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Repair cycle of the Photosystem II complex in cyanobacteria

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Summary

Photosystem II (PSII) is a membrane pigment-protein complex which utilizes light energy for oxidation of water to molecular oxygen and emits electrons into the electron transfer chain of oxygenic phototrophs, i.e. plants, algae and cyanobacteria. Most of pigments and cofactors involved in electron transfer within PSII are bound to the central D1 and D2 reaction center (RC) subunits forming the heterodimer. On its either side are large proteins CP43 and CP47 that bind chlorophyll (Chl), deliver energy to RC pigments and, in the case of CP43, also participate (together with the D1 protein) in the ligation of the $CaMn_4O_5$ cluster involved in water oxidation. The complex also contains at least 13 small membrane subunits bound at the periphery of the complex, and three extrinsic lumenal subunits that are thought to stabilize the $CaMn_4O_5$ cluster. The unique photochemical properties of PSII RC pigments allowing withdrawal of electrons from water are directly related to high vulnerability of PSII to a light-induced damage (photoinactivation). This damage is irreversible and to restore its photochemical activity, the PSII complex must undergo a series of events comprising so called repair cycle. The presented thesis represents a collection of 25 articles dealing with all aspects of PSII repair cycle in cyanobacteria, which are excellent model organisms to study oxygenic photosynthesis.

The PSII repair process includes a partial disassembly of the complex, its recognition by the protease, selective replacement of the D1 protein, re-assembly of the complex and activation of electron transfer. The photodamage seems to primarily occur at the $CaMn_4O_5$ cluster and results in modified binding of lumenal proteins leading to monomerization of the PSII dimeric structure and release of CP43 antenna. Resulting PSII subcomplex lacking CP43 is recognized by a protease that selectively degrades the D1 protein. The obtained data support a model in which damaged D1 is removed by homologues of a bacterial-type metalloprotease FtsH, which degrade the protein from the N-terminus in a processive reaction without accumulation of degradation fragments. Deg proteases are not required for D1 degradation in *Synechocystis* 6803 but might play an auxiliary role in D1 degradation under extreme conditions when D1 forms various cross-linking products with other PSII subunits. We also postulate that a similar N-terminal mechanism of D1 degradation also operates in chloroplasts. It can also explain the existence of several forms of the D1 protein which were found in many cyanobacteria and which differ in the N-terminal amino acid sequences and in the rate of the light-induced degradation. A typical example of such a cyanobacterium is *Synechococcus* PCC 7942 which contains two different forms of the D1 protein. Under increased irradiance the so called "low light" form D1:1 is replaced by "high light" form D1:2 which is able to

better cope with increased rate of PSII photoinactivation by accelerating the D1 replacement process. Both forms of the protein kept the different ability to repair PSII even in the cyanobacterium *Synechocystis* in which the coding region for original D1 was replaced by either the D1:1 or D1:2 form from *Synechococcus*.

The rate and specificity of the repair-related D1 degradation is dependent on the ongoing protein synthesis suggesting requirement for de novo synthesized protein factor(s). The direct proportion between the rate of D1 degradation and the level of transcript of the *psbA* gene encoding the D1 protein shows that the required factor is the D1 protein alone. It indicates that the D1 protein is rapidly and selectively degraded only when a copy of the newly synthesized protein is prepared for the prompt insertion into the PSII complex. When the copy is not available, the FtsH protease may degrade D1 slower and together with D2.

It is not clear which is the fate of Chl molecules released during the degradation of D1. Identification of tentatively Chl-binding small high light-induced proteins (HLIPs) in PSII complex suggests that these proteins with similarity to plant light-harvesting CAB proteins (synonym SCPs, small CAB-like proteins) may transiently bind Chl released during the degradation of D1. Nevertheless, the D1 replacement process seems to consume large amount of newly synthesized Chl and its rate is dependent on Chl availability.

The efficient replacement process requires the fast synthesis of D1. The newly synthesized protein becomes stabilized by association with a small subunit PsbI and also requires interaction with the auxiliary lumenal factor YCF48. The mutant of *Synechocystis* lacking YCF48 exhibits deficiency in the de novo assembly of PSII as well as in the PSII repair. Binding of YCF48 to D1 is stabilized by its interaction with the cleavable C-terminal extension of the precursor form of D1 and stabilization effect of the extension on the interaction between D1 and YCF48 may represent the primary function of the extension in oxygenic phototrophs. In contrast to YCF48, another PSII auxiliary factor Psb27 bound to the antenna CP43 does not influence significantly the PSII repair.

When the FtsH protease cannot operate (extreme stress conditions, mutants lacking particular FtsH protease, *in vitro* experiments) non-repaired PSII complexes generate reactive oxygen species (ROS) that attack D1 and other PSII proteins and inhibit the repair process. Singlet oxygen was shown to cause oxidation of the D1 protein reflected by shift of its electrophoretic mobility and oxidized D1 also cross-links with other Photosystem II subunits like alpha subunit of cytochrome b-559. On the other hand action of hydroxyl radicals results in generation of specific fragments substantiating the ability of ROS to cause direct scission of the D1 peptide bonds without the assistance of proteases.

Souhrn

Fotosystém II (PSII) je membránový pigment-proteinový komplex, který využívá světelnou energii pro oxidaci vody na molekulární kyslík a vysílá elektrony do fotosyntetického elektrontransportního řetězce oxygenních fototrofů, tj. rostlin, řas a sinic. Většina pigmentů a kofaktorů účastnících se elektronového transportu v PSII je vázána na centrální podjednotky reakčního centra (RC) D1 a D2, které tvoří heterodimer. Na každé straně tohoto heterodimeru se nacházejí anténní proteiny CP43 a CP47, které váží chlorofyl (Chl), dodávají energii pigmentům RC a v případě CP43 se také účastní spolu s proteinem D1 vazby klastru $CaMn_4O_5$ účastnícího se oxidace vody. PSII dále obsahuje nejméně 13 malých membránových podjednotek vázaných na periferii komplexu a tři vnější lumenální proteiny, které stabilizují klastr $CaMn_4O_5$. Unikátní fotochemické vlastnosti pigmentů RC PSII umožňující vytržení elektronů z vody mají přímý vztah k vysoké citlivosti PSII k světlem indukovanému poškozování (fotoinaktivaci). Toto poškozování je ireversibilní a pro obnovení fotochemické aktivity musí komplex PSII podstoupit sérii kroků, které tvoří takzvaný opravný cyklus. Předkládaná práce je souborem 25 článků zabývajících se všemi aspekty opravného cyklu PSII u sinic, které představují vynikající modelový organismus pro studium oxygenní fotosyntézy.

Proces opravy PSII zahrnuje částečný rozpad komplexu, jeho rozpoznání proteázou, selektivní výměnu proteinu D1, zpětné složení komplexu a aktivaci elektronového transportu. K primárnímu poškození komplexu světlem nejpravděpodobněji dochází v okolí klastru $CaMn_4O_5$ a má za následek modifikovanou vazbu lumenálních podjednotek, což vede k monomerizaci dimerní struktury PSII a uvolnění CP43. Vzniklý subkomplex s chybějícím CP43 je rozpoznán proteázou, která selektivně degraduje protein D1. Námi získaná data podporují model, ve kterém poškozený protein D1 je odstraněn homology bakteriální metaloproteázy FtsH, která degraduje protein kontinuálně od N-konce bez akumulace degradačních fragmentů. Proteázy Deg nejsou pro degradaci proteinu D1 v sinici potřebné, i když mohou hrát pomocnou úlohu za extrémních podmínek, když protein D1 tvoří různé zesítěné produkty s jinými proteiny PSII. Předpokládáme také, že podobný N-terminální mechanismus degradace D1 rovněž funguje v chloroplastech. Mechanismus také dokáže vysvětlit existenci několika forem proteinu D1, které byly nalezeny u mnoha sinic a které se liší sekvencí u N-konce a rychlostí světlem indukované degradace. Typický příklad takové sinice je *Synechococcus* PCC 7942, který obsahuje dvě odlišné formy proteinu D1. Za zvýšené ozářenosti tzv. „nízkosvětelná“ forma D1:1 je nahrazena „vysokosvětelnou“ formou D1:2, která je schopna se lépe vyrovnat s vyšší rychlostí fotoinaktivace díky rychlejší

výměně proteinu D1. Je zajímavé, že tato odlišná schopnost obou proteinů je zachována po jejich vnesení do sinice *Synechocystis*, u níž byla kódující oblast pro originální protein nahrazena kódující oblastí pro D1:1 nebo D1:2 ze sinice *Synechococcus*.

Rychlost a specifičnost degradace D1 je závislá na funkční proteosyntéze, což naznačuje potřebu nově syntetizovaného proteinového faktoru. Přímá závislost mezi rychlostí degradace D1 a hladinou transkriptu genů *psbA* kódujících D1 ukazuje, že potřebným faktorem je nově syntetizovaný protein D1. To naznačuje, že protein D1 je rychle a selektivně degradován pouze pokud je nově nasyntetizovaná kopie připravena k vsunutí do komplexu namísto staré. Pokud tato kopie není dostupná, proteáza FtsH degraduje protein D1 pomaleji a s ním degraduje i protein D2.

Není jasné, jaký je osud molekul Chl uvolněných během degradace D1. Identifikace malých, vysokým světlem indukovaných proteinů hypoteticky schopných vázat Chl (HLIPs) v komplexu PSII ukazuje, že tyto proteiny podobné rostlinným světlosběrným anténám CAB (od toho synonymum SCPs, small CAB-like proteins) mohou dočasně vázat Chl uvolněný během degradace D1. Nicméně se ukazuje, že proces výměny D1 spotřebovává velké množství nově syntetizovaného Chl a rychlost výměny je závislá na jeho dostupnosti.

Efektivní proces výměny vyžaduje rychlou syntézu proteinu D1. Nově syntetizovaný protein D1 je stabilizován malou podjednotkou PSII označovanou PsbI a také vyžaduje interakci s pomocným lumenálním faktorem YCF48. Mutant sinice *Synechocystis* s chybějícím faktorem YCF48 není schopen tvořit dostatečné množství nových komplexů PSII a rovněž není schopen tyto komplexy efektivně opravovat. Vazba YCF48 na D1 je stabilizována interakcí s odštěpitelnou C-terminální extenzí prekurzoru proteinu D1 a právě stabilizace vazby mezi D1 a YCF48 může představovat primární funkci extenze u oxygenních fototrofů. Na rozdíl od YCF48, jiný pomocný lumenální protein Psb27, který se váže na anténu CP43, nemá významný vliv na proces opravy PSII.

Pokud proteáza FtsH nemůže pracovat (např. za extrémních stresových podmínek, u specifických mutantů nebo *in vitro*), neopravené komplexy PSII tvoří reaktivní formy kyslíku (ROS), které napadají protein D1 i ostatní podjednotky PSII a inhibují opravný proces. Singletní kyslík způsobuje oxidaci proteinu D1 detekovatelnou díky posunu elektroforetické pohyblivosti proteinu a oxidovaný D1 tvoří zesítěné produkty a agregáty se sousedními podjednotkami jako je např. podjednotka alfa cytochromu b-559. Naproti tomu hydroxylové radikály indukují tvorbu specifických fragmentů, což dokládá schopnost ROS přímo štěpit peptidové vazby v proteinu D1 bez asistence proteáz.

Introduction - the structure, assembly and function of the Photosystem II complex in cyanobateria

Photosystem II (PSII) is a multisubunit chlorophyll-binding protein complex belonging to photosynthetic reaction center (RC) complexes which perform the light-induced primary charge separation. This reaction represents the driving force for photosynthetic electron transfer and formation of the transmembrane proton gradient used to generate NADPH and ATP, respectively. Oxygenic photosynthesis performed by plants, algae and cyanobacteria is characterized by the presence of two RC protein complexes, PSII, which catalyzes electron transfer from water to plastoquinone, and Photosystem I (PSI) mediating electron transfer from plastocyanin or cytochrome c553 to ferredoxin. PSII is a very complex assemblage of polypeptide chains, pigments, lipids and other cofactors. The native functional form of the PSII complex in both cyanobacteria and plants is most probably dimer. The latest, most detailed structural model of the cyanobacterial PSII showed that it contains 17 intrinsic and three extrinsic protein subunits, 35 chlorophyll a (Chl a) molecules, two pheophytins, 12 carotenoids, two haem molecules, one non-haem iron, two plastoquinones, 25 lipid molecules, one calcium ion and a $CaMn_4O_5$ metal cluster that oxidizes water to dioxygen (Umena et al. 2011; Fig. 1). At the center of the complex there are two homologous RC subunits called D1 and D2, which both contain five transmembrane helices and which form heterodimer binding cofactors involved in the primary charge separation. On either side of the heterodimer are CP43 and CP47 inner antennae that bind Chl and β-carotene. CP43 in addition participates (together with D1) in ligating the oxygen evolving $CaMn_4O_5$ cluster (Ferreira et al. 2004, Umena et al., 2011). On the periphery of these large subunits there are 13 small, mostly single helix subunits and the lumenal part of the complex is shielded by three extrinsic subunits PsbO, PsbU and PsbV which stabilize the $CaMn_4$ cluster (reviewed by Roose et al. 2007). The genomes of cyanobacteria also contain genes homologous to ones coding for the PsbP and PsbQ proteins found on the lumenal side of PSII in chloroplasts. Mutagenesis experiments indicated their requirement for the optimal oxygen evolving activity (Thornton et al., 2004) although these proteins are not present even in the latest model structures of the cyanobacterial PSII (Ferreira et al., 2004, Guskov et al., 2009, Umena et al., 2011).

The stromal side of the cyanobacterial PSII also binds peripheral light-harvesting antennae called phycobilisomes that deliver energy to the RC and consist of a large number of water-soluble proteins with covalently attached chromophores phycobilins (Sidler 1994; Fig. 1). This type of antenna

significantly differs from antennae of higher plants that are embedded in the membrane and bind Chl *a* and *b* molecules and carotenoids (Liu et al. 2004).

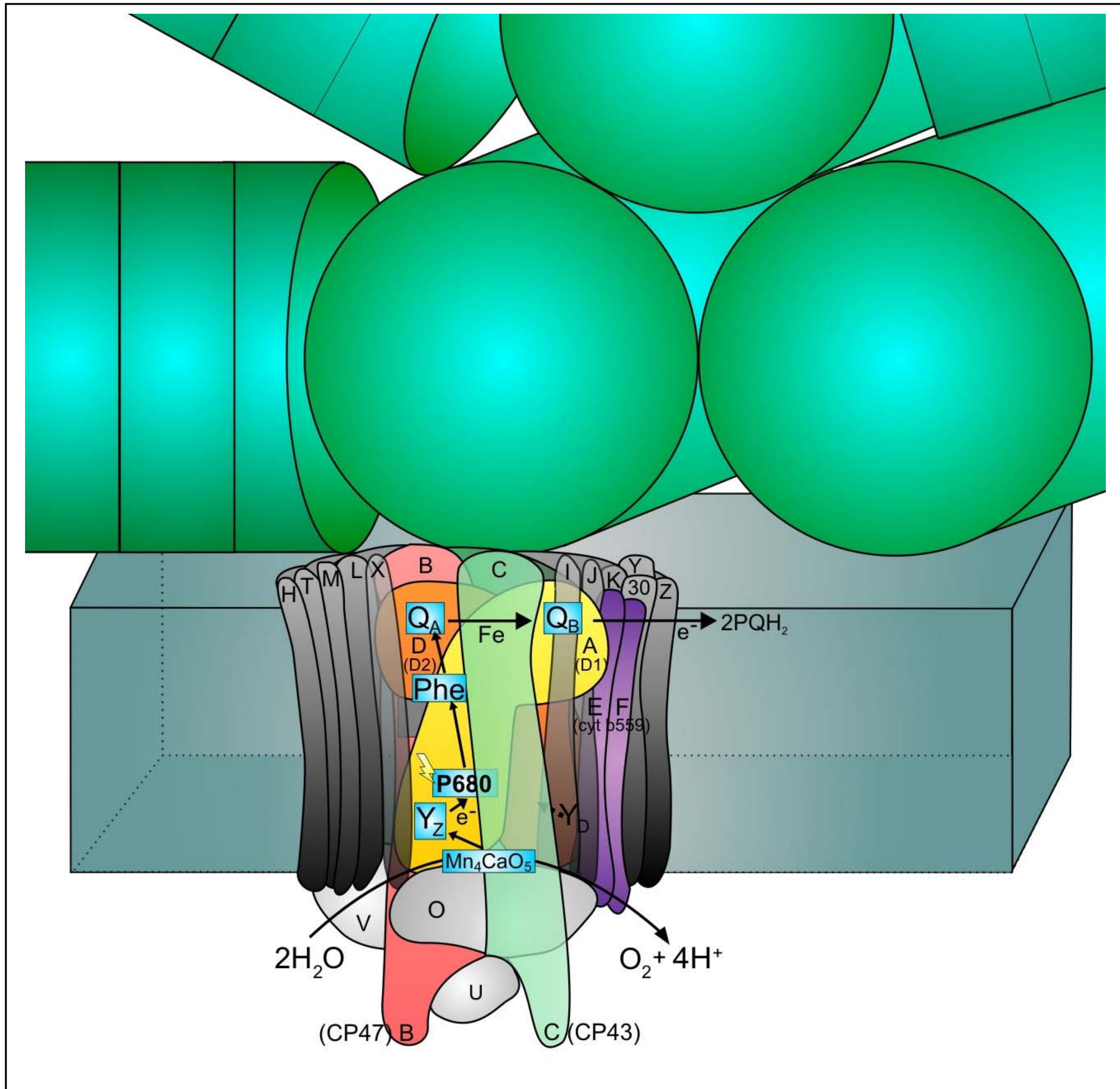

Fig 1. Scheme of the cyanobacterial Photosystem II complex showing approximate arrangement of electron transfer chain components and protein subunits including peripheral phycobilisome antenna (blue green).

Taking into account necessity to avoid the presence of free Chl, which can quickly generate highly destructive reactive oxygen species (ROS), biosynthesis of Chl and synthesis of the corresponding apoproteins must be precisely coordinated during PSII biogenesis. However, the exact mechanism, how this is achieved, remains unknown. For characterization of the PSII assembly process in cyanobacteria the strain *Synechocystis PCC* 6803 has proved to be an excellent model organism due to the ease of generating its specific mutants and its ability to grow on glucose in the absence of the functional PSII (Williams, 1988). Characterization of PSII mutants lacking specific large or small subunits

of the PSII complex in combination with modern biochemical tools have provided support for the stepwise assembly process involving a number of PSII assembly intermediate sub- or precomplexes. As in higher plants (Müller and Eichacker, 1999), cytochrome b-559 and D2 protein initiate PSII assembly to form a D2–Cyt b-559 precomplex (Komenda et al. 2004 and 2008) and then, after addition of another precomplex containing at least D1 and PsbI, (Dobákova et al. 2007), a PSII RC subcomplexes are formed (Komenda et al. 2008). Formation of these subcomplexes in *Synechocystis* is facilitated by a homologue of the plant HCF136 (YCF48, Slr2034) which stabilizes the newly synthesized D1 protein (Komenda et al. 2008). Then, CP47 subcomplex formed by attachment of small subunits PsbH, PsbL and PsbT to CP47 is attached to the PSII RC subcomplex to form the so-called RC47 complex (Komenda et al. 2004). Also formation of this subcomplex is regulated by an auxiliary protein factor termed Psb28 (Kazusa designation Sll1398), which is required for the efficient synthesis of CP47 (Dobáková et al. 2009).

Subsequent attachment of the CP43 subcomplex formed by binding of PsbK, PsbZ and Psb30 to CP43 and stabilized by the auxiliary factor Psb27 (Komenda et al. 2012), leads to formation of the monomeric PSII core complex (RCC1). At the level of RCC1 the assembly of the $CaMn_4O_5$ cluster and attachment of the lumenal extrinsic subunits, PsbO, PsbU and PsbV, occurs. Finally, two monomers form a dimer representing the native form of the complex. During the biogenesis of PSII, the D1 subunit is synthesized in most organisms as a precursor protein (pD1) with a C-terminal extension that in most cyanobacteria consists of 16 amino acid residues. This extension must be cleaved to allow assembly of a functional $CaMn_4O_5$ cluster (Nixon et al., 1992; Anbudurai et al., 1994) but its real function remains unclear.

Despite the presence of many proteins in PSII, majority of redox cofactors and prosthetic groups necessary for electron transfer from water to plastoquinone are bound to the D1 and D2 polypeptides (Ferreira et al. 2004). These includes four Chl molecules comprising the primary electron donor P680, the transient acceptor pheophytin (Pheo), the quinone acceptors Q_A and Q_B , transient donor Y_Z and the $CaMn_4O_5$ cluster (for approximate localization see Fig. 1). The light energy captured by the outer and inner antennae is transferred to four Chl molecules forming the primary electron donor P680. The excitation of P680 leads to the transfer of an energized electron from P680 to a nearby Pheo molecule within a few picoseconds forming the initial charge separation pair $P680^+Pheo^-$. Subsequently, the electron is transferred from $Pheo^-$ to the primary electron acceptor Q_A (plastoquinone molecule bound to D2) and subsequently to the secondary plastoquinone acceptor Q_B bound to D1. After the second light-induced charge separation event Q_B^- receives a second electron,

becomes protonated and it is replaced by a new oxidized plastoquinone molecule. The oxidized form of P680 is reduced by a redox-active tyrosine residue TyrZ (Y161 of the D1 protein (Debus et al. 1988)) within nano- to microseconds and this oxidized tyrosine is reduced by the $CaMn_4O_5$ cluster coordinated by the several histidine, aspartate and glutamate residues of D1 protein, and by the residue E354 of CP43 (Ferreira et al. 2004). The Mn cluster binds intermediates of water oxidation and after withdrawal of four electrons it releases molecular oxygen.

The unique ability of PSII to withdraw electrons from water seems to be directly related to its high vulnerability to a light-induced damage (photoinactivation) which occurs with certain frequency under all light conditions. Since the primary target of this damage is the D1 protein, it is probable that the PSII photoinactivation is caused by a loss of function of an electron transfer component bound to this central subunit of PSII. Since this loss of function is irreversible, the oxygenic phototrophs developed a sophisticated mechanism for continuous maintaining the PSII functionality. And just all aspects of this so called PSII repair cycle in cyanobacteria represent the topic of this thesis. The following chapter contains an overview of the present knowledge on the PSII repair cycle with emphasis on the contribution of the author to this subject. The references of articles included in the thesis are designated by the reference number in bold and these references are listed separately.

Repair cycle of the Photosystem II complex with emphasis on the contribution of the author to this research field

The Photosystem II repair cycle consists of a number of steps which are schematically illustrated in Fig. 2 (for review, see Nixon et al. 2005 and 2010, **Refs. 10 and 23**). Briefly, the repair cycle consists of partial disassembly of the dimeric PSII core complex, degradation of photodamaged D1 and insertion of the newly synthesized copy, re-assembly of the PSII core complex and finally activation of the electron-transport processes. Molecular details of most of these steps remain unknown and some of them are shared with the *de novo* assembly process. The repair cycle is assumed to be mostly initiated by the light-induced damage to PSII and *in vitro* studies suggested several damage mechanisms which differ in the primary site in which the PS II electron transport is interrupted. This interruption can occur at the donor or the acceptor side, or directly in RC (for reviews see Prášil et al. 1992; Aro et al. 1993; Mattoo et al. 1999). However, it is not clear yet which mechanism is dominant *in vivo*. The

recent studies more and more point out at the central role of the $CaMn_4O_5$ cluster which can be impaired by blue light directly absorbed by the cluster (recently reviewed in Murata et al. 2007). In this respect there is an interesting question concerning the nature of the signal that triggers the repair process. The study of PSII repair following photoinhibition under low temperature in the thermophilic cyanobacterium *Synechococcus elongatus* showed that PSII inactive in oxygen evolution, but still fully assembled, is already marked for the D1 replacement (Komenda and Masojídek 1995, **ref. 1**). Many reports suggested control of the D1 degradation process by the conformation of the Q_B binding pocket (Kirilovsky et al. 1988; Gong and Ohad 1991; Komenda and Barber 1995, **ref. 2**). However, more recent analyses of *Synechocystis* PCC 6803 mutants with large D1 deletions did not support requirement of specific sequences nearby the Q_B pocket for the fast D1 degradation (Nixon et al. 1995, **ref. 3;** Mulo et al. 1998). In contrast, our recent study of *Synechocystis* mutants with various defects at lumenal side of PSII causing improper function of the $CaMn_4O_5$ cluster, showed that all these defects were effectively recognized by the repair machinery leading to the fast D1 replacement (Komenda et al. 2010, **ref. 22**). On the other hand, certain mutations on the acceptor side of PSII either did not change the rate of D1 degradation (deletion of the PEST sequence, Nixon et al. 1995, **ref. 3**) or inhibited it (missing PsbH protein, Komenda and Barber, 1995, **ref. 2;** or replacement of the D1 amino acid residue Ser264 by Pro, Dalla Chiesa et al. 1997). Thus, the available data indicate that the PSII repair process is most probably triggered at lumenal side of PSII by changes around the $CaMn_4O_5$ cluster.

The triggering event is accompanied or quickly followed by the monomerization of the PSII core complex (Barbato et al. 1992c; Komenda et al. 2007b; **ref. 17**). In this respect it is important to note that in all strains with defects at the PSII lumenal site the dimeric form of PSII core complex is destabilized (Komenda et al. 2010, **ref. 22**). This finding is in agreement with the role of lumenal subunit PsbO in the stabilization of the dimer inferred from the structural model of the cyanobacterial PSII (Umena et al. 2011) showing interaction of PsbO from one monomer with CP47 of the second monomer within the single dimer. In this context it is easy to imagine that any change at the lumenal part of PSII (including that caused by photoinactivation) may have an adverse effect on the strength of the interaction between PsbO and CP47 and may cause destabilization of the dimer.

In order to get access to the D1 protein in the inactive PSII core complex for its proteolysis, the detachment of CP43 antenna is required as the next step in the cycle. This photoinactivation-induced detachment was observed in both higher plants (Barbato et al. 1992c) and cyanobacteria (Komenda and Masojídek

1995; **ref. 1**) and resulting sub-core complex RC47 becomes immediately the target for the protease which removes the impaired D1 protein. In analogy, the same complex was suggested as a target for the D1 degradation and as an acceptor of the newly synthesized D1 protein in green algae (Adir et al. 1990) and in higher plants (Zhang et al. 2000).

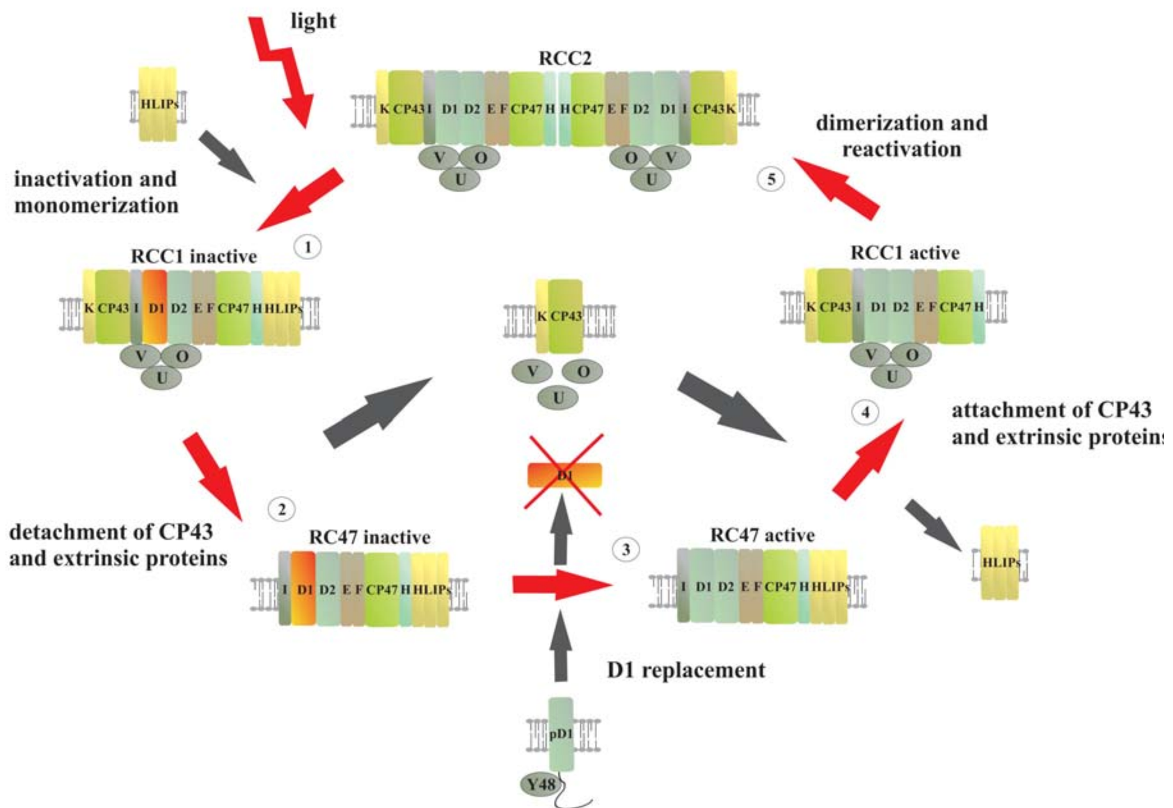

Fig. 2. The repair cycle of Photosystem II in cyanobacteria. PSII repair cycle starts with inactivation of PSII dimer RCC2 and its monomerization (step 1). At this stage HLIPs are most probably attached. Then CP43 and extrinsic proteins are detached (step 2) and the old D1 is selectively replaced by a newly synthesized copy in the RC47 complex (step 3). Finally, CP43 and extrinsic proteins are re-attached, HLIPs detached (step 4), and the repaired PSII monomer is dimerized with concomitant reactivation of the oxygen evolving machinery (step 5).

The mechanism of CP43 detachment is not known. However the surprising finding that the $CaMn_4O_5$ cluster is partly liganded by residues of CP43 (Ferreira et al. 2004) tempts to hypothesis that damage to the cluster directly leads to such a destabilization of binding between D1 and CP43 that CP43 is finally released. It is interesting that such a destabilization of CP43 binding is caused in *Synechocystis* by deletion of the *psbI* gene that codes for a small PSII subunit PsbI bound to the D1 protein at the interface with CP43 (Dobáková et al. 2007, **ref. 18**). In *Synechocystis* the RC47 complex usually

does not accumulate (Komenda et al. 2006, **ref. 12**) unless the photoinactivation occurs at low temperature decreasing the proteolytic activity of the enzyme (Komenda and Masojídek 1995, **ref. 1**), the gene encoding the protease is inactivated (Komenda et al. 2006, **ref. 12)** or the availability of the D1 for protease is lowered by its N-terminal truncation (Komenda et al. 2007b, **ref. 17**). The idea that the D1 protein is selectively replaced just in the RC47 complex is supported on one hand by very fast and selective D1 turnover in this complex observed in the strain lacking the *psbC* gene coding for CP43, and on the other hand by concomitant degradation of both D1 and D2 in the RC complexes accumulating in the strain lacking CP47 (Komenda et al. 2004**;** Komenda, unpublished data).

The selective replacement of the D1 protein is one of the most important steps of the PSII repair cycle (for reviews see Nixon et al. 2005 and 2010, **Refs. 10 and 23**). It can be documented experimentally by the fast incorporation of radioactively labeled amino acids into the newly synthesized protein (radioactive pulse) followed by their fast replacement with the "cold" ones during the degradation process (radioactive chase, Komenda and Barber 1995, **ref. 2**). Using this technique, the unusually fast turning-over chloroplast protein was for the first time identified in plants and algae in early eighties of the previous century (Mattoo et al. 1981, Kyle et al. 1984) and later also in cyanobacteria (Goloubinoff et al. 1988). Alternatively, the existence of such a fast turning-over photosynthetic protein was inferred from experiments that monitored a fast decline of the photosynthetic activities when chloroplast protein synthesis had been stopped (Šetlík et al. 1981). Interestingly, the combination of both above mentioned approaches revealed that the degradation of radioactively labeled D1 in the presence of protein synthesis inhibitors is slower than that without inhibitors. It showed requirement for ongoing synthesis of a protein factor needed for maximal rate of the D1 degradation (Komenda and Barber 1995, ref. 2; Komenda *et al.* 1999, **ref. 4**). Based on this result we hypothesized that this requirement in fact reflects synchronization between the processes of D1 synthesis and degradation and that the required factor is the newly synthesized D1 protein. The hypothesis was subsequently supported by our study of *Synechocystis* mutants that primarily differed in the type and level of the *psbA* transcript (Komenda *et al.* 2000, **ref. 6**). The study showed that strains with higher level of the *psbA* transcript and higher synthesis of D1 also faster degrade the D1 protein. The synchronization of the D1 degradation and synthesis seems to prevent premature degradation of the D1 protein (without possibility of immediate insertion of the D1 copy) that would lead to the extensive disassembly of the PSII complex and a release of pigments that generate ROS (Komenda and Masojídek 1995, **ref. 1**). Despite the apparent

importance of the synchronization process, its exact mechanism remains unknown. In higher plant, the synchronization process is complicated by the structural heterogeneity of chloroplast thylakoids that consist of stacked granal membranes and unstacked stromal lamellae. Most of functional PSII is located in grana and also the light-induced damage occurs in this membrane compartment which is inaccessible to protein synthesis machinery. Therefore, the potential degradation of D1 in grana cannot be immediately followed by insertion of its newly synthesized copy. To cope with this problem, plants phosphorylate the D1 protein which drives the whole PSII complex into stromal membranes in which the protein is dephosphorylated and subsequently replaced (Rintamäki et al. 1996). In agreement with this scenario, the stromal membranes were shown to contain protease which is assumed to degrade D1 (Komayama et al 2007, see below).

In cyanobacteria the synchronization between degradation and synthesis is expected to be simpler since their thylakoid membranes are rather uniform without granal regions (Stanier 1988). However, the cyanobacterial thylakoids are organized in concentric layers and our data indicate that the D1 replacement can occur just in membranes close to the cell surface nearby the cytoplasmic membrane (Komenda, unpublished results). Since the cyanobacterial D1 protein (and any other PSII proteins) cannot be phosphorylated (Komenda et al. 2002), there should be different mechanism which directs movement of PSII complexes destined for repair.

For long time the crucial question concerning the D1 replacement process was the identity of the protease responsible for the degradation of the impaired copy of D1, and the mechanism how is the protein selectively removed from the complex. Cyanobacteria and chloroplasts contain many homologues of bacterial proteases and among them Clp, Deg and FtsH proteases have been proposed to participate in the degradation of the plant D1 (Adam and Clarke, 2002). While there is no recent, really solid support for the role of Clp protease in the D1 replacement, studies of *Arabidopsis* mutants revealed that chloroplast ATP-dependent metalloproteases FtsH and ATP independent serine protease Deg play an important role in the effective removal of the impaired D1 protein (for recent review see Kato and Sakamoto, 2009).

For long time it has been believed that the D1 protein is initially cleaved in the vicinity of the Q_B binding pocket giving rise to 23 kDa N-terminal (Greenberg et al. 1997) and 10 kDa C-terminal (Barbato et al. 1991) fragments that are subsequently completely removed. Based on *in vitro* experiments using isolated *Arabidopsis* stromal protease DegP2 Haussühl et al. (2001) proposed that the initial cleavage is performed by this protease and the FtsH proteases are subsequently responsible for disappearance of the fragments. This was

demonstrated *in vitro* using the isolated overexpressed FtsH1 from *Arabidopsis* that was able to degrade the light-induced 23 kDa N-terminal fragment of D1 in the ATP and Zn-dependent manner (Lindahl *et al.* 2000). However, we challenged the concept of coordinated action of Deg and FtsH proteases in the cyanobacterium *Synechocystis* which contains three Deg proteases designated HtrA, HhoA and HHoB. Deletion of genes encoding all three proteases did not lead to the inhibition of the D1 turnover and in fact even a certain acceleration of the degradation process was observed (Barker at al. 2006, **ref. 13**). This result nearly excluded involvement of this type of protease in the D1 degradation during PSII repair process in cyanobacteria. Later, deletion of the genes for *Arabidopsis* Deg proteases provided evidence that neither the chloroplast stromal (Huesgen et al. 2006), nor lumenal (Sun et al. 2007 and 2010) Deg proteases play a crucial role in the repair related degradation of D1 *in vivo*. In contrast, the efficiency of the PSII repair process and the rate of the D1 degradation is drastically reduced in an *Arabidopsis* mutant lacking the chloroplast FtsH variant Var2 (Bailey et al. 2002) and in analogous *Synechocystis* strain lacking the homologous protease FtsH2 (Silva et al. 2003). FtsH2 is one of four FtsH protease homologues present in *Synechocystis* (Mann et al. 2000). While FtsH1 and FtsH3 seem to be indispensable for the cyanobacterium, the remaining FtsH4 does not seem to be important under common cultivation conditions (Mann et al. 2000). In the following, a more detailed study of the FtsH2 function in *Synechocystis* we provided evidence that inactivation of the *ftsH2* gene indeed results in inhibition of the D1 proteolysis which is accompanied by accumulation of the RC47 complex (Komenda et al. 2006, **ref. 12**). Moreover, FtsH2 was found to be essential for removal of the non-functional mutated D1 protein and also for removal of unassembled PSII proteins D2 and CP47. This latter function of FtsH2 can be used for isolation and characterization of assembly intermediate complexes like RC47 or CP47 since these complexes overaccumulate and could be more easily isolated after inactivation of the *ftsh2* gene (Boehm et al. 2011, **ref. 24**). In contrast, accumulation of the unassembled D1 was hardly affected by inactivation of FtsH2 in the strain lacking D2 indicating that the level of unassembled D1 is controlled by other proteases (Komenda et al. 2010, **ref. 22**).

The identification of FtsH protease as the key player in the PSII repair cycle also pointed out to a possible mechanism of the D1 degradation. The model FtsH protease from *Escherichia coli* contains two transmembrane segments, a short N-terminal part involved in oligomerization and a long C-terminal region in which a zinc-metalloproteinase active site and the ATPase domain are located (Langer 2000). The protease forms hexameric complex with central cavity containing proteolytic sites into which are protease substrates translocated by the

action of ATPase domains (Bieniossek et al 2006). The protease also forms supercomplexes with specific members of the Band 7 superfamily of proteins that are bound to periplasmic loop of the protease between transmembrane segments and regulate its proteolytic activity (for review see Ito and Akiyama 2005). Genome of *Synecocystis* also contains several genes encoding proteins homologous to Band 7 proteins but we did not obtained any results that would evidence their association with FtsH or regulation of FtsH activity and substrate specificity (Boehm et al., 2009; **ref. 21**). In *Escherichia coli* the protease mostly initiates degradation of the target proteins at its N- or less frequently C-terminus and then continues in the processive way up to the end of the molecule (Ito and Akiyama 2005). The requirement for the protein to be efficiently degraded by this mechanism is sufficient length of the terminus, at least 20 amino acid residues (Chiba et al., 2000). In the case of the of D1, only the N-terminal tail fulfills this length requirement and, in addition, it is also exposed to the same stromal side of the membrane as the proteolytic domain of FtsH and sufficiently protrudes from the overall structure of the cyanobacterial PSII complex (Umena et al. 2011). We experimentally supported the N-terminal model of the D1 degradation *in vivo* by constructing *Synechocystis* mutants with the N-terminus truncated to various extent (Komenda et al. 2007b, **ref. 17**). Their characterization confirmed that shortening of D1 by 20 residues inhibits D1 degradation and PSII repair documenting that the processive degradation of D1 by FtsH alone starting from the N-terminus seems to be a convincing alternative to earlier models that emphasized initial cleavage of the protein (Lindahl et al., 2000; Haussühl et al., 2001). The N-terminal mechanism is also able to explain selectivity of the D1 degradation and reason why the PSII subunits, which are in the contact with the D1 protein, are not degraded either. This especially concerns the small subunit PsbI that is very closely associated with D1 and seems to stabilize D1 already during its synthesis. Unlike D1, PsbI is stable and is not turned-over with D1 (Dobáková et al. 2007, **ref. 18**). The cyanobacterial structural model of PSII showed that the N-terminus of PsbI is oriented into lumen and therefore, it cannot be drawn into the stromal proteolytic cavity of FtsH. In the case of D2, its N-terminus is much less protruding from the PSII structure in comparison with D1 and is therefore less accessible to the protease (Umena et al. 2011). The N-terminal mechanism of the D1 degradation seems to be also related to the presence of several D1 forms in many cyanobacterial strains. These forms usually differ in the amino acid sequence at the N-terminus, and depending on the momentary presence of the particular form, the cells differ in the ability to repair PSII under high light conditions. A typical representative of cyanobacteria having two forms of the D1 protein is *Synechococcus* PCC 7942. The genome of this strain contains one *psbA* copy (*psbAI*) which encodes

so called low light D1:1 form while remaining two gene copies *psb*AII and *psb*AIII encode high light D1:2 form. The variants differ in 25 aminoacid residues and there are 12 differences localized among first 16 residues (Golden et al. 1986). When exposed to increased irradiance, the D1:1 form synthesized under low irradiance is quickly exchanged by the D1:2 form and the reverse process occurs after transfer of the cells from high to low irradiance (Schaefer and Golden 1989; Clarke et al. 1993; Komenda et al. 1999, **ref. 4**). Our study showed that under increased irradiance the D1:2 variant exhibits a faster turnover and provides better protection of PSII against photoinactivation than D1:1 (Komenda 2000, **ref. 5**). It is interesting that the higher ability of D1:2 to repair PSII in comparison to D1:1 is retained even when the genes for these D1 variants from *Synechococcus* are expressed in *Synechocystis* (Tichy et al. 2003, **ref. 8**). We assume that the N-terminal model is valid also for D1 degradation in higher plants and it could easily explain the inhibitory effect of N-terminal phosphorylation on the D1 degradation (Rintamäki et al. 1996).

The fast and selective FtsH-mediated removal of the D1 protein during PSII repair is observed when immediate replacement by the newly synthesized D1 copy occurs. If synthesis of D1 is limiting factor, FtsH protease can degrade also the D2 protein. The light intensity saturation curve for the degradation of plant D2 *in vivo* resembles that for the D1, except that half-life of D2 is about 3 times longer than those of D1 while the degradation ratio D2/D1 is irradiance independent (Jansen *et al.* 1999). However, under extreme stress conditions, in FtsH-less mutants or *in vitro* when FtsH-mediated degradation is too slow or cannot proceed, the inactive PSII complex generates ROS that can attack most of PSII proteins and cofactors. This attack is manifested by slow disappearance of D1 which is preceded by smearing and by decrease in electrophoretic mobility of the original D1 protein band (e.g. Shipton and Barber 1992, Miyao 1994). This is most probably a consequence of conformational changes in protein secondary structure caused by oxidation (He et al. 1991). The D1 oxidation under these conditions was confirmed by detection of carbonyl groups on such a smeared D1 protein band (Komenda et al. 2002). Our study dealing with the direct effect of various ROS on the D1 protein isolated from *Synechocystis* provided evidence that singlet oxygen was the predominant species causing this modification (Lupínková and Komenda 2004, **ref. 9**). The other manifestation of ROS attack on the D1 protein was a formation of cross-linked protein species consisting of the oxidized D1 protein and neighbor PSII protein subunits. The most frequently observed cross-linking products detected in illuminated or ROS-treated PSII preparations are the D1-D2 heterodimer, the D1-cytochrome b-559 adduct and the D1-CP43 cross-linking products (Yamamoto 2001). We clarified origin of the adduct of the D1 protein with the

α-subunit of cytochrome b_{559}. This cross-linking product had been described for the first time by Barbato *et al.* (1992b) in illuminated thylakoid membranes from higher plants and using site-specific mutant of *Synechocystis* and by chemical modification of histidine residues and free amino groups we identified the adduct as a product of reaction between the oxidized residue His252 of the D1 polypeptide and the N-terminal amino group of the cytochrome alpha subunit (Lupínková et al. 2002, **ref. 7**). Although the relationship between the degradation and cross-linking of the D1 protein is not clear, it is probable that the cross-linking does not allow fast FtsH-mediated D1 degradation and therefore, the formation of the D1 adducts should compete with the regular degradation of the D1 protein typical for the PSII repair cycle (Yamamoto 2001). In agreement with this hypothesis there is a slower degradation of the D1 protein in cyanobacterial mutants in which the 41 kDa adduct is detected (Dalla Chiesa et al. 1997, Lupínková and Komenda 2004, **ref. 9**). Action of ROS on the D1 protein may also result in formation of discrete protein fragments which were originally ascribed to the action of proteases (Aro et al. 1992). Our observation that hydrogen peroxide can directly cleave the D1 protein (Lupínková and Komenda 2004, **ref. 9)** supports the model that under certain circumstances ROS chemically cleave the peptide bonds of the D1 protein without assistance of proteases (Miyao 1994, Miyao *et al.* 1995).

ROS generated in damaged PSII attack also other PSII proteins, especially D2 and to lesser extent the inner antenna CP43. They may undergo the same type of ROS-induced modification as D1 (Barbato *et al.* 1992a; Shipton and Barber 1994; Sharma *et al.* 1997). Interestingly, the second PSII inner antenna CP47 seems to be more resistant to attack by ROS than the other large membrane subunits of PSII. The high stability of CP47 is also manifested by the lowest incorporation of the radioactive methionine among large PSII subunits during radioactive pulse labeling (Komenda et al. 2004). A possible reason for CP47 stability may be its association with so called small high-light induced proteins (HLIPs) (Dolganov et al. 1995) which are also designated as small CAB-like proteins (Scps) due to their sequence similarity to plant light-harvesting antennae encoded by CAB genes (Funk and Vermaas 1999). The *Synechocystis* genome contains five genes encoding Scps, four of them *scpB*, *scpC*. *scpD* and *scpE* encode small proteins in the range of 5-7 kDa, the last *scp* gene named *scp*A encodes the enzyme ferrochelatase consisting of a Scp-like membrane domain merged with the enzymatic heme-synthesizing part (Funk and Vermaas 1999). We have found three of the four small proteins, namely ScpB, ScpC and ScpD in the PSII core complexes (Yao et al. 2007, **ref. 15**) and ScpD was shown to bind to CP47 antenna in the vicinity of the small PSII subunit PsbH (Promnares et al. 2006, **ref. 14**). Moreover, our recent unpublished

data show that ScpB and ScpC are also bound to CP47 and remaining two Scps, ScpE and ScpA (ferrochelatase) can also under certain circumstances bind to PSII complexes (Komenda and Sobotka, unpublished results). Function of these stressed induced proteins is not quite clear yet, a role in regulation of Chl (Xu et al. 2002) or recycling Chl released from degraded Chl-proteins including the D1 protein was suggested (Vavilin et al. 2007). We have found out that under the extremely high irradiance (about 2000 μE m^{-2} s^{-1}) the absence of ScpD and/or ScpE (but not ScpC or ScpB) leads to the ROS-induced damage to PSII subunits including CP47 (Komenda, unpublished results) which is in agreement with the role of Scps in scavenging and possibly recycling Chl molecules released during the stress-induced damage to PSII.

There are currently very scarce data on the fate of cofactors and especially Chl molecules that are released from the degraded D1 protein. These molecules can be either immediately bound to the newly inserted D1 molecule (we do not know whether the newly synthesized D1 molecule already contains Chl before its insertion into PSII), or it is released and degraded or reused for synthesis of other Chl-binding proteins. Our recent study of the D1 turnover in PSII donor side mutants showed that the fast D1 turnover might be a crucial consumer of Chl and for instance in the strain lacking PsbO proteins D1 is turned-over in low light so quickly that probably consumes most of synthesized Chl which consequently becomes unavailable for other Chl-binding proteins and the strain stops growth even in the presence of glucose (Komenda et al. 2010, **ref. 22**). Our data also show that availability of Chl significantly affects efficiency of the PSII repair. In the strain lacking a small protein Psb28 which is important for efficient synthesis of CP47 and PSI, an increased amount of Chl available for the D1 replacement leads to the faster PSII repair than in the control wild-type strain (Dobáková et al. 2009, **ref. 20**). On the other hand, we have identified the cyanobacterial homologue of the chloroplast factor HCF136 (YCF48) as the key regulator of the D1 accumulation and in its absence D1 synthesis is diminished while PSI overaccumulates (Komenda et al. 2008, **ref. 19**), most probably due to the large quantity of available Chl that can be used for synthesis of PSI. In *Synechocystis* the D1 protein is synthesized as a precursor with the C-terminal extension consisting of 16 amino acid residues that is cleaved in two steps after residues Ala352 (Komenda et al. 2007a, **ref. 16**) and Ala344 (Nixon et al. 1992). The extension is at least a part of the domain that binds the YCF48 factor although it is not essential for the binding (Komenda et al. 2008, **ref. 19**). The mutants of *Synechocystis* lacking the extension (or just its half) exhibits less efficient PSII repair (Kuviková et al. 2005, **ref. 11**), most probably due to the limitation in the D1 synthesis.

It is assumed that other protein factors that are not components of the fully active dimeric PSII complex are required for the correct process of PSII repair. Among them, the Psb27 lipoprotein associated with the lumenal part of PSII had been postulated to participate in the process (Nowaczyk et al. 2006). However, our very recent results did not confirm this participation and instead, they supported the function of the protein in the *de novo* biogenesis of the antenna CP43 and its stabilization (Komenda et al., 2012, **ref. 25**).

Conclusion

The requirement for PSII repair is definitely a weak link in maintaining stable photosynthetic activity in the light, namely when combined with adverse environmental conditions, and is therefore considered a potential target for crop improvement. Especially, the recently discovered importance of FtsH complexes for the degradation of damaged PSII proteins during the PSII repair suggested that manipulating FtsH levels could represent one route leading to the improved resistance of plants to photoinhibition and consequently to the increased crop. Such a prospect could not be accomplished without the detailed knowledge on the PSII repair that progressed tremendously since the discovery of the repair in the late seventies of the last century. The work presented in this thesis significantly contributed to this progress. This is documented by frequent citation of the papers included in the thesis (nearly 500 citations) and by several invitations of the author to give plenary lectures on this topic at important international conferences like Gordon Research Conference on Photosynthesis and Photosynthesis Research Congress. The work on PSII repair also attracted fruitful collaborations with foreign academic institutions (Imperial College London, LMU Munich) and allowed several students to complete their PhD studies.

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List of publications presented for the thesis

1. Komenda, J., Masojídek, J. Structural changes of Photosystem II complex induced by high irradiance in cyanobacterial cells. Eur. J. Biochem. 233, 677–682, 1995; IF 3.13, number of citations 22
2. Komenda, J., Barber, J. Comparison of *psb*O and *psb*H deletion mutants of *Synechocystis PCC* 6803 indicate that degradation of D1 protein is regulated by the Q_B site and is dependent on protein synthesis. Biochemistry 34, 9625–9631, 1995; IF 3.23, number of citations 57
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