the presence of lincomycin.

In intact *Synechocystis* PCC6803 cells 120 min of UV-B irradiation results in a gradual inhibition of oxygen evolution which decreases to about 50% of the original activity in the WT and Δ*deg* and to about 80% in the ΔFtsH/*slr0228* mutant. In order to check the ability of UV-B inhibited cells to restore their photosynthetic activity, the cell suspension was transferred to visible light and the oxygen evolution rate was measured. Fig. 4.1 A and B shows that in the WT and Δ*deg* the original activity is completely restored within the recovery period (2h). In the ΔFtsH/*slr0228* cells the recovery is substantially retarded as compared with the WT and Δ*deg* cells (Fig. 4.1 C). Restoration of PSII activity following UV-B exposure is also affected differentially in the two mutants: in the absence of all three Deg proteases, recovery proceeds like in the WT; however, the lack of the FtsH/Slr0228 protease suppresses the recovery although does not block it completely (Fig. 4.1 C).

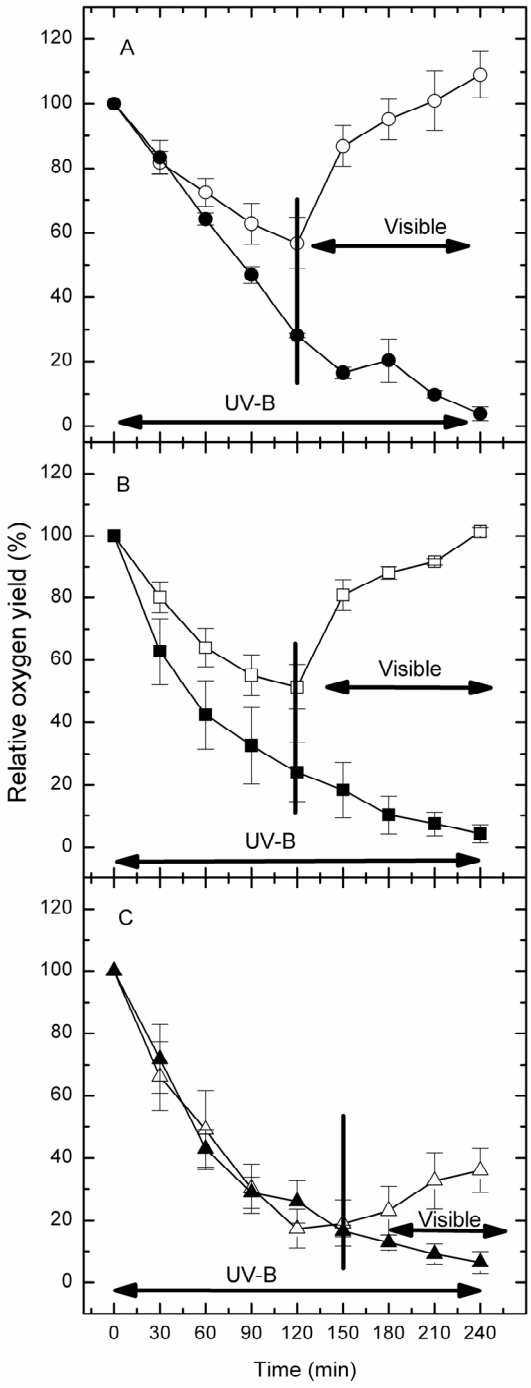

Fig.4.1. The effect of UV-B illumination on PSII activity in the Δdeg and ΔftsH/slr0228 mutants. WT (A), ΔDeg (B) and ΔFtsH/slr0228 (C) cells were exposed to UV-B light. The experiments were performed either in the presence (full symbols) of a protein synthesis inhibitor (lincomycin for WT and ΔFtsH/slr0228, and spectinomycin for ΔDeg), or in the absence (empty symbols) of it. In the presence of protein synthesis inhibitors cells were exposed only to UV-B light, whereas in the absence of protein synthesis inhibitors 120 min UV-B exposure was followed by a recovery period under visible light of 40 $\mu Em^{-2}s^{-1}$ as indicated on the horizontal arrows. PSII activity was followed by oxygen evolution measurements in the presence of 0.5 mM DMBQ as an artificial electron acceptor. The data represent the average of three independent experiments and shown after normalization to the oxygen evolution rates measured in the non-irradiated control cells.

In the presence of a protein synthesis inhibitor (lincomycin), the WT and Δ*deg* cells showed an accelerated loss of oxygen evolution under UV-B exposure resulting in about ~ 70% activity decrease after 120 min, and almost complete loss of activity after 240 min exposure (Fig. 4.1 A and B). However, in the ΔFtsH/*slr0228* strain, inhibition of protein synthesis did not accelerate further the loss of oxygen evolution (Fig. 4.1 C), so that the

kinetic was similar to that seen in the WT and Δ*deg* cells in the presence of a protein synthesis inhibitor. The fast rates of damage obtained in the presence of lincomycin represent the true rate of PS II inactivation in the absence of any repair process. The slower rates of PSII inactivation observed in the absence of lincomycin represent the balance between UV-B induced inactivation and its continuous repair. The kinetics of UV-B induced inhibition in the presence of protein synthesis inhibitor is the same for all three cultures, revealing that the WT, ΔDeg and ΔFtsH are equally susceptible to UV-B. The same pattern of oxygen evolution inhibition in the ΔFtsH/*slr0228* mutant, with or without lincomycin, demonstrates that the loss of FtsH protease interrupts the PSII repair cycle. It is known that the UV-B radiation damages the D1 protein and enhances the turnover of this subunit *in vivo*. If the removal of damaged D1 is impaired the repair process is blocked and the PSII function cannot be rehabilitated as shown by the loss of oxygen activity. Inactivation of the FtsH protease prevents the replacement of UV-B damaged PSII subunits with newly synthesized copies. Previous results with a *slr0228* insertion mutant have revealed that this protein is needed for the photoprotection of PSII activity during high light stress (Silva et al. 2003). In *Arabidopsis*, mutation of the *var2-2* gene, a close homologue of *slr0228* in *Synechocystis* PCC6803 renders PSII more susceptible to photoinhibition (Bailey et al. 2002). It seems that even though high light and UV-B light damage PSII through different mechanisms, the point of convergence is the damage of D1 protein and the participation of the same protease in the repair process. Moreover, the function of FtsH protease in PSII repair appears to be conserved in both cyanobacteria and in higher plants.

The effect of UV radiation on the function of PSII can also be followed by measuring the kinetics of flash-induced chlorophyll fluorescence relaxation (Sicora et al. 2003). In dark-adapted samples illumination with a single saturating flash forms Q_A^-, which results in a rapid rise of variable fluorescence. The initial amplitude of the fluorescence signal is proportional to the number of PSII centers capable of reducing Q_A (Vass et al. 1999). The relaxation kinetics reflects the reoxidation of Q_A^- via various pathways in the dark and exhibits three main decay phases (not shown). The fast (few hundred μs) phase reflects electron transfer from Q_A^- to a PQ molecule bound to Q_B quinone binding site. The middle (few ms) phase also arises from electron transfer from Q_A^- to Q_B, but in such PSII centers which bind PQ molecules after the light pulse, i.e. the time constant of this phase shows the rate constant of PQ binding to the Q_B site. Finally the slow (few s) phase arises from back reaction of Q_B^- with the oxidized S_2 state of the water-oxidizing complex.

Since it is known that the main target of UV-B radiation is the CaMn cluster of water oxidation, we have chosen to measure the flash induced fluorescence in the conditions which give us information about this component of PSII. In the presence of DCMU, which occupies the Q_B^- binding site and inhibits Q_A^- to Q_B electron transfer, the fluorescence relaxation reflects the recombination of Q_A^- with positively charged donor components of PSII. In non-irradiated cells the relaxation follows hyperbolic kinetics with about 1 s time constant, which arises from the recombination of Q_A^- with the S_2 state of the water-oxidizing complex (Vass et al. 1999) (Fig. 4.2 A–C, squares). As a consequence of UV-B irradiation a faster component (with a 5–10 ms time constant) appears (Fig. 4.2 A–C, circles), reflecting the recombination of Q_A^- with Tyr-Z^{ox} in PSII centers in which the electron transport between the CaMn cluster and Tyr-Z has been inactivated (Vass et al. 1999). The fraction of PSII centers showing the fast decaying component was about the same in the WT and Δ*deg* cells, and substantially higher in the ΔFtsH/*slr0228* cells. In the WT and Δ*deg* cells the fast phase completely disappeared during recovery under visible light (Fig. 4.2 A and B, triangles) demonstrating the restoration of normal electron transfer in the PSII complex. However, in the ΔFtsH/*slr0228* cells there was only a partial restoration and the relaxation kinetics were dominated by the fast component even after 120 min recovery. The fast decaying phases in the chlorophyll induced fluorescence in the presence of DCMU demonstrates the accumulation of PSII centers in which the CaMn cluster is inactivated (Vass et al. 1999). Low- intensity-visible light has been shown to alleviate the damaging effects of UV-B irradiation when applied both during and after an UV-B treatment (Sicora et al. 2003).

In our experiments, low-light treatment following UV-B irradiation restored the integrity of the electron transport chain (Fig. 4.2 A and B) and also the number of PSII active centers (not shown) in the WT and Δ*deg* cells. In these cells the ongoing PSII repair driven by visible light replaced the nonfunctional CaMn clusters and this is obvious in the disappearance of the fast phase of flash induced chlorophyll fluorescence. On the contrary, in the ΔFtsH/*slr0228* mutant the fast phase was persistent during the recovery period (Fig. 4.2, C) and also contained less PSII active centers than at the beginning of the treatment (not shown). In case of the ΔFtsH/*slr0228* mutant light was ineffective in repairing UV-B damaged PSII centers and this could be linked with the missing protease and the failure to remove the damaged centers. Furthermore, the simultaneous measurements of oxygen evolution and chlorophyll fluorescence relaxation demonstrate that FtsH/*slr0228* is required for restoring electron transfer between the CaMn cluster and the acceptor side of PSII via Tyr-Z (Fig. 4.1 and 4.2).

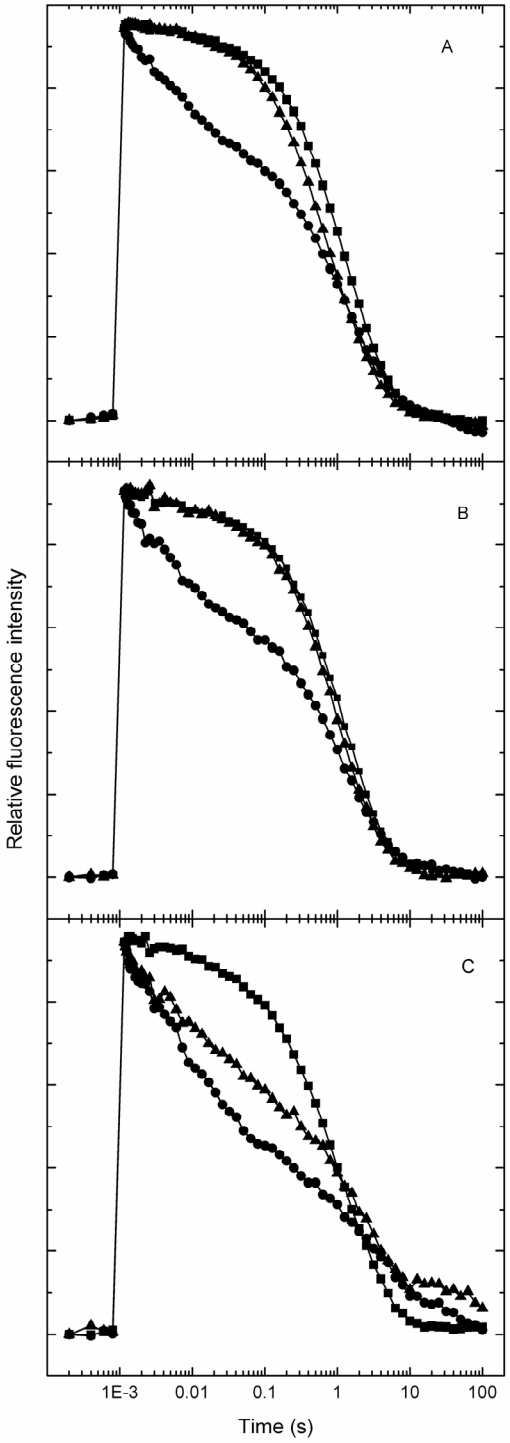

Fig. 4.2. Damage and recovery of flash-induced chlorophyll fluorescence in UV-B illuminated cells of ΔDeg and ΔFtsH. Cells were exposed to UV-B light followed by recovery under visible light of 40 $\mu Em^{-2} s^{-1}$. PSII function was followed by measuring flash-induced chlorophyll fluorescence in the presence of DCMU. The kinetics of fluorescence relaxation are shown for WT (A), ΔDeg (B) and ΔFtsH/slr0228 (C) cells before (squares) and after 120 min UV-B treatment (circles), as well as after 60 min recovery (triangles) after normalization to the same initial value

Overall, these data indicated that PSII repair was functioning almost equally well in the Δ*deg* mutant as in WT, but was severely inhibited in the ΔFtsH/*slr0228* mutant. The lack of significant effect of deleting the three Deg homologues shows that in contrast to FtsH/Slr0228 the Deg protease family is not essentially required for repair of UV-damaged PSII.

4.1.2. UV-induced degradation of the D1 and D2 proteins in the Δ-Deg and Δ-FtsH mutants

In the case of visible-light damage the FtsH and Deg proteases have both been implicated in PSII repair *in vivo*. The persistence of full-length D1 protein in the FtsH/*slr0228* mutant, the co-purification of Slr0228 with His-tagged PSII (Silva et al. 2003), and the exclusion of the functional role of other cyanobacterial proteases in the cleavage of damaged D1 protein has led to a general model for PSII repair in which FtsH complexes alone are able to degrade visible-light damaged D1 (Nixon et al. 2005). FtsH protease activity has also been associated with the degradation of oxidatively damaged D1 protein *in vivo* in higher plants (Bailey et al. 2002; Sakamoto et al. 2003). In contrast, an alternative view emphasizes the involvement of the DegP/HtrA or Deg proteases in PSII repair and D1 degradation following visible light stress, both in chloroplasts (Haussuhl et al. 2001) and cyanobacteria (Huesgen et al. 2005). In the model of Huesgen et al. (2005), which is partially supported by *in vitro* data (Kanervo et al. 2003), D1 is proposed to be cleaved in periplasmic-exposed loops by the HhoA protease. However, it has been recently reported that although the Deg proteases are required for photo-tolerance, they are not involved in D1 turnover following visible-light stress (Nixon et al. 2005; Barker et al. 2006).

Whether FtsH and Deg proteases have a role in the response to UV-B damage was unclear before our studies.

Recent microarray data have indicated that UV-B radiation strongly induces the transcript levels of the *ftsH*/*slr0228* gene in *Synechocystis* PCC6803 (Huang et al. 2002; Cadoret et al. 2005). This observation points to the possibility that the FtsH/Slr0228 protease could be involved in the repair of UV-damaged PSII complex similarly to its previously documented role in visible light stress (Silva et al. 2003; Komenda et al. 2006).

In order to clarify the role of the Deg and FtsH proteases in D1 protein degradation we followed the time course of D1 protein levels during UV-B light treatment and subsequent recovery in visible light. In WT and Δ*deg* cells, the amount of D1 decreased during the UV-B illumination to about 65% of the initial value, but its amount was restored to the original level in visible light. In contrast, in the ΔFtsH/*slr0228* cells the amount of D1 was practically unchanged (Fig. 4.3.) despite the severe inhibition of PSII activity shown in Fig. 4.1.

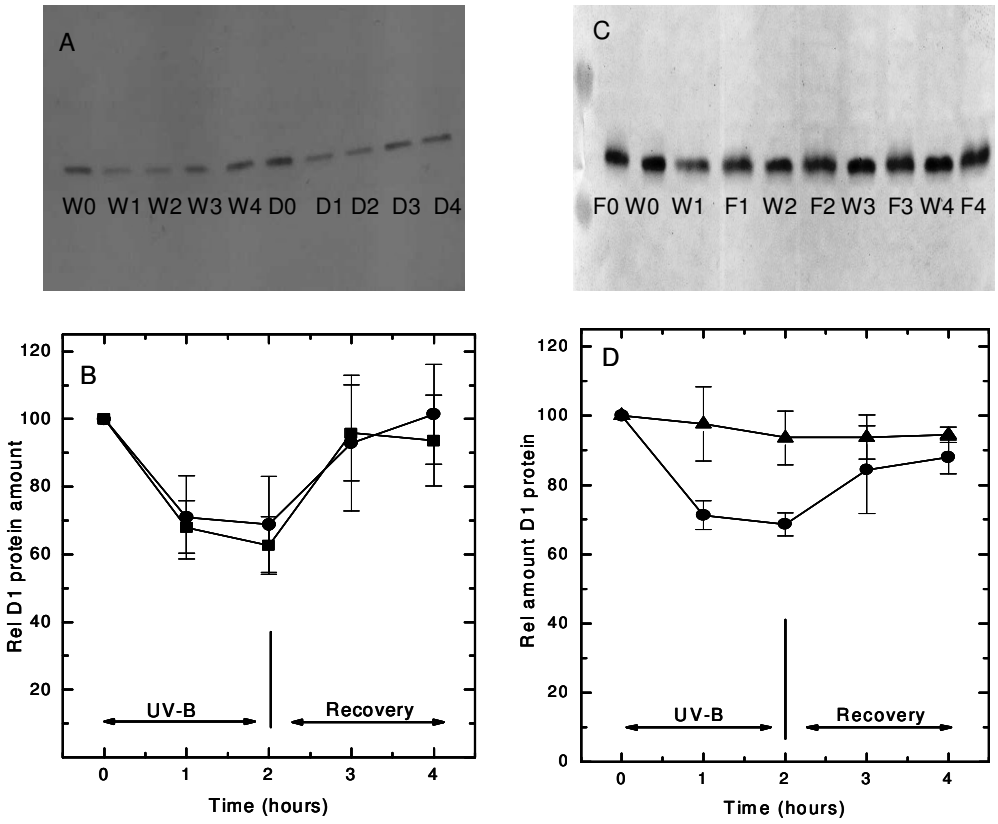

Fig. 4.3. D1 protein content in the absence of protein synthesis inhibitor in UV-B illuminated WT and mutant Synechocystis PCC6803 cells. Cells were exposed to UV-B radiation followed by recovery under visible light of 40 μEm^{-2}s^{-1}. Thylakoids were isolated at the indicated time points and D1 protein amount was determined by immunoblotting. (A and C) Immunoblots of D1 obtained in WT (W0,..,W4), ΔDeg (D0, …, D4) and ΔFtsH/slr0228 (F0,…,F4) cells after 0 h (W0, D0, F0), 1 h (W1, D1, F1) and 2 h (W2, D2, F2) UV-B exposure followed by 1 h (W3, D3, F3) and 2 h (W4, D4, F4) of recovery. (B and D) Changes in the D1 protein amount obtained from densitograms of blots of WT (circles), ΔDeg (squares) and ΔFtsH/slr0228 (triangles) thylakoids. The data are shown after normalization to the value at the 0 time point.

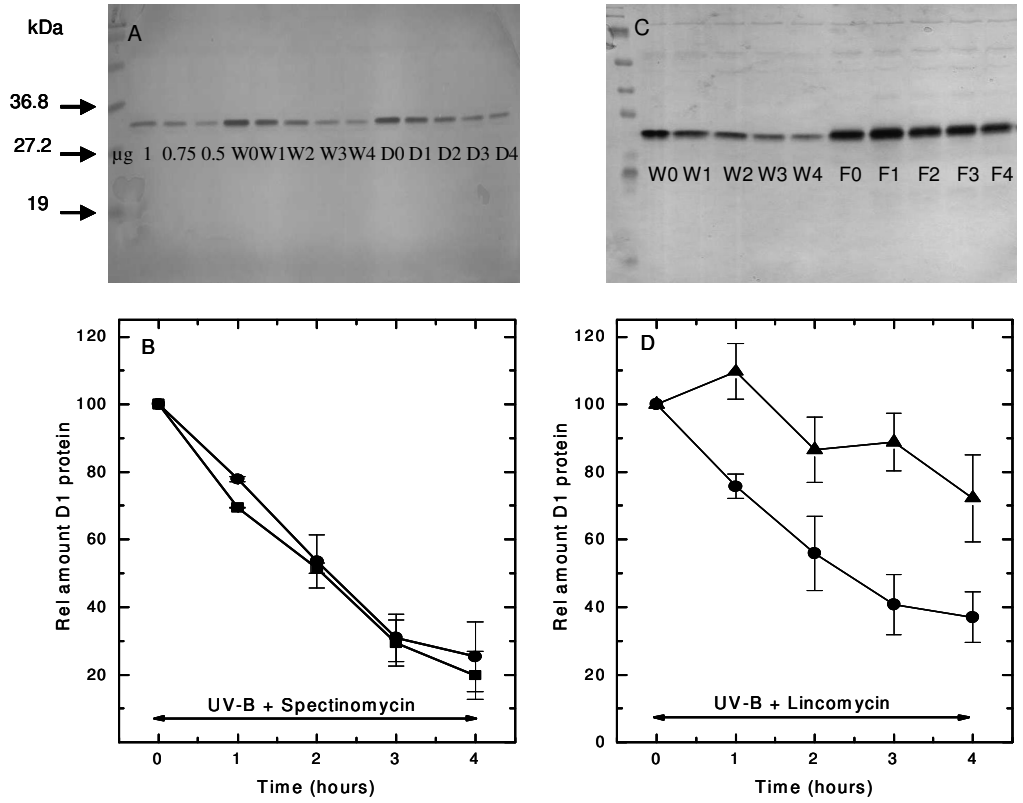

Fig. 4.4. D1 protein content in the presence of a protein synthesis inhibitor in UV-B illuminated WT and mutant Synechocystis PCC6803 cells. Cells were exposed to UV-B irradiation in the presence of protein synthesis inhibitors (spectinomycin for ΔDeg and its WT control, or lincomycin for ΔFtsH/slr0228 and its WT control). Thylakoids were isolated at the indicated time points and D1 protein amount was determined by immunoblotting. (A and C) Immunoblots obtained in WT (W0,..,W4), ΔDeg (D0, …, D4) and ΔFtsH/slr0228 (F0,…,F4) cells after 0 h (W0, D0, F0), 1 h (W1, D1, F1),…,4 h (W4, D4, F4). On the left side of panel A, a dilution series is shown with 0.5, 0.75 and 1.0 µg Chl/lane loading, whereas, the other samples contained 1,5 µg Chl. (B and D) Changes in the D1 protein amount obtained from densitograms of blots of WT (circles), ΔDeg (squares) and ΔFtsH/slr0228 (triangles) thylakoids. The data are shown after normalization to the value at the 0 time point.

To study the rate of D1 degradation without the compensating effect of *de novo* protein synthesis the experiments were also performed in the presence of a protein synthesis inhibitor. Under these conditions the UV-B-induced loss of the D1 protein was strongly

accelerated in the WT and ΔDeg cells, but their kinetics were practically identical (Fig. 4.4A,B). However, in the ΔFtsH/*slr0228* cells the rate of D1 loss was significantly slower than in the WT or ΔDeg (Fig. 4.4C,D).

The major consequence of deleting the *slr0228* gene was a reduced ability to repair UV-B damaged PSII centers *in vivo* (Fig. 4.1 C). In accordance with the PSII repair cycle, this effect could be attributed to one of the following steps: monomerization of PSII, removal of the damaged subunits, synthesis and integration into the membrane of a new D1 protein. During PSII repair *in vivo*, it is hard to discriminate between D1 degradation and D1 synthesis due to the high coordination of the two processes: impaired synthesis might block degradation and *vice versa* (Komenda 2000). Protein synthesis inhibitors like lincomycin and spectinomycin enable the study of protein degradation independently of protein synthesis and are a good tool for examining the kinetics of proteolysis. Clear difference between the ΔDeg and ΔFstH mutants was found in the rate of D1 degradation in the presence of protein synthesis inhibitors, which reflects different roles of the proteases in this process. Summing up the results of the PSII activity and D1 degradation a new model of PSII repair under UV-B radiation is contouring in which FtsH protease plays the main role.

The involvement of specific proteases in the degradation of the reaction center proteins during PSII repair has so far been studied in detail only for D1, although the D2 subunit can also be degraded under extreme conditions (Sass et al. 1997; Jansen et al. 1998). UV-B irradiation provides a convenient tool to study the role of proteases in D2 degradation since UV-B light induces D2 protein loss to an extent comparable with that of D1 protein. To this end we followed the kinetics of D2 abundance under the same conditions as was done for D1. The data in Fig. 4.5 show that in the absence of protein synthesis inhibitors the D2 protein was lost to the same extent as seen for D1 and was restored during the recovery period. The kinetics of D2 loss and recovery were almost identical for the WT and ΔDeg cells (Fig. 4.5 B). However, loss of D2 during UV-B irradiation was retarded in ΔFtsH/*slr0228* as compared to the WT, and its recovery in visible light was severely inhibited (Fig. 4.5 D). Experiments performed in the presence of protein synthesis inhibitors confirmed the differential effect of inactivating the Deg proteases and FtsH/Slr0228 on D2 protein degradation, since the kinetics of D2 loss were similar for the WT and ΔDeg cells in the presence of spectinomycin (Fig. 4.6 A, B), whereas, the loss of D2 was much slower in ΔFtsH/*slr0228* than in WT in the presence of lincomycin (Fig. 4.6 C, D).

In contrast to visible light, which induces preferential damage and repair of D1, UV-B light leads to a similar extent of damage of both D1 and D2 (Sass et al. 1997). Acceleration of

D1 and D2 degradation under UV-B exposure and retardation of their resynthesis both under UV-B and visible light in the ΔFtsH/*slr0228* mutant, as compared to the WT, show that FtsH/Slr0228 is involved in the degradation of not only the D1 but also of the D2 protein.

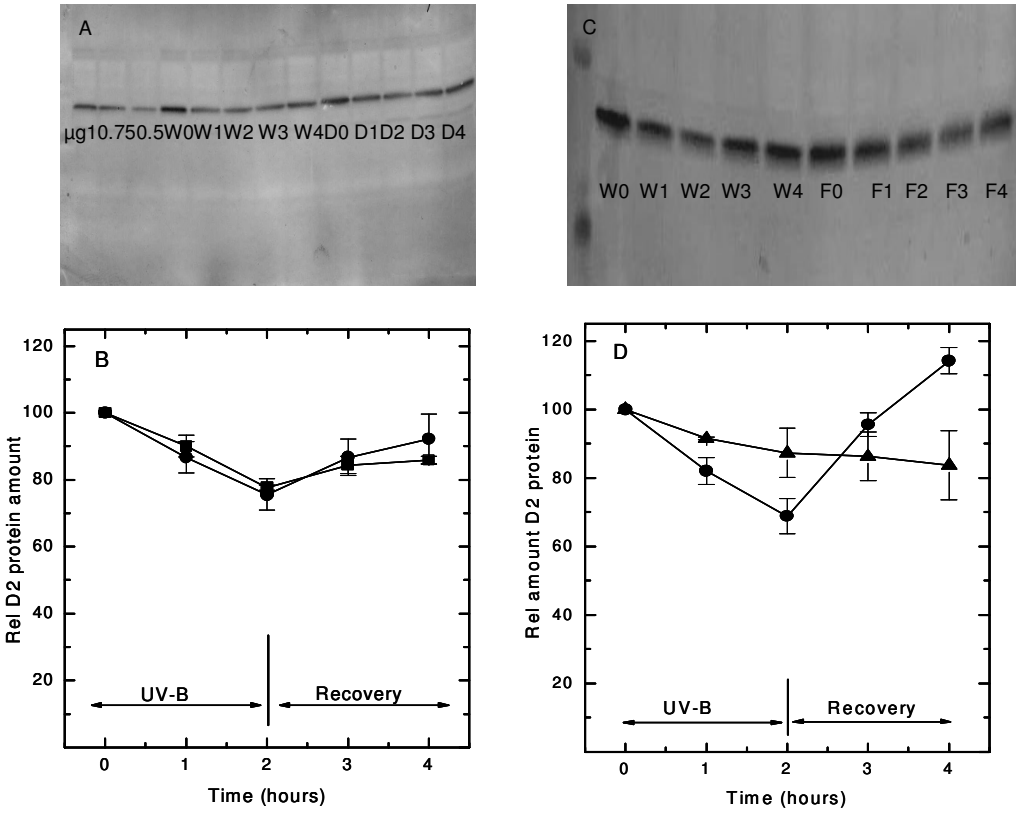

Fig. 4.5. D2 protein content in the absence of protein synthesis inhibitor in UV-B illuminated WT and mutant Synechocystis PCC6803 cells. Cells were exposed to UV-B irradiation followed by recovery under visible light of 40 µEm^{-2}s^{-1}. Thylakoids were isolated at the indicated time points and levels of D2 were determined by immunoblotting (A and C). Immunoblots of D2 obtained in WT (W0,..,W4), ΔDeg (D0, …, D4) and ΔFtsH/slr0228 (F0,…,F4) cells after 0 h (W0, D0, F0), 1 h (W1, D1, F1) and 2 h (W2, D2, F2) UV-B exposure followed by 1 h (W3, D3, F3) and 2 h (W4, D4, F4) of recovery. On the left side of panel A, a dilution series is shown with 0.5, 0.75 and 1.0 µg Chl/lane loading, whereas, the other samples contained 1,5 µg Chl. (B and D) Changes in D2 levels obtained from densitograms of blots of WT (circles), ΔDeg (squares) and ΔFtsH/slr0228 (triangles) thylakoids. The data are shown after normalization to the value at the 0 time point.

Our present results demonstrate that FtsH/Slr0228 is involved in the removal of light damaged D2 subunit embedded in the PSII complex providing example for the more general role of this protease besides the selective degradation of D1. In contrast, the lack of a significant effect upon inactivating the three Deg homologues shows that the Deg proteases are not absolutely required for degradation of UV-B damaged D1 and D2.

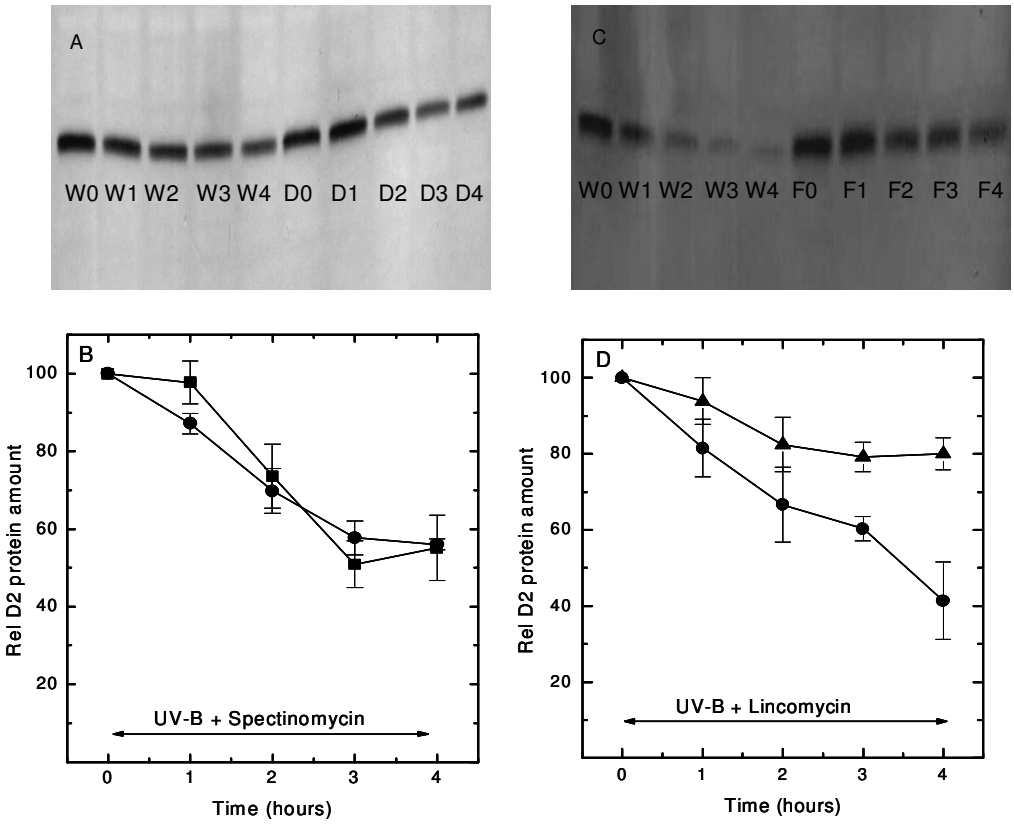

Fig. 4.6. D2 protein content in the presence of a protein synthesis inhibitor in UV-B illuminated WT and mutant Synechocystis PCC6803 cells. Cells were exposed to UV-B irradiation in the presence of protein synthesis inhibitors (spectinomycin for ΔDeg and its WT control, or lincomycin for ΔFtsH/slr0228 and its WT control). Thylakoids were isolated at the indicated time points and D2 levels were determined by immunoblotting. (A and C) Immunoblots obtained in WT (W0,..,W4), ΔDeg (D0, …, D4) and ΔFtsH/slr0228 (F0,…,F4) cells after 0 h (W0, D0, F0), 1 h (W1, D1, F1),…,4 h (W4, D4, F4). (B and D) Changes in D2 levels were obtained from densitograms of blots of WT (circles), ΔDeg (squares) and ΔFtsH/slr0228 (triangles) thylakoids. The data are shown after normalization to the value at the zero time point.

Detection of D1 protein fragments with a molecular mass smaller than that of the original protein have led to the conclusion that the degradation process occurs in a multi-step proteolytic reaction. In plants, D1 degradation following the acceptor-side photoinhibition produced 2 fragments: a 10-kDa C-terminal fragment and a 23-kDa N-terminal fragment (Greenberg et al. 1987). In the donor-side photoinhibition of isolated PSII complexes from plants a C-terminal 24-kDa fragment has been detected (De Las Rivas et al. 1992). *In vitro* studies showed the formation of a 20-kDa C-terminal D1 fragment via a non-enzymatic mechanism when isolated thylakoid membrane particles were treated with UV-B light suggesting that this particular cleavage site of D1 is located in the middle of the 2nd transmembrane helix of D1 (Friso et al. 1994b). In case of the D2 protein a 22-kDa N-terminal D2 fragment was observed when isolated D1/D2 reaction center complexes were exposed to UV-B light in the presence of the quinone analogue DBMIB (Friso et al. 1994a). However, these D1 and D2 fragments were formed only in a minor amount in the isolated membrane particles, and were not observed in the present study at all when whole *Synechocystis* PCC6803 cells were irradiated with UV-B light. In addition, fragments did not accumulate in the absence of FtsH or Deg proteases either (Figs. 4.4A,C and 4.6A,C). Therefore, we must conclude that the degradation pathways involving non-proteolytic D1 and D2 fragment formation are not significant in intact *Synechocystis* PCC6803 cells.

According to a recent model of Nixon et al. (2005), in cyanobacteria and chloroplasts FtsH is proposed to form a hexameric ring in the membrane, and the damaged protein is translocated through a central pore in an ATP-driven process and subsequently degraded at a Zn^{2+}-activated center in a highly processive reaction (Nixon et al. 2005). Based on what is known about the orientation of FtsH in chloroplasts (Lindahl et al. 1996), the protease domain of FtsH/Slr0228 is likely to be located on the cytoplasmic side of the thylakoid membrane rather than the lumenal side (Komenda et al. 2006). The N-termini of both D1 and D2 are located at the periphery of the PSII complex (Ferreira et al. 2004) and so ideally placed for engaging with FtsH. This would mean that removal and degradation of UV-B damaged D1 and D2 also proceeds from the cytoplasmic side of the PSII complex. This hypothesis has been indeed demonstrated by Komenda et al. providing a clear support for the FtsH-mediated degradation of damaged D1 via its N-terminus (Komenda et al. 2007).

Although our results demonstrate that the Deg homologues of *Synechocystis* PCC6803 are not required for the degradation of UV damaged D1 and D2, the approximately 2-fold UV-B induction of the *sll1679* gene encoding HhoA (Cheregi et al. 2007) indicates some role for this protein as well in the UV-B stress response other than the involvement in

D1 and D2 turnover. A recent study by Barker et al. (2006) using double and triple mutants of the same *deg* genes demonstrated that these proteases show overlap in function and are involved in a number of key physiological responses ranging from protection against light and heat stresses to phototaxis. As it was previously shown, the Deg proteases are needed for photoprotection of *Synechocystis* PCC6803, but they do not play an essential role in D1 turnover and PSII repair *in vivo* (Barker et al. 2006). Homology studies show that the three Deg proteases in *Synechocystis* PCC6803 are closely related to the lumenal Deg1, Deg5, Deg8 and Deg14 in *Arabidopsis*. Since the transcript levels of *deg1*, *deg2* and *deg8* were increased following exposure of *Arabidopsis* seedlings to high light (Sinvany-Villalobo et al. 2004) it was of interest to verify the role of these genes. Recently it was demonstrated that the double mutant *deg5deg8* showed increased sensitivity to photoinhibition and reduced rates of D1 degradation in high light compared with the single mutants of *deg5* and *deg8* (Sun et al. 2007). A simultaneously conducted study by Kapri-Pardes et al. (2007) investigated the role of Deg1 in *Arabidopsis*. Taking advantage of the RNA interference method, they created a mutant which had reduced levels of Deg1 and followed the levels of D1 and FtsH proteins in this mutant. The D1 protein is stabilized in Deg1 mutants and the level of a 5.2 kDa C-terminal fragment seems to be lower than in the WT (Kapri-Pardes et al. 2007). The results of this last study lead to an interesting model of D1 degradation in plants: a transmembrane multiproteolytic complex in which Deg proteases from both sides of thylakoid membrane make single cleavages and FtsH protease makes complete proteolysis of these fragments.

Whether the other FtsH homologues besides Slr0228 are involved in PSII repair in *Synechocystis* PCC6803 is unclear. Recent work has demonstrated that two different types of FtsH subunit participate in PSII repair in *Arabidopsis thaliana*. These so called type-A and type-B subunits are suggested to form a hetero-hexameric complex (Zaltsman et al. 2005b). Both types of subunit are made of a pair of redundant copies (type-A: FtsH1 and FtsH5, type-B: FtsH2 and FtsH8) and the complete absence of either the A- or B-types is lethal. Based on a recent phylogenetic analysis, FtsH/*slr0228* and FtsH/*slr1390* in *Synechocystis* PCC6803 would correspond to type-B subunits, and FtsH/*slr1604* to a type-A subunit (Yu et al. 2005). Thus, by analogy to chloroplasts, FtsH complexes in *Synechocystis* PCC6803 might also be composed of various heterocomplexes including an FtsH/*slr1604*-FtsH/*slr0228* complex, which would fit well with the observed UV-B induction of these two genes (Cheregi et al. 2007). Our data also show that some PSII repair (Fig. 4.1C, D) and D1 and D2 degradation persists in the ΔFtsH/*slr0228* mutant (Fig.4.4 and 4.6). This residual repair activity might be because FtsH/*slr1390* can operate as a type-B subunit or that other types of proteases are able

to catalyze PSII subunit degradation, albeit with reduced effectiveness. Clarification of interplay of the different FtsH copies in the turnover of the D1 and D2 subunits of PSII will be an important challenge of research in the near future.

4.2. The role of Slr2100 cGMP phosphodiesterase in acclimation to UV-B

4.2.1. PSII activity in the ΔSlr2100 mutant

The cyclic nucleotides signaling pathway is known to play an important role in the regulation of various biological activities by controlling gene expression level. While cAMP and cGMP are modulating the function of molecular switches in the cell (cAMP binds to CRP, cGMP interacts with the phytochrome) (Kolb et al. 1993; Bowler et al. 1994), the cNMP phosphodiesterases are controlling the duration and the intensity of these cellular responses by reducing the levels of cAMP and cGMP .

In *Synechocystis* PCC6803 the open reading frames *slr2100* and *slr1614* are proposed to be putative cNMP phosphodiesterases because they each carry a HD domain (de Alda et al. 2000b). The phosphodiesterases carrying a HD domain (named after the conserved doublet of predicted catalytic residues) catalyze the degradation of cNMP to inorganic phosphate and AMP/GMP. To get information about the possible implication of *slr2100* gene in the metabolism of cGMP, the gene was inactivated and the corresponding mutant was characterized in terms of growth, cyclic nucleotide content under control, high light and UV-B stressed conditions (Cadoret et al. 2005). The Δ*slr2100* mutant and the WT exhibited similar growth rates but the mutant had higher steady-state level of cGMP (Cadoret et al. 2005). UV-B stress affects specifically the level of cGMP: following 10 min of UV-B exposure, in the WT the cGMP concentration drops to ~60% of original concentration. In the Δ*slr2100* mutant the level of cGMP does not vary after UV-B exposure. This is also the case when the WT is exposed to UV-B in the presence of dipyridamole, an inhibitor of phosphodiesterase activity. There is also a notable difference in the transcription profiles of 400 genes following 30 minutes UV-B exposure of WT and Δ*slr2100* mutant cells. These series of experiments brought physiological evidence that the *Synechocystis* open reading frame *slr2100* could be a cGMP phosphodiesterase (Cadoret et al. 2005). The consequences of high light treatment on the PSII activity, measured as oxygen evolution, were the same in the WT and ΔSrl2100 mutant. In contrast, differences were observed in the response to a UV-

B stress. Following these results, our aim was to see if Slr2100 is involved in the UV-B signal transduction pathway that affects the repair process of PSII.

Figure 4.7 shows that when exposed to UV-B for 2 hours, the extent of the damage is higher in the ΔSlr2100 mutant (~40%) than in the WT (~29%). Following one hour recovery in visible light both cultures recover with the same rate showing that the PSII inactivation is fully reversible for the two strains.

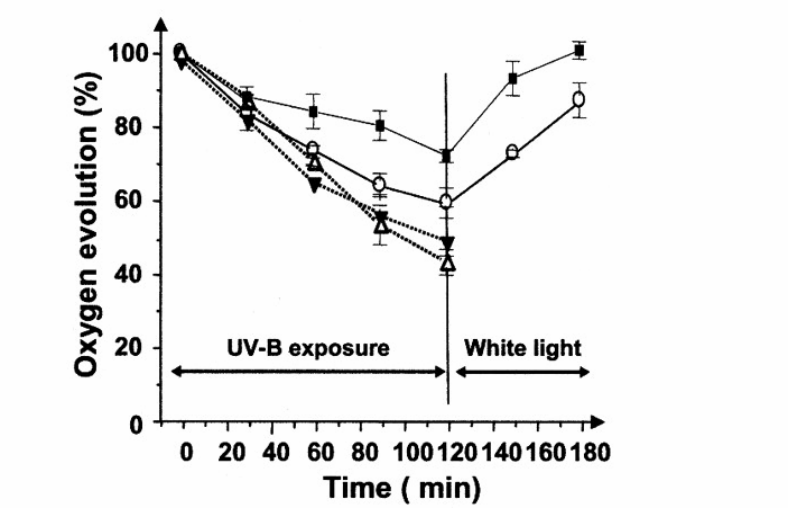

Fig. 4.7. PSII inactivation during UV-B stress. Synechocystis PCC6803 wild type (filled symbols) and Δslr2100 mutant (open symbols) cells were exposed to UV-B (6 μmol $m^{-2}s^{-1}$) at time 0 for 2h, and then transferred back to white light (50 μmol $m^{-2}s^{-1}$). Experiments were performed in the absence (squares and circles) or presence (triangles) of lincomycin (translation inhibitor). Plotted values represent mean of 3 independent measurements performed with cell suspensions at 6.5 μg Chla ml^{-1}.

To determine the origin of the increased sensitivity of the ΔSlr2100 mutant to UV-B radiation, the same experiment was repeated in the presence of lincomycin. The impairment of oxygen evolution under these conditions (UV-B +lincomycin) is similar in the WT and the ΔSlr2100 mutant, an evidence of the equal sensitivity of the two strains to UV-B induced damage in the absence of any repair process. A straightforward explanation of the increased UV-B sensitivity of the ΔSlr2100 mutant is that the mutation has an effect on the cascade of events required for the repair of the damaged PSII centers, which is known to require *de novo* protein synthesis (Aro et al. 1993). UV-B damage of PSII electron transport is revealed by

the changes in the shape of the relaxation of flash fluorescence curve when measured in the presence of DCMU. Such measurements give information on the functional integrity of the redox components on the donor side of PSII. Samples of the WT and ΔSlr2100 mutant were measured in untreated cells, after 2 hours of UV-B treatment, and subsequent 1 hour recovery in visible light.

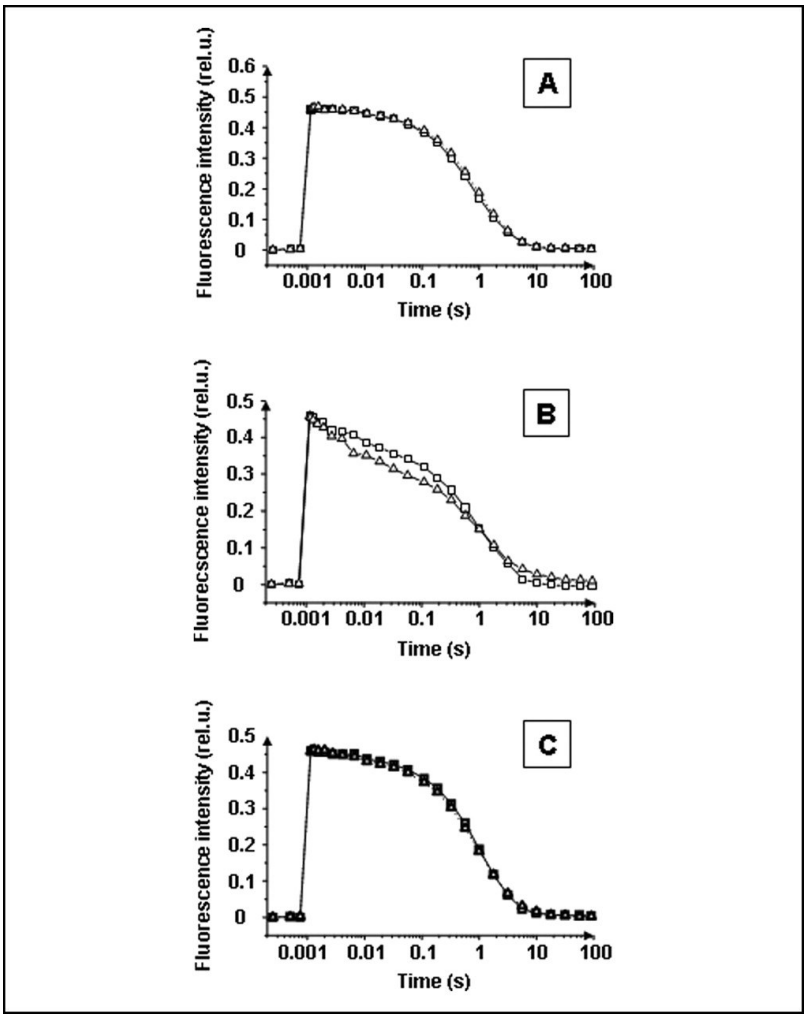

Figure 4.8. Relaxation of the variable fluorescence induced by a single saturating flash applied to Synechocystis PCC6803 cells: wild type strain (squares), Δslr2100 mutant (triangles). A, cells before the UV-B treatment. B, after 2h of UV-B irradiation. C, after a 1h recovery under 50 µmol.m^{-2}.s^{-1} white light. Cells at 6.5µg Chla/ml were dark-adapted for 10 min before exposure to the saturating flash and recording of the variable fluorescence. Experiments were performed in the presence of 10µM DCMU, and repeated three times. The curves were normalised to the same amplitude.

When plotted on a logarithmic time scale, the fluorescence relaxation curve has a sigmoid shape and it reflects the recombination of Q_A^- with the S_2 states of the water-oxidation complex. The half-lifetime for relaxation is 0.6 s for the two strains when grown under standard conditions (Fig. 4.8 A). After 2h of UV-B treatment, the overall decay was faster for the wild type (half-lifetime 0.4 s) and a fast phase also appeared (Fig. 4.8 B). This indicates that Q_A^- cannot recombine with the S_2 state in part of the centers, instead less stable donor components like the redox-active tyrosine (Tyr-Z) act as recombination partner for Q_A^- (Vass et al. 1999; Tichy et al. 2003). The acceleration of fluorescence relaxation was even more pronounced for the ΔSlr2100 mutant (half-lifetime 0.3 s). This result indicates that the Mn-cluster of the PSII centres was more damaged by the UV-B treatment in the mutant than in the wild type. After the recovery phase, the fluorescence relaxation profiles are similar for both strains (Fig. 4.8 C) confirming that the mutation likely affects the PSII repair process that takes place during the UV-B exposure.

4.2.2. D1 protein degradation in the Δ *slr2100* mutant

After clarifying that the absence of the Slr2100 protein affects the PSII repair cycle we wanted to identify at which level the PSII repair cycle is impaired. We took advantage of our SDS-Page and Western Blot set-up and followed the levels of D1 protein in the WT and Δ*slr2100* strains, in control and UV-B treated samples. As shown in Figure 4.9, there is a significant difference between the two strains, showing faster loss of the D1 protein in the mutant, which confirms an impairment of D1 turnover in Δ*slr2100* mutant. The decreased amount of D1 protein in the thylakoids of the mutant strain exposed to UV-B compared to that of the wild type (Fig. 4.9) demonstrates that the degradation part of the repair cycle is not affected by the lack of the *slr2100* gene.

A correlation between cyclic nucleotides, UV-B stress and D1 degradation cannot be made without taking into consideration the effects of the ΔSlr2100 mutation on the gene expression level. As found by our collaborators (Cadoret et al. 2005), the induction patterns of *slr0228* (coding for the FtsH protease) and *psbA3* (coding for D1 protein) support our hypothesis that cGMP participates in UV-B signaling during PSII repair. The level of *slr0228* and *psbA3* is twofold lower in Δ*slr2100* than in the wild type under standard conditions. The mutant cells are thus partly deprived of essential components for the PSII repair when exposed to UV-B radiation.

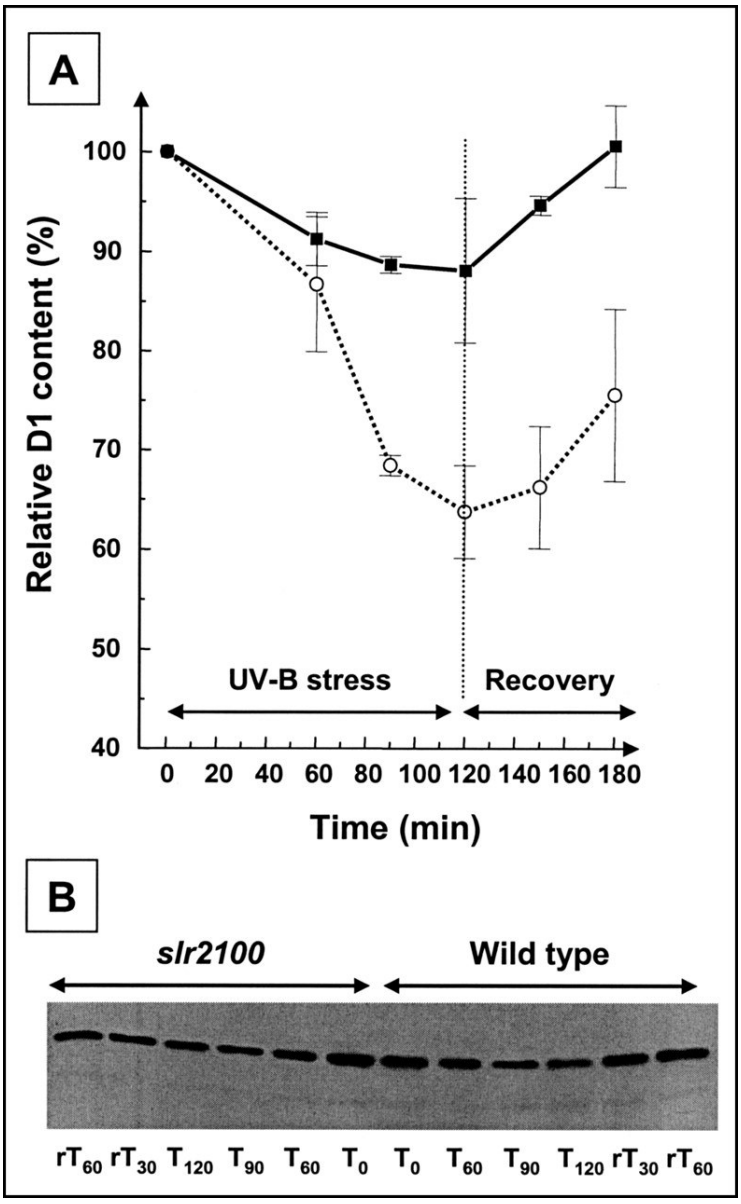

Fig. 4.9. D1 content of thylakoid membranes. Membranes were prepared at the given time points and the D1 protein content was determined by immunodetection using an anti-D1 antiserum. Wild type, squares;Δslr2100, circles. Data are means of 3 experiments.

However, the transcription of *ftsH* increases more in the mutant than in the wild type so that the *ftsH* mRNA level in Δ*slr2100* mutant reaches that of the wild type after fifteen minutes of UV-B treatment, ensuring efficient D1 degradation (Cadoret et al. 2005).

The replacement of UV-damaged and degraded D1 by new copies requires an enhanced transcription of *psbA* genes, especially of *psbA3* (Máté et al. 1998), followed by translation and incorporation of new D1 copies into the PSII reaction centre complex.

Although the *psbA* mRNA levels are lower in the mutant than in the wild type under standard conditions, the higher extent of UV-B induction compensates for this effect in the *slr2100* mutant under UV-B exposure. The similar and higher mRNA amounts in UV-B stressed wild type and mutant cells show that it is not the abundance of *psbA* transcripts that limits D1 synthesis. Therefore, the unregulated cGMP concentration observed in the mutant under the conditions of UV-B exposure should affect either the translation of *psbA* mRNA or the incorporation of newly synthesized D1 into the PSII reaction centre.

Altogether our data are consistent with the observation that the repair of damaged PSII differs depending on whether the inhibition was induced by UV-B or high intensity visible light (Giacometti et al. 1996), since the difference in the repair efficiency between the Δ*slr2100* mutant and wild type strains only exists when cells are exposed to UV-B and not during photoinhibition by visible light.

4.3. Small heat shock proteins and their role in PSII repair

4.3.1. Effect of the Q16R-Hsp17 mutation on the UV-B sensitivity of oxygen evolving activity

In search for features of Hsp17 that are essential for its *in vivo* function, a screening for random mutation that cause a loss of thermotolerance was performed in *Synechocystis* PCC6803 (Giese et al. 2005). The majority of mutations studied was in the conserved α-crystalline domain and were found to disrupt Hsp17 oligomerization and to reduce both *in vitro* chaperone activity and *in vivo* thermotolerance. Single residue mutations on the N-terminus like L9P (change of leucine 9 to proline) and Q16R (change of glutamine 16 to arginine) were found to have only minor effects on the oligomeric state of Hsp17, with L9P oligomers being somewhat less stable than WT and Q16R more stable than WT. Although these mutant sHSPs retain the ability to protect a model substrate they did not support *in vivo* thermotolerance when compared with the WT. It is possible that in L9P and Q16R mutations a crucial site for binding cellular substrates *in vivo* has been affected. Another possible explanation for the failure of the N-terminal mutants to provide thermotolerance *in vivo* is that L9P and Q16R could have altered lipid interaction properties (Giese et al. 2005). This hypothesis was further investigated and it was demonstrated that mutant L9P protein showed reduced association with the thylakoid membrane, while Q16R protein was almost exclusively associated with the thylakoid fraction (Balogi et al. unpublished).

Lipid interaction of L9P-Hsp17 and Q16R-Hsp17 proteins was studied with the monolayer technique and the results of it were striking: of the three sHsp species tested, Q16R-Hsp17 displayed the highest interaction with monolayers formed both from TPL (total polar lipids) and each individual lipid classes. Moreover, the highest degree of insertion was recorded with a negatively charged lipid, SQDG (Balogi et al, unpublished). In the structure of PSII from *Thermosynechococcus elongatus* SQDG has contacts with proteins from both monomers and is very close to the Q_B site, localization with potential impact on the processes that involve D1 protein and Q_B site: the monomerization/dimerization of PSII during PSII repair and electron transport chain.

Due to the preferential and selective association of the Q16R-Hsp17 protein with the thylakoid membrane after heat shock our goal was to verify if this event confers increased resistance to PSII damage or facilitates the recovery/repair from UV-B damage.

The consequences of photodamage by UV-B radiation are the irreversible inactivation of the electron transport chain and the degradation of the D1 and D2 proteins of the PSII reactions center. One way to follow the UV-B induced inhibition of PSII complex is to measure the rate of oxygen production with the help of a Clark-type electrode. Heat hardened cells (42˚C, 3h) of the WT and the two mutant strains, Q16R-Hsp17 and L9P-Hsp17, were exposed to UV-B radiation for two hours at 30˚C and then shifted to one hour recovery in visible light. As a consequence of UV-B exposure, the oxygen evolving activity in the WT and L9P-Hsp17 mutant decreased continuously during the treatment and after 2 hours reached 60% of original activity. Astonishingly, the rate of oxygen evolution in the Q16R-Hsp17 mutant showed no significant inhibition during the 2 hours of UV-B treatment (Fig.4.10). During the consequent recovery in visible light and normal growth temperature (30˚C), all three strains recovered with the same rate and reestablished the initial activities. The rate of the oxygen evolution reflects the interplay of the damage and repair processes; when the rates of the repair processes match the rate of the damaging processes, there is no loss of the oxygen producing activity. If the oxygen activity is decreasing, it might come either from an enhanced sensitivity to the damaging factor or from an impaired repair process. The next step in elucidating which of the two processes are predominant when oxygen activity is decreasing is to block one of them.

To this end, we exposed heat hardened cells of the WT and Q16R-Hsp17 mutant to UV-B radiation, in the presence of lincomycin. Under these conditions both the WT and the Q16R-Hsp17 mutant showed an accelerated and similar loss of oxygen evolution, which reached 30-40% of the original activity after 2 hours of irradiation (Fig. 4.10 B).

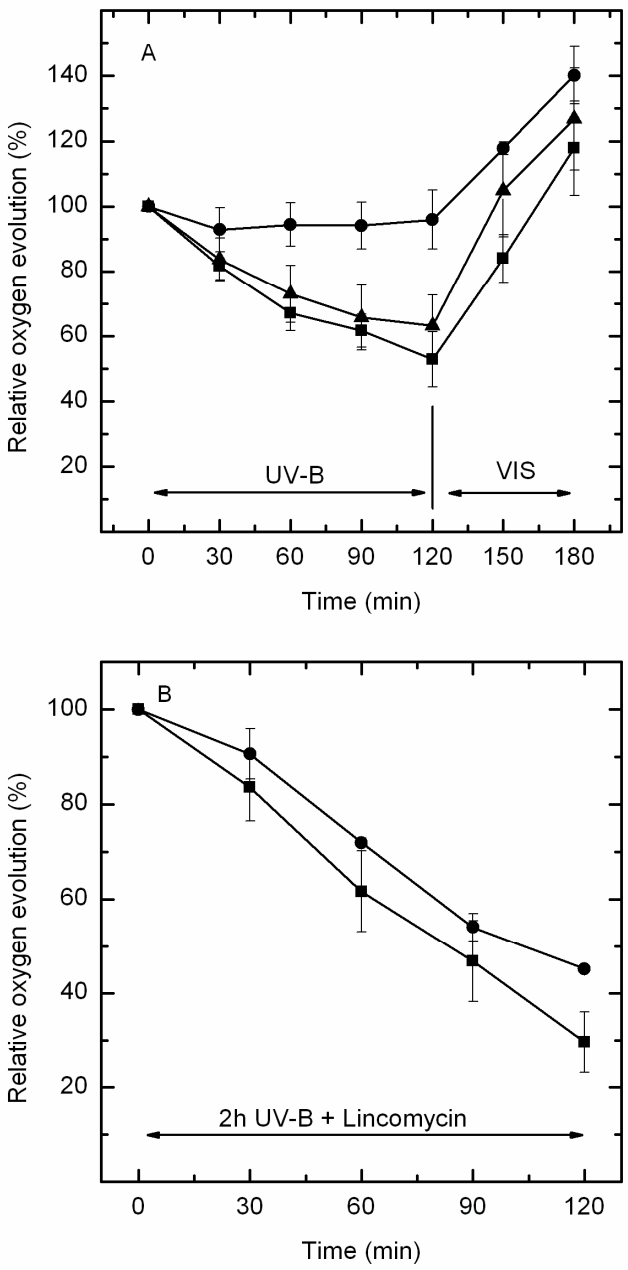

Fig. 4.10. Effect of Hsp17 mutations on oxygen evolving activity. Heat preconditioned (42 ºC, 3 hours) WT (squares), Q16R-Hsp17 (circle) and L9P-Hsp17 (up triangle) strains were exposed to UV-B radiation for 2 h followed by a recovery period in visible light for 1 h. Oxygen evolving activities were measured at the indicated time points. Results are expressed as a percentage of the oxygen evolution rate measured at time zero. Error bars represent standard error obtained from three repetitions. Experiments were performed (A) in the absence or (B) presence of the protein synthesis inhibitor lincomycin.

Since the WT and the Q16R-Hsp17 mutant exhibit the same UV-B sensitivity, the remarkable difference between their oxygen activities (Fig. 4.10 A) comes from different repair rates. It seems that the Q16R mutation in the Hsp17 protein not only modifies the Q_B site but also protects PSII activity against the UV-B photodamaging radiation by facilitating the repair process.

4.3.2. Effect of the Q16R-Hsp17 mutation on PSII electron transport

In order to explore the possible consequence of the exclusive thylakoid association and enhanced lipid binding properties of Q16R-Hsp17 protein on PSII electron transport characteristics, we measured the kinetics of flash-induced chlorophyll fluorescence, which monitors forward and backward electron transfer processes at the reducing side of PS II. In the Q16R-Hsp17 mutant the fast and middle phases, which reflect forward electron transport between Q_A^- and Q_B in PSII centers with and without bound Q_B, respectively were faster than in the WT (Fig. 4.11 B and Table 1) indicating faster oxidation of Q_A^- by bound Q_B, and faster binding of PQ to Q_B in the Q16R mutant.

Together these findings demonstrate a modification of the Q_B site in the mutant, which could be a consequence of a structural change induced by the association (or the different mode of association) of mutated Hsp17 with the PSII complex. In addition, the slow phase of fluorescence decay became slower in the mutant than in the WT (Table 1) showing an enhanced stability of electrons on bound Q_B^-.

When the Q_A to Q_B electron transfer step is blocked by DCMU, the reoxidation of Q_A^- proceeds via charge recombination with the S_2 state of the water oxidizing complex. The Q16R-Hsp17 mutation had practically no effect on fluorescence relaxation in the presence of DCMU, showing that characteristics of the S_2 state and of the Q_A acceptor are left unaffected in the Q16R mutant. This indicates that association of Q16R-Hsp17 protein with PSII has no effect on Q_A and the water-oxidizing complex, which are shielded by the protein matrix in contrast to the more exposed Q_B acceptor. The free energy (redox) gap, which determines the rate of electron transfer between the Q_A and Q_B acceptors, can be calculated from the ratio of the time constants of the slow phases of fluorescence in the absence and presence of DCMU (Allahverdiyeva et al. 2004). This calculation resulted in 52 ±4 and 69 ±5 meV in the WT and Q16R strains, respectively, in agreement with the observed acceleration of electron transfer between Q_A and Q_B in the Q16R-Hsp17 mutant strain. Taken together, the above data

demonstrate a specific modification of the Q_B binding site in cells which have the Hsp17 protein with the Q16R mutation.

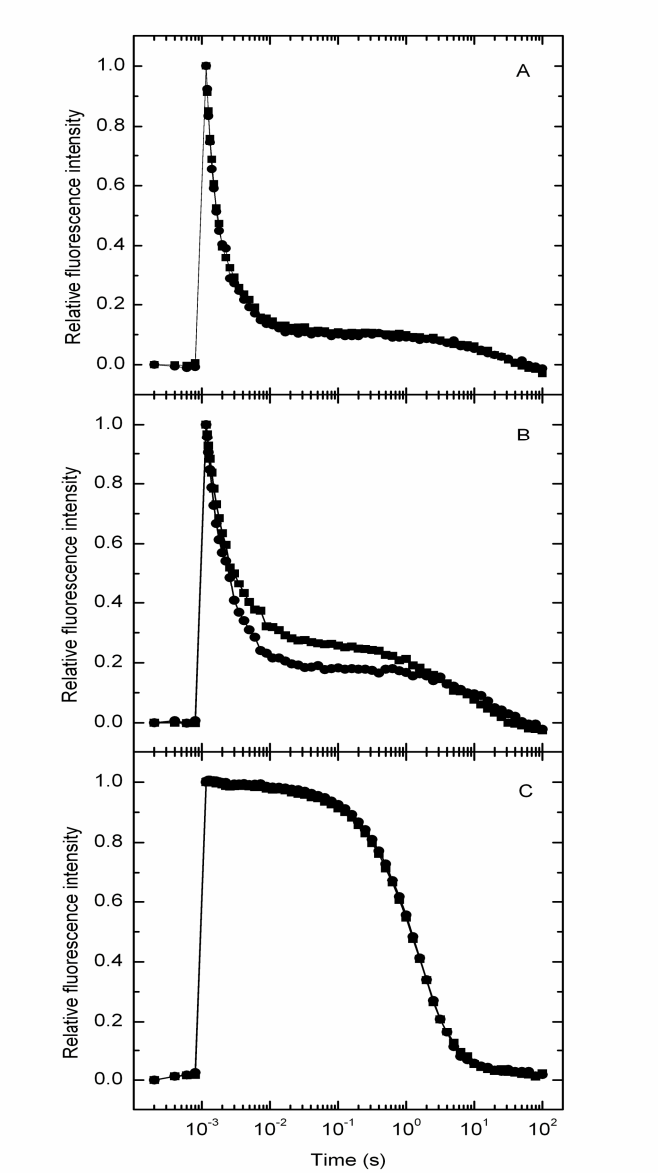

Fig.4.11. Relaxation of flash induced chlorophyll fluorescence in non-heat and heat preconditioned (42˚C, 3 hours) in WT (squares) and Q16R-Hsp17 (circles) cells. Fluorescence excitation was achieved with a single turnover saturating flash at t= 1 ms. Subsequent fluorescence relaxation was measured in the absence (A, B) of electron transport inhibitors and in the presence (C) of 10 μM DCMU. Curves shown were normalized to the same initial amplitude.

Photosynthetic electron transport between the thylakoid embedded PSII and cytb$_6$f complexes is mediated by plastoquinone (PQ) molecules via diffusion in the lipid phase. The chlorophyll fluorescence relaxation measurements in this study show that the rate of PQ binding to the Q_B site of PSII, where exchange of these mobile electron carrier molecules with bulk PQ occurs, is accelerated in the Q16R-Hsp17 mutant. This finding indicates that the mutated sHsp interacts not only with the thylakoid membrane in general, but specifically with the PSII complex.

Strain	Fast phase: T1(ms) (Amplitude, %)	Middle phase: T2(ms) (Amplitude, %)	Slow phase: T3(ms) (Amplitude, %)
No addition[a]			
WT	0.67±0.04 (52±2)	5.67±0.4 (28±2)	8.8±1 (19±2)
Q16R	0.43±0.04 (53±3)	3.46±0.2 (30±3)	17±2 (16±0.6)
With DCMU[b]			
WT	- (0)	- (0)	1.15±0.05 (100±0.004)
Q16R	- (0)	- (0)	1.14±0.03 (100±0.004)

[a] The curves were analyzed in terms of two exponential components (fast and middle phases) and one hyperbolic component (slow phase). [b] The curves were analyzed in term of one hyperbolic component (slow phase).

Table 1. Characteristics of chlorophyll fluorescence yield relaxation in Synechocystis PCC6803 cells. Heat treated samples were dark adapted for 3 min, excited with a single turnover flash and fluorescence relaxation was measured. T1-T3 and A1-A3 refer to the time constants and relative amplitudes of the decay phases, respectively. Standard errors show the reliability of the obtained kinetic parameters.

The Q_B site is partly constituted by a large cytosol exposed loop of the D1 protein (Loll et al. 2005). Thus Q16R Hsp17, which is expected to bind at the cytosolic side of the

thylakoid membrane, may affect this region. This idea is supported by recent structural data, which show the presence of protein-associated lipids, including negatively charged SQDG, at the cytosolic side of the PSII complex around the Q_B binding site (Loll et al. 2005; Loll et al. 2007). Since a preferential interaction of the Q16R-Hsp17 protein with SQDG has been demonstrated *in vitro* (Balogi et al. submitted), similar interaction in the PSII complex is a possible explanation for the specific effect of the mutated protein on the Q_B electron acceptor observed in the mutant cells. Although this hypothesis sounds reasonable, we cannot exclude an interaction between Q16R-Hsp17protein and other lipid classes. PG has been shown to be specifically associated with the D1 protein (Kruse et al. 1995) and is essential for maintaining the structure of Q_B site (Gombos et al. 2002; Sakurai et al. 2007). PSII complexes that were treated with phospholipase A_2 were converted from dimers to monomers suggesting that PG might be involved in the dimerization of PSII complex (Kruse et al. 2000). Experiments with a *pgsA* mutant of the *Synechocystis* PCC6803 defective in the synthesis of PG suggest that PG plays an important role in the maintenance of the photosynthetic machinery through the dimerization and reactivation of the PSII core complex (Sakurai et al. 2003).

SQDG, with a negative sulfo-group and PG, with a negative phosphate-group, exert their role in the structure and functionality of PSII complex through their particular interaction with the subunits and cofactors of PSII complex (Sato 2004).

4.3.3. PSII activity measured in the presence of different quinones

The modification on the Q_B was further investigated by measuring the PSII activities in the presence of various quinones: 2,5 DCBQ (2,5 dichlorobenzoquionone), 2,5 DMBQ (2,5 dimethylbenzoquinone) and p-Benzoquinone. PSII activities in the WT and Q16R-Hsp17 mutant were almost the same when measured in the presence of the three different acceptors before the heat shock (Fig 4.12 A). The highest activity (~ 400 μM O_2/mg Chla/h) was measured in the presence of DMBQ and about half of this activity was measured with p-BQ. A three hours treatment at 42˚C, required for the induction of sHSP, had no effect on the activity of PSII in the WT in the presence of DMBQ and DCBQ. In contrast, in the Q16R-Hsp17 mutant the recorded activities decreased to 55 and 71%, respectively of the activities before the heat shock (Fig. 4.12 B). In the presence of p-BQ, the heat shock caused a 25% reduction in the activity, both in the WT and Q16R-Hsp17 mutant.

The benzoquinone electron acceptors can take electrons via direct binding to the Q_B site, and/or via interacting with the PQ molecules dissolved in the lipid phase of the thylakoid

membrane. The three acceptors used in our measurements have different affinities for the Q_B site, in the DCBQ > DMBQ >> pBQ order (Satoh et al. 1995). The decreased oxygen evolution rates in the heat treated Q16R-Hsp17 mutant cells relative to the WT, when measured in the presence of DCBQ and DMBQ, indicate altered accessibility of these acceptors to the Q_B site after association of the Q16R-Hsp17 protein to the PSII complex. The lack of this effect in the presence of pBQ can be explained by the low affinity of pBQ to the Q_B site and preferential uptake of electrons via interaction with the PQ molecules in the lipid phase of the membrane (Satoh et al. 1995). Taken together the fluorescence and oxygen evolution data demonstrate that the accessibility of the Q_B site to plastoquinone and benzoquinone molecules is altered due to a specific interaction of the Q16R-Hsp17 protein with the PSII complex.

The amplitude of the fluorescence signal, which reflects the number of active PSII centers, showed insignificant variation when measured in the WT and Q16R-Hsp17 mutant, before and after heat shock (Fig. 4.12 C, insert). This confirms that the heat shock does not decrease the amount of functional PSII centers in the WT or the mutant cells, thus the decreased oxygen yield in the presence of DCBQ affects the efficiency in transferring the electrons from Q_A to the artificial acceptor bound to the Q_B site. In the WT, where we do not observe the strong association of Q16R-Hsp17 proteins to the thylakoid membrane following the heat shock, the number of PSII active centers and their activity remained unchanged.

4.3.4. Effect of the Q16R- Hsp17 mutation on the repair of UV-B damaged D1 protein

D1 protein quantification during UV-B exposure revealed a higher steady state level of the protein in the Q16R-Hsp17 mutant compared with the WT (Fig. 4.13 A). This finding, in parallel with the oxygen evolution results, points to an involvement of the Q16R-Hsp17 protein in PSII repair. If the experiments are performed in the presence of a protein synthesis inhibitor (lincomycin), the degradation kinetics of D1 protein is the same in the WT and Q16R-Hsp17 mutant (Fig. 4.13 B), indicating that the Q16R mutation in Hsp17 does not affect the rate of D1 protein damage, but could enhance the rate of D1 repair.

Our data demonstrate that association of Q16R-Hsp17 with the thylakoid membrane does not prevent the UV induced damage of the already existing PSII complex; therefore, the protective effect of the Q16R-Hsp17 protein must be exerted at the level of PSII repair. The functional PSII unit is a dimeric complex of two monomeric reaction centers (Rhee et al. 1997).

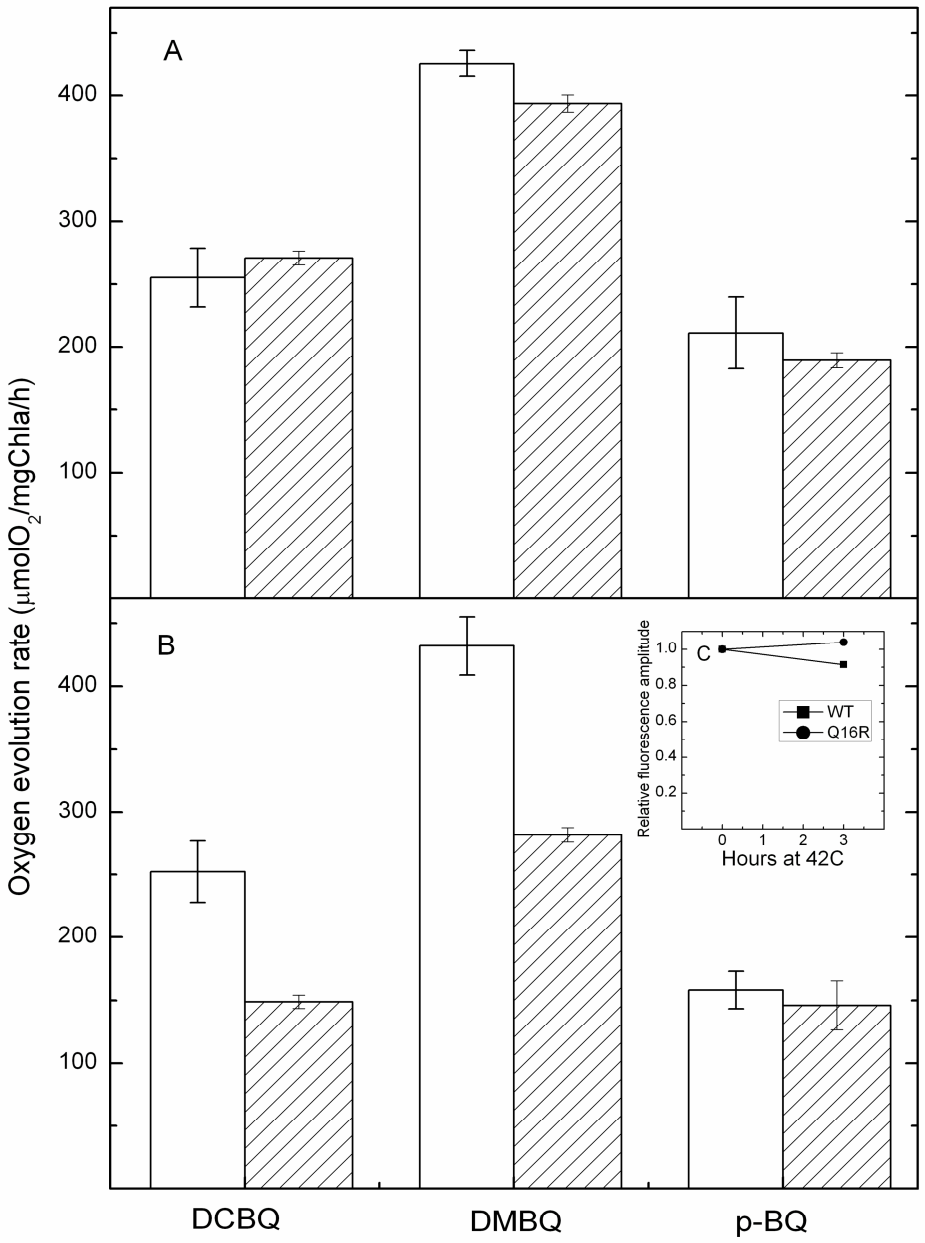

Fig. 4.12. PSII activity measured as the rate of oxygen evolution in the WT (empty columns) and Q16R-Hsp17 (hatched columns) mutant before (A) and after 3 hours at 42˚C (B). The activity was measured in the presence of different electron acceptors: 2,5 DMBQ, 2,5 DCBQ and p-BQ (0.5 mM). Error bars represent standard error obtained from three repetitions. C, the relative amplitude of the flash fluorescence signal, measured before and after 3 hours at 42˚C.

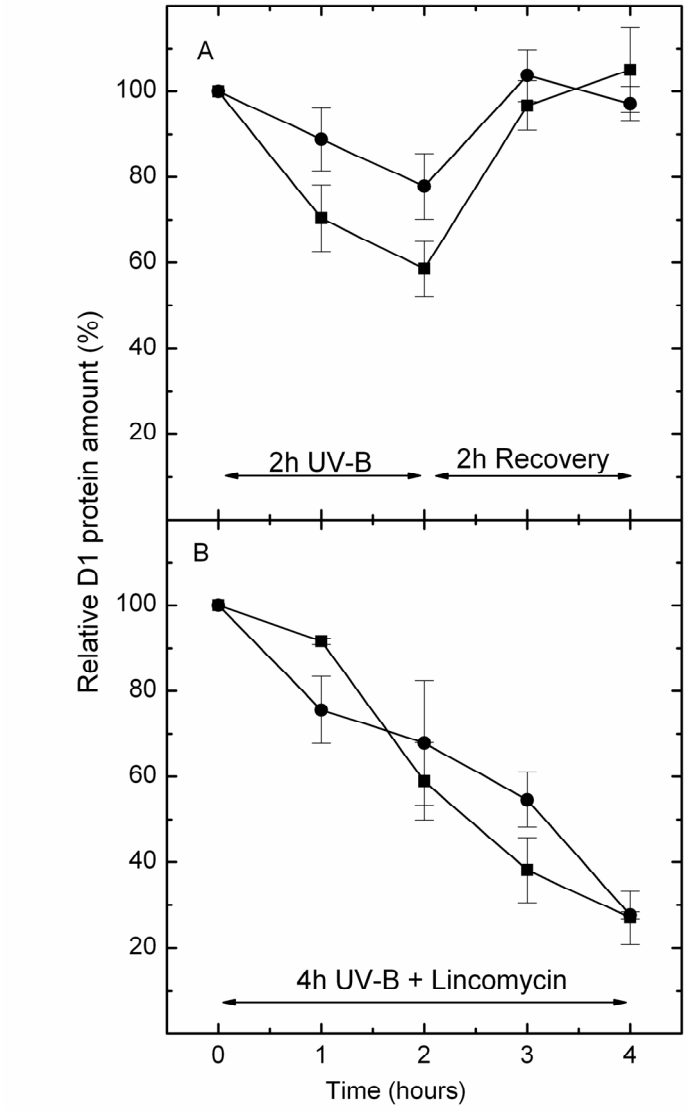

Fig. 4.13. Change in the total D1 protein content in heat preconditioned WT (squares) and Q16R-Hsp17 (circle) mutant cells challenged by UV-B stress. D1 content in samples was followed during UV-B stress and subsequent recovery (A) or tested upon prolonged UV-B treatment in the presence of lincomycin (B). Data are obtained by densitometry of the Western blots and are means of three parallel experiments. Results are expressed as a percentage of the initial D1 protein level.

At the interface between the two monomeric PSII there are four SQDG and two MGDG molecules, which are expected to have an important role in the monomer–monomer interaction (Loll et al. 2007). The dynamic process of D1 replacement was shown to involve the monomerization of the PSII dimer structure, and likewise, the dimerization of repaired PSII monomers to form fully functional PSII (Barbato et al. 1992).

The preferential *in vitro* interaction of the Q16R-Hsp17 protein with SQDG lipids – besides altering the binding of PQ to the Q_B site – indicates that the Q16R-Hsp17 may facilitate the dimer to monomer and/or the monomer to dimer reorganization of PSII during the repair cycle. However, we can not exclude that the beneficial effect of the Q16R-Hsp17 is related to other factors, such as chaperoning the insertion of newly synthesized D1 copies into the PSII complex or otherwise modulating overall protein synthesis.

5. CONCLUSIONS

1. Our work demonstrates the participation of the FtsH/Slr0228 protease in the repair of UV-B damaged PSII reaction center. The FtsH protease is involved in the *in vivo* proteolytic removal of both D1 and D2 proteins of the PSII complex. Deg proteases do not seem to have a role in PSII repair following UV-B induced damage either in D1 and D2 proteins proteolysis (Cheregi et al. 2007).

2. We have demonstrated that when the *slr2100* gene is inactivated, the repair of UV-B damaged PSII is retarded. In the ΔSlr2100 mutant the level of cGMP is unregulated and this affects the adaptation of PSII apparatus to UV-B stress. This work points to the participation of the Slr2100 in the regulatory network by which *Synechocystis* senses UV-B light (Cadoret et al. 2005).

3. We have found that the Q16R-Hsp17 mutant, with an enhanced lipid-mediated thylakoid membrane interaction, is able to protect PSII functions under UV-B photoinhibitory conditions. The protection of PSII function is exerted at the level of PSII repair through a facilitated D1 repair cycle. Besides this effect, the Q16R mutation in the Hsp17 modifies the acceptor side of the PSII complex at the level of Q_B (Balogi et al. submitted).

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PhD ÉRTEKEZÉS KIVONATA

PROTEÁZOK, CIKLIKUS NUKLEOTIDOK ÉS KIS HŐSOKK FEHÉRJÉK SZEREPE A MÁSODIK FOTOKÉMIAI RENDSZER HELYREÁLLÍTÁSÁNAK SZABÁLYZÁSÁBAN *SYNECHOCYSTIS* sp. PCC6803 CIANOBAKTÉRIUMBAN

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A Magyar Tudományos Akadémia Szegedi Biológiai Központja
Növénybiológiai Intézet
Molekuláris Stressz- és Fotobiológiai Csoport

BEVEZETÉS

A cianobaktériumok, mint a legelterjedtebb és a legnagyobb számban fellelhető fotoszintetikus prokarióták, számos környezeti stresszhatásnak vannak kitéve. Az elvékonyodó ózonréteg miatt a napfény UV-B komponensének fotoszintézisre gyakorolt hatása is egyre nagyobb. A második fotokémiai rendszerben (PSII) az UV-B sugárzás által okozott károsítás fő következményei: az elektrontranszportlánc inaktivációja (Vass, 1999), és a D1, D2 reakcióközpont-fehérjék lebomlása (Friso és mtsai, 1994; Friso és mtsai, 1995). A fotoszintetizáló szervezetek a PSII hatékony helyreállítási ciklusa révén tudják kompenzálni az UV-B okozta károsodást a sérült alegységek kicserélésével. A helyreállítási ciklus szorosan összehangolt lépései a következők: a PSII dimerek monomerizációja, a károsodott alegységek lebomlása, a riboszomális fehérje szintézis, az újonnan szintetizált fehérjék beillesztése a tilakoid membránba, a PSII monomerek összeállása és fotoaktivációja, és végül a monomerek összekapcsolódása PSII dimerekké (Aro és mtsai 1993; Nixon és mtsai 2005). A jelen dolgozatban az UV-B károsítást követő PSII helyreállítási ciklus néhány lényeges kérdését vizsgáltuk meg.

Ismert tény, hogy a D1 és a D2 fehérjék lebomlása egy proteolitikus folyamat, de a folyamatban résztvevő proteázok azonosítása érdekében jelenleg is intenzív kutatás folyik. Az FtsH-proteázok a PSII komplexekben helyezkednek el, a katalitikus alegységükkel a tilakoid membrán citoplazmatikus oldala felé mutatva (Silva és mtsai 2003). A négy *ftsH* gén közül kettő inaktiválása letális a cianobaktérium számára, egynek nincs jellemző fenotípusa, míg az *slr0228* gén mutációja megnövekedett fényérzékenységet és sérült PSII helyreállítást okozott (Silva és mtsai 2003). A *Synechocystis* PCC6803 *slr0228* génje közeli homológja az *Arabidobsis var2* génjének, ez utóbbinak a PSII helyreállításában és a kloroplasztisz biogenézisében van szerepe (Bailey és mtsai 2002). A Deg-proteázok családjának három tagja lelhető fel a *Synechocystis* PCC6803-ban (Sokolenko és mtsai 2002), melyek mindegyikét a magasabb rendű növények *deg* génjeinek homológjai kódolják. Az utóbbiak szerepe a PSII helyreállításában intenzív kutatások tárgyát képezte, részben ellentmondó adatokat eredményezve (Lindahl és mtsai 2000; Kanervo és mtsai 2003; Huesgen és mtsai 2005; Barker és mtsai 2006). Jelen kutatásunk során az *slr0228,* valamint a három *deg* gén inaktivációjának hatását vizsgáltuk UV-B károsítás alatt és után.

Annak ellenére, hogy az UV-B sugárzás hatásai és következményei részletekbe menően ismertek, a sugárzást érzékelő mechanizmusok és válaszreakciók többnyire ismeretlenek. A ciklikus nukleotidok, cAMP (3',5'- cyclic adenosine monophosphate) és cGMP (3',5'- cyclic guanosine monophosphate), jellemzően másodlagos jelátvivők; a sejten belül, a külső környezetből érkező jelek felfogására képesek és a felfedezésüket Nobel-díjjal jutalmazták. A ciklikus nukleotidok sejten belüli szintjét az adenilil- és guanilil-ciklázok (a cAMP és a cGMP ATP-ből történő szintézisét katalizálják) valamint a ciklikus nukleotid-foszfodiészterázok (a két ciklikus nukleotid lebontását katalizálják) tartják egyensúlyban.

Az *slr2100* és az *slr1614* génszakaszok feltehetően cNMP-foszfodiészterázokat kódolnak, mivel mindegyikük hordozza a HD domént (de Alda és mtsai 2000b). Kutatásunk célja az UV-B sugárzást érzékelő szabályozó hálózat vizsgálata volt az *slr2100* ciklikus nukleotid mutáns felhasználásával.

A Hsp17 kis hősokk fehérje a PSII hőstresszel szembeni ellenállását növeli (Nakamoto és mtsai 2000). A *Synechocystis* PCC6803 cianobaktérium csak egy hősokk fehérjével rendelkezik, amelynek szintézisét számos stressz indukálja, és az újonnan szintetizált fehérjék a tilakoid membránnal lépnek kapcsolatba (Horvath és mtsai 1998). A Hsp17 szerkezetében a 16-os pozíciójú glutamin aminósav argininnal való kicserélése erős kapcsolódást hoz létre a tilakoid membrán és a mutáns Hsp17 fehérje között. Munkánk során

szerettük volna megvizsgálni, hogy ez a kölcsönhatás hogyan befolyásolja a PSII aktivitását UV-B sugárzás folyamán.

CÉLKITŰZÉSEK

A kísérleteinkben célul tűztük az UV-B sugárzás által károsított PSII helyreállításában résztvevő faktorok azonosítását és szerepük jellemzését. A *Synechocystis* PCC6803 cianobaktérium mutánsok felhasználásával végzett munkánk pontos céljai a következők voltak:

1. A PSII reakciócentrum komplex UV-B sugárzás által károsított, D1 és D2 fehérje komponenseinek lebontásában résztvevő proteáz(ok) azonosítása. Ennek érdekében a Deg és FtsH proteáz családok mutánsait vizsgáltuk.
2. Az *slr2100* gén egy feltételezett cNMP-foszfodiészteráz, mivel magában hordozza a HD domént. A kérdések, amelyek ezzel kapcsolatban felmerültek, a következők: mi az *in vivo* szerepe ennek a génnek, illetve, hogy van-e szerepük a ciklikus nukleotidoknak a PSII helyreállításához kapcsolódó jelátviteli utakban?
3. Mivel a mutáns Q16R-Hsp17 hősokk fehérje szelektíven és elsődlegesen a tilakoid membránnal lép kapcsolatba, célunk volt annak tisztázása, hogy ez a folyamat hatással van-e az UV-B által károsított PSII komplex helyreállítási folyamataira.

ANYAGOK ÉS MÓDSZEREK

- *Synechocystis* cianobaktérium nevelése
- Tilakoid membránok izolálása
- Klorofill-tartalom meghatározása
- Látható- és UV-B fénnyel való kezelés
- A PSII elektrontranszport aktivitásának mérése
- Gélelektroforézis és immunoblotting

EREDMÉNYEK ÉS MEGVITATÁSUK

Az FtsH és a Deg proteáz-családok szerepét vizsgáltuk az UV-B sugárzás károsította D1 és D2 fehérjék lebontásában *Synechocystis* PCC6803 cianobaktériumban. A ΔFtsH/*slr0228* mutánsban megnövekedett UV-B érzékenységet tapasztaltunk, valamint a károsítást követő gyenge intenzitású fehér fényben történő helyreállítás is gyengébb volt. Ezzel ellentétben a ΔDeg sejtekben, melyekben mindhárom *deg* gén inaktivált, mind a károsodás, mind az aktivitás visszaállása a vad típusával megegyező kinetikát mutatott. Az immunoblot vizsgálatokkal megállapítottuk, hogy UV-B sugárzás hatására mind a D1, mind a D2 fehérje lebomlása kisebb mértékű volt a ΔFtsH/*slr0228* esetében, mint a vad típusban. Ugyanezen fehérjék helyreállítódása is lassabbnak bizonyult a mutánsban, mint a vad típusban. A ΔDeg sejtekben azonban, a D1 és D2 fehérjék helyreállításának kinetikája megegyezett a vad típusával. Mindezek mellett, semmilyen D1 vagy D2 fehérje fragmentumok nem voltak kimutathatóak a FtsH és Deg proteázoktól mentes sejtekben, ami arra enged következtetni, hogy a nem proteolítikus fehérjehasító folyamatok, amelyek D1 és D2 fragmentumok keletkezéséhez vezethetnének, nem számottevőek a *Synechocystis* PCC6803 sejtekben.

Annak érdekében, hogy adatokat gyűjtsünk a feltételezett foszfodiészteráz, az *slr2100* gén UV-B sugárzás jelátviteli útjában betöltött szerepéről, lemértük a mutáns sejtek aktivitását nagy intenzitású fehér fény és UV-B stressz alatt. A nagy intenzitású fehér fény hatása a PSII aktivitására, amit a termelt oxigén értékeiből állapítottunk meg, ugyanaz volt a vad típusnál és a Srl2100 mutánsnál egyaránt. Ezzel ellentétben, az *slr2100* mutáns nagyobb mértékben károsodott UV-B stressz alatt és aktivitása kevésbe állt helyre, mint a vad típusú sejteké. Egy lehetséges magyarázat az *slr2100* mutáns megemelkedett UV-B érzékenységére az lehet, hogy a mutáció befolyásolja a PSII helyreállítást szabályzó jelátviteli folyamatokat, amelynek elengedhetetlen része a *de novo* fehérje szintézis. A tilakoid membránok UV-B kezelés alatt és után mért D1 fehérjeszintjének összehasonlítása is ezt támasztja alá. Az *slr2100* mutáns sejtekben kapott alacsony D1 mennyiség azt mutatja, hogy az *slr2100* gén hiánya gátolja a D1 fehérje helyreállítását.

Mivel a mutáns Q16R-Hsp17 hősokk fehérje szelektíven és elsődlegesen a tilakoid membránnal lép kapcsolatba, a célunk az volt, hogy kiderítsük, ez a kapcsolat okoz-e emelkedett szintű hőrezisztenciát a PSII számára, illetve elősegíti-e az UV-B által okozott károsodások kijavítását. Fényimpulzusok által indukált klorofill-fluoreszcencia mérések során kiderült, hogy a PSII elektrontranszportja sajátos módon megváltozik, ami a

plasztokinon molekuláknak az ún. Q_B kötőhelyre való gyorsított kötődését jelenti. Ezen hatás további viszgálata érdekében lemértük a PSII aktivitást különböző kinonok jelenlétében hősokk előtt és után, a vad típusban és a Q16R-Hsp17 mutánsban egyaránt. Három órányi 42˚C-os kezelés nem befolyásolta az aktív PSII komplexek számát, az elektrontranszport sebessége azonban különbséget mutat a vad típus és a Q16R-Hsp17 mutáns esetében különböző kinon akceptorok jelenlétében mérve. A Hsp17 fehérjék kapcsolata a tilakoid membránnal és ugyanezen fehérjék preferenciális kölcsönhatása a tilakoid membrán SQDG lipidjeivel UV-B védelmet biztosíthat azáltal, hogy több szinten is segíti a PSII repair ciklusát: a D1 fehérje hasításánál, a fehérje szintézisénél és a membránba való beillesztésénél, a PSII monomerizációjánál illetve dimerizációjánál.

KÖVETKEZTETÉSEK

1. A kutatásaink kimutatták, hogy az FtsH/Slr0228 proteáz részt vesz az UV-B által károsított PSII komplexek helyreállításában, a D1 és D2 fehérjék PSII komplexekből való *in vivo*, proteolítikus eltávolításában. A Deg proteázok nem játszanak szerepet az UV-B által károsított PSII helyreállítási folyamatában, sem a D1 fehérje, sem a D2 fehérje proteolízisében (Cheregi és mtsai 2007).
2. Kimutattuk, hogy az slr2100-as gén inaktiválása esetén, az UV-B által károsított PSII helyreállítás csökkent mértékű. A ΔSlr2100 mutánsban a cGMP szint szabályozatlan és ez befolyásolja a PSII UV-B stresszhez való alkalmazkodását. Ezek az eredmények arra engednek következtetni, hogy a Slr2100 részt vesz abban a szabályozó hálózatban, amely által a *Synechocystis* 6803 érzékeli az UV-B sugárzást (Cadoret és mtsai 2005).
3. Kimutattuk, hogy a Q16R-Hsp17 mutánsoknál fellelhető erős, lipid által közvetített tilakoid membrán kölcsönhatás képes megvédeni a PSII funkcióit az UV-B sugárzás fénykárosító hatásaival szemben. A PSII működésének védelme a helyreállítás szintjén valósul meg azáltal, hogy hatékonyabbá válik a D1 fehérje kicserélődése. Ezen hatása mellett a Hsp17 fehérjében lévő Q16R mutáció módosítja a PSII akceptor oldalát is a Q_B szintjén (Balogi és mtsai, beküldve).

ABSTRACT OF THE THESIS

INVOLVEMENT OF PROTEASES, CYCLIC NUCLEOTIDES AND SMALL HEAT SHOCK PROTEINS IN PSII REPAIR IN *SYNECHOCYSTIS* sp. PCC6803

PhD Thesis

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INTRODUCTION

Cyanobacteria, the most widespread and abundant oxygenic photosynthetic prokaryotes, are exposed to various types of environmental stresses. With the recent thinning of the ozon layer, the UV-B component of solar radiation is of particular importance. The main consequences of UV-B induced damages are the inactivation of electron transport chain (Vass, 1999) and the degradation of the D1 and D2 reaction center proteins (Friso et al. 1994; Friso et al. 1995). Photosynthetic organisms can cope with the UV-B induced damages because of an efficient PSII repair cycle that replaces the damaged subunits. The events of the repair cycle are highly coordinated are require monomerization of PSII dimers, proteolytic degradation of damaged subunits, the ribosomal protein synthesizing machinery, reinsertion of newly synthesized proteins into the thylakoid membrane, reassembly of PSII monomers and photoactivation, and finally dimerization of monomers into oxygen-evolving PSII centers (Aro et al. 1993;Nixon et al. 2005).

In this thesis, several aspects concerning the PSII repair cycle following UV-B induced damage have been studied.

The degradation of D1 and D2 proteins is known to be a proteolytic process but the identity of proteases involved is a field of intense research. FtsH (*F*ilamentation *t*emperature-*s*ensitive) proteases are localized in PSII complexes, with the protease domain oriented toward the cytoplasmic side of the thylakoid membrane (Silva et al. 2003). Inactivation of two of the four *ftsH* genes in *Synechocystis* PCC6803 proved to be lethal, one had no obvious phenotype and the mutation of *slr0228* caused an increased sensitivity to light and impaired PSII repair (Silva et al. 2003). The *slr0228* gene in *Synechocystis* PCC6803 is a very close homologue of the *var2* of *Arabidopsis* which is involved in PSII repair and chloroplast biogenesis (Bailey et al. 2002). The Deg family of proteases has three members in *Synechocystis* PCC6803 (Sokolenko et al. 2002), which are encoded by homologues of d*eg* genes of higher plants. Their role in PSII repair has been a subject of intense research leading to partly contradictory data (Lindahl et al. 2000; Kanervo et al. 2003; Huesgen et al. 2005; Barker et al. 2006). Here we have studied the effect of inactivating the *slr0228* gene and the three *deg* genes of *Synechocystis* PCC6803 on PSII repair during and after UV-B damage.

Although the effects and consequences of UV-B radiation are known in detail the mechanisms for sensing and responding to UV-B are largely unknown. The cyclic nucleotides cAMP (3',5'- cyclic adenosine monophosphate) and cGMP (3',5'- cyclic guanosine monophosphate) are typical second messengers, intracellular receptors of extracellular signals and their discovery was awarded with a Nobel prize. The cellular level of cyclic nucleotides is an equilibrium between the activities of adenylyl-, guanylyl-cyclases (which catalyze the synthesis of cAMP and cGMP from ATP, respectively) and cyclic nucleotide phosphodiesterases (which catalyze the degradation of the two cyclic nucleotides). The open reading frames *slr2100* and *slr1614* are proposed putative cNMP phosphodiesterases because they each carry a HD domain (de Alda et al. 2000b). Our work aimed to investigate the regulatory network by which *Synechocystis* PCC6803 senses UV-B, through the analysis of a cyclic nucleotide phosphodiesterase mutant, Δ*slr2100*.

The heat shock protein 17 (Hsp17) confers thermal resistance to PSII apparatus during heat shock (Nakamoto et al. 2000). *Synechocystis* PCC6803 has only one small heat shock protein (sHSP) which is induced by many stress conditions; the newly synthesized proteins are associated with the thylakoid membrane (Horvath et al. 1998). A glutamine to arginine residue change in the structure of Hsp17 induces a strong association of mutant protein with the lipids of the thylakoid membrane. We wanted to verify if this interaction modifies the activity of PSII under UV-B stress conditions.

AIMS OF THE STUDY

In our experiments we were aiming to identify factors and their contribution to the repair of UV-B damaged PSII reaction center in the cyanobacterium *Synechocystis* PCC6803. The particular goals of our work were:

1. To find which protease is involved in the degradation of the UV-B damaged D1 and D2 proteins of the PSII reaction center complex. Therefore, we took advantage of a series of mutants for two families of proteases: Deg and FtsH.
2. The open reading frame *slr2100* is a proposed cNMP phosphodiesterase because it carries a HD domain. The questions we addressed were: what is the *in vivo* function of this gene? Do cyclic nucleotides play a role in the signaling pathways of PSII repair?
3. Due to the preferential and selective association of the Q16R-Hsp17 protein with the thylakoid membrane after heat shock our goal was to verify if this event confers increased resistance to PSII damage or facilitates the recovery/repair from UV-B damage.

MATERIALS AND METHODS

- *Synechocystis* growth conditions
- Thylakoid isolation
- Chlorophyll content determination
- Visible and ultraviolet light treatment
- Measurement of photosystem II electron transport activity
- Gel electrophoresis and immunoblotting

RESULTS AND DISCUSSION

We have investigated the involvement of the FtsH and Deg protease families in the degradation of UV-B-damaged PSII reaction center subunits, D1 and D2, in the cyanobacterium *Synechocystis* PCC6803. PSII activity in a ΔFtsH/*slr0228* strain, measured as the rate of oxygen evolution, showed increased sensitivity to UV-B radiation and impaired recovery of activity in visible light after UV-B exposure. In contrast, in Δ*deg* cells, in which all the three *deg* genes were inactivated, the damage and recovery kinetics were the same as in WT. Immunoblotting showed that the loss of both the D1 and D2 protein was retarded in ΔFtsH/*slr0228* during UV-B exposure, and the extent of their restoration during the recovery period was decreased relative to the WT. However, in the ΔDeg cells the damage and recovery kinetics of D1 and D2 were the same as in the WT. Moreover, we have not detected any fragments of the D1 or D2 proteins in the absence of FtsH or Deg proteases which indicates that degradation pathways involving non-proteolytic D1 and D2 fragment formation are not significant in intact *Synechocystis* PCC6803 cells.

To get information about the possible implication of *slr2100* gene, a putative phosphodiesterase, in the UV-B transduction pathway we measured the activity of the mutant cells in the conditions of high light and UV-B stress. The consequences of high light treatment on the PSII activity, measured as oxygen evolution, were the same in the WT and Δ*slr2100* mutant. In contrast, differences were observed in the response to a UV-B stress. A possible explanation of the increased UV-B sensitivity of the Δ*slr2100* mutant is that the mutation has an effect on the cascade of events required for the repair of the UV-B damaged PSII centers, which is known to require *de novo* protein synthesis. Indeed, the decreased amount of D1 protein in the thylakoids of the mutant strain exposed to UV-B compared to that of the wild type demonstrates that the degradation part of the repair cycle is not affected by the lack of the *slr2100* gene.

Due to the preferential and selective association of the Q16R-Hsp17 protein with the thylakoid membrane after heat shock our goal was to verify if this event confers increased resistance to PSII damage or facilitates the recovery/repair from UV-B damage. Measurements of flash-induced chlorophyll fluorescence in the mutant cells revealed a specific modification of the thylakoid embedded PSII: acceleration of plastoquinone binding to the Q_B site. The modification on the Q_B binding site was further investigated by measuring the PSII activities in the presence of various quinones, in the WT and Q16R-Hsp17 mutant, before and after the heat shock. The number of PSII active centers is not affected by the 3

hours treatment at 42˚C but the electron transfer efficiencies of these centers, measured with three different quinones acceptors, are different for the WT and Q16R-Hsp17 mutant. The Q16R-Hsp17 mutant shows reduced UV-B damage of PSII activity in comparison with the WT. The association of Q16R-Hsp17 protein with the thylakoid membrane and the preferential interaction with the SQDG lipids could confer UV-B protection by facilitating the PSII repair cycle at one or more levels: D1 degradation, resynthesis/reinsertion into the membrane, PSII monomerization/dimerization.

CONCLUSIONS

1. Our work demonstrates the participation of the FtsH/Slr0228 protease in the repair of UV-B damaged PSII reaction center. The FtsH protease is involved in the *in vivo* proteolytic removal of both D1 and D2 proteins of the PSII complex. Deg proteases do not seem to have a role in PSII repair following UV-B induced damage either in D1 and D2 proteins proteolysis (Cheregi et al. 2007).

2. We have demonstrated that when the *slr2100* gene is inactivated, the repair of UV-B damaged PSII is retarded. In the ΔSlr2100 mutant the level of cGMP is unregulated and this affects the adaptation of PSII apparatus to UV-B stress. This work points to the participation of the Slr2100 in the regulatory network by which *Synechocystis* PCC6803 senses UV-B light (Cadoret et al. 2005)

3. We have shown that the Q16R-Hsp17 mutant, with an enhanced lipid-mediated thylakoid membrane interaction, is able to protect PSII functions under UV-B photoinhibitory conditions. The protection of PSII function is exerted at the level of PSII repair through a facilitated D1 repair cycle. Besides this effect, the Q16R mutation in the HSP17 modifies the acceptor side of the PSII complex at the level of Q_B (Balogi et al. submitted).

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LIST OF PUBLICATIONS

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