DOCTORAL THESIS

GENERATION OF REACTIVE OXYGEN SPECIES BY PHOTOSYSTEM II

BY

RAKESH KUMAR SINHA

DEPARTMENT OF BIOPHYSICS
CENTER OF THE REGION HANÁ FOR BIOTECHNOLOGICAL AND AGRICULTURAL RESEARCH
FACULTY OF SCIENCE, PALACKÝ UNIVERSITY
OLOMOUC, CZECH REPUBLIC

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Declaration

Hereby I declare that the Ph.D. thesis is my original work and that I have written it by myself using the literature listed in the section “References”.

In Olomouc, ……

Rakesh Kumar Sinha

List of papers

The thesis is based on the following papers. These papers are referred in the text by the corresponding roman numerals and are enclosed at the end of the thesis.

I. **Sinha RK**, Tiwari A, Pospíšil P (2010) Water-splitting manganese complex controls light-induced redox changes of cytochrome b_{559} in Photosystem II, Journal of Bioenergetics and Biomembranes 42 (4), 337-344.

II. Yadav DK, Kruk J, **Sinha RK**, Pospíšil P (2010) Singlet oxygen scavenging activity of plastoquinol in photosystem II of higher plants: Electron paramagnetic resonance spin-trapping study. Biochimica et Biophysica Acta 1797, 1807–1811.

III. **Sinha RK**, Komenda J, Knoppová J, Sedlářová M, Pospíšil P (2012) Small CAB-like proteins prevent formation of singlet oxygen in the damaged Photosystem II complex of the cyanobacterium *Synechocystis sp*. PCC 6803. Plant, Cell and Environment 35, 806–818**.**

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Abbreviations

BSA	bovine serum albumin
CAT	catalase
cyt b_{559}	cytochrome b_{559} in photosystem II
cyt b_{559} LP, HP	low- and high-potential form of cytochrome b_{559}
D1, D2	D1 and D2 proteins of photosystem II
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DEPMPO	diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide
DEPMPO-OH	hydroxyl radical adduct of DEPMPO
DEPMPO-OOH	superoxide anion radical adduct of DEPMPO
DM	β-n-dodecyl maltoside
DMPO	5,5-dimethyl-1-pyrroline N-oxide
DMPO-OH	hydroxyl radical adduct of DMPO
DMPO-OOH	superoxide adduct of DMPO
DMSO	dimethylsulphoxide
EDTA	ethylenediamine-N,N,N',N'-tetraacetic acid
EMPO	2-ethoxycarbonyl-2-methyl-3,4-dihydro 2H-pyrrole-1oxide
EMPO-OH	hydroxyl radical adduct of EMPO
EMPO-OOH	superoxide anion radical adduct of EMPO
EPR	electron paramagnetic resonance
etOH	ethanol
Fd	ferredoxin
FeS	iron-sulphur center
G	gauss
His	histidine
Fe^{3+}	ferric iron
HLIPs	high-light-induced-proteins
H_2O_2	hydrogen peroxide
$HO^{\bullet}$	hydroxyl radical

$H O_2^{\bullet -}$	hydroperoxyl radical(s)
LHCII	light-harvesting complex of photosystem II
OEC	oxygen evolving complex
P680	special chlorophyll pair in the reaction centre of PSII
^{3}P680*	excited triplet state of P680
Pheo	pheophytin
POBN	α-phenyl N-tert-butylnitrone
POBN-CH(OH)CH3	α-hydroxyethyl radical adduct of POBN
PQ	plastoquinone
PSI	photosystem I
PSII	photosystem II
PSI-less	strains of *Synechocystis sp.* PCC 6803 lacking photosystem I
PS I-less/*scp*ABCDE$^-$	strains of *Synechocystis sp.* PCC 6803 lacking photosystem I and *scp*ABCDE$^-$
PVDF	polyvinylidene difluoride
Q_A, Q_B	primary and secondary quinone acceptors in PSII
ROS	reactive oxygen species
SCPs	small CAB-like proteins
*scp*ABCDE$^-$	strains of *Synechocystis sp.* PCC 6803 lacking (*scp*A, *scp*B, *scp*C, *scp*D, *scp*E) gene
SDS	sodium dodecyl sulfate
$^1O_2$	singlet oxygen species
SOD	superoxide dismutase
SOSG	singlet oxygen sensor green
$O_2^{\bullet -}$	superoxide anion radical
TEMP	2,2,6,6-tetramethylpiperidine
TEMPD	2,2,6,6-tetramethylpiperidone
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
TEMPONE	4-oxo-2,2,6,6-tetramethylpiperidine-1-oxyl

^{3}Chl*	triplet excited chlorophyll
$^3O_2$	molecular oxygen
TyrD	redox active tyrosine-161 of the D2 protein in PSII
Tyr_Z	redox active tyrosine-161 of the D1 protein in PSII
UV	ultra-violet

Abstract

In nature, photosynthesis is the only key process responsible for production of biomass, food and molecular oxygen as well as reducing the level of atmospheric carbon dioxide. Light, as an energy source is an absolute prerequisite for photosynthesis process. However, excess of light is harmful, which may lead to the destruction of photosynthetic apparatus. A number of mechanism have been evolved by photosynthetic machinery to dissipate excess absorbed light energy, however, it still remains a fragile system and vulnerable to damage by light. Excessive absorbed light brings system under the oxidative stress and enhances the formation of reactive oxygen species (ROS) in thylakoid membrane. This thesis examines the generation of potentially damaging ROS during light stress in the Photosystem II (PSII) membrane *in vivo* and *in vitro.*

Photosystem II is a transmembrane protein in plants, algae and cyanobacteria, absorbs light and catalyzes the oxidation of water, liberating electrons that are used to form reducing power. The active site of PSII, where oxidation of water occurs, contains tetranuclear water-spliting Mn complex and redox active tyrosyl residue. The participation of water-splitting Mn complex on light-induced redox changes of cytochrome b_{559} (cyt b_{559}) was studies in Mn-depleted and Mn-intact PSII membranes. It was observed that light-induced redox change of cyt b_{559} proceeds via a different mechanism in the intact and the Mn-depleted PSII membranes. In the intact PSII membranes, photoreduction of the heme iron is mediated by plastoquinol. However, in the Mn-depleted PSII membranes, photoreduction and photooxidation of the heme iron was mediated by superoxide anion radical ($O_2^{\bullet -}$). On the other hand, singlet oxygen ($^1O_2$) scavenging activity of plastoquinol was studied in PQ-depleted PSII membranes and it was observed that the addition of the exogenous plastoquinol causes an efficient suppression of singlet oxygen in the PSII of higher plants.

We also studied the role of small CAB-like proteins (SCPs) in PSII membrane and investigated how these proteins were involved in the protection of PSII membrane from oxidative stress. Our *in vivo* study in cyanobacteria using confocal microscopy and *in vitro* study using electron paramagnetic resonance (EPR) spectroscopy confirms an enhancement in $^1O_2$ level in the absence of SCPs. Furthermore, it was concluded that during degradation of the damaged D1 protein, chlorophyll released from the protein complexes enhances the risk of

formation of triplet chlorophylls. These triplet chlorophylls react with the molecular oxygen resulting in the formation of $^1O_2$. Under stress conditions, cyanobacteria accumulate single-helix SCPs known to temporarily bind with some of the chlorophyll molecules that were released due to damage caused by light stress and reduced the formation of $^1O_2$.

CHAPTER 1

1. Introduction

It is very intricate to imagine how the Earth would have been without the occurrence of aerobic photosynthesis. Photosynthesis is a unique process in nature which converts light energy into chemical energy along with generation of molecular oxygen ($^3O_2$) allowing existence of all oxygen- consuming life on Earth (Wydrzynski and Satoh 2005).

1.1. Evolution of photosynthesis

Photosynthesis is the only major source of solar energy storage on Earth and is the source of all our food and energy. A perceptive idea of the origin and evolution of photosynthesis is helpful to understand the progression of photosynthesis.

The formation of Earth is expected to be around 4.6 billion years ago (Fig.1.1). Early atmosphere of Earth was reducing, thus only allowed those form of life, which used H_2, H_2S and other organic compounds as an electron donors. Around three billion years ago (Catling et al. 2001, Kasting and Siefert 2002) (Fig.1.1) due to the exhaustion of these kinds of resources, primitive form of life was forced to shift, and thus, started to adopt the mechanism of using sunlight as a source of energy (Olson 2001, Blankenship and Hartman 1998, Tice and Lowe 2004). The adoption of an oxygenic photosynthesis was the only favorable choice for survival for autotrophic organisms around 3.5 billion years ago (Larkum et al. 2007, Blankenship 2002). Actual photosynthesis is thought to have evolved about 3 billion years ago. For the first time, organisms were able to produce their own food using solar energy and water as a source of electrons with concomitant evolution of molecular oxygen ($^3O_2$) on Earth. The evolution of $^3O_2$ becomes precursor for all the subsequent evolution on Earth. Despite the fact that $^3O_2$ is a toxic compound, protective mechanisms against reactive oxygen species (ROS) came into existence, stimulating the evolution of aerobic respiration. The evolution of oxygenic photosynthesis radically altered the Earth's atmosphere. Increase in $^3O_2$ enhanced the efficiency of aerobic respiration and allowed the evolution of extremely efficient organisms. The evolutionary relationships of the reaction centers (RCs) found in all the classes of currently existing organisms have been analyzed using sequence analysis (Blankenship 1992). The evolution of RCs was started from primitive RC having single

special chlorophyll (Chl), performing as a light-driven oxidoreductant which leads to remarkably-specialized descendants (Larkum et al. 2007) (Fig. 1.2).

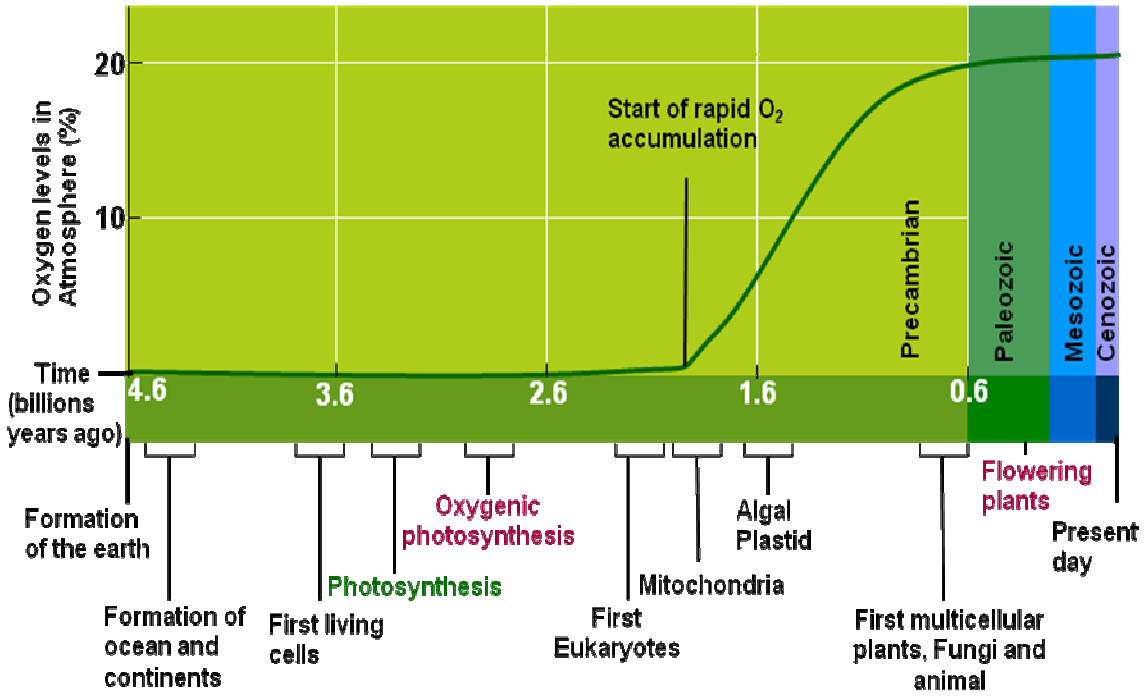


Figure 1.1. Systematic arrangements of important evolutionary events of photosynthesis

Zhang and co-workers (1997) speculated that the ancestral RC might be formed from five transmemmbrane-helices (TMH) protein in association with the light-harvesting protein and provide the ability to capture sunlight. The evolution of dimeric RC originated due to gene duplication which contains two molecules of Chls. Initially dimeric RC was a homodimer (as found in chlorobium and heliobacteria), which latter developed into a heterodimer (Fig. 1.2) (Larkum et al. 2007). The heterodimer RCs are found in all other organisms are likely to be derived forms that correspond to specialization in function. Each dimer subunit contains Chls that participate in charge separation and as electron acceptors.

Reaction centre are divided into two classes on the basis of first stable electron acceptor. In class I type, the terminal electron acceptor is iron-sulphur clusters (FeS-type), and it is found as homodimer in green sulfur bacteria and helicobacteria; while as

heterodimer in photosystem I (PSI) of cyanobacteria, algae and higher plants (Blankenship 1992). The class II type of RC is present in the purple and in the green filamentous non-sulfur bacteria as well in photosystem II (PSII) of cyanobacteria, algae and higher plants which contain quinone (Q-type) molecules as terminal electron acceptor.

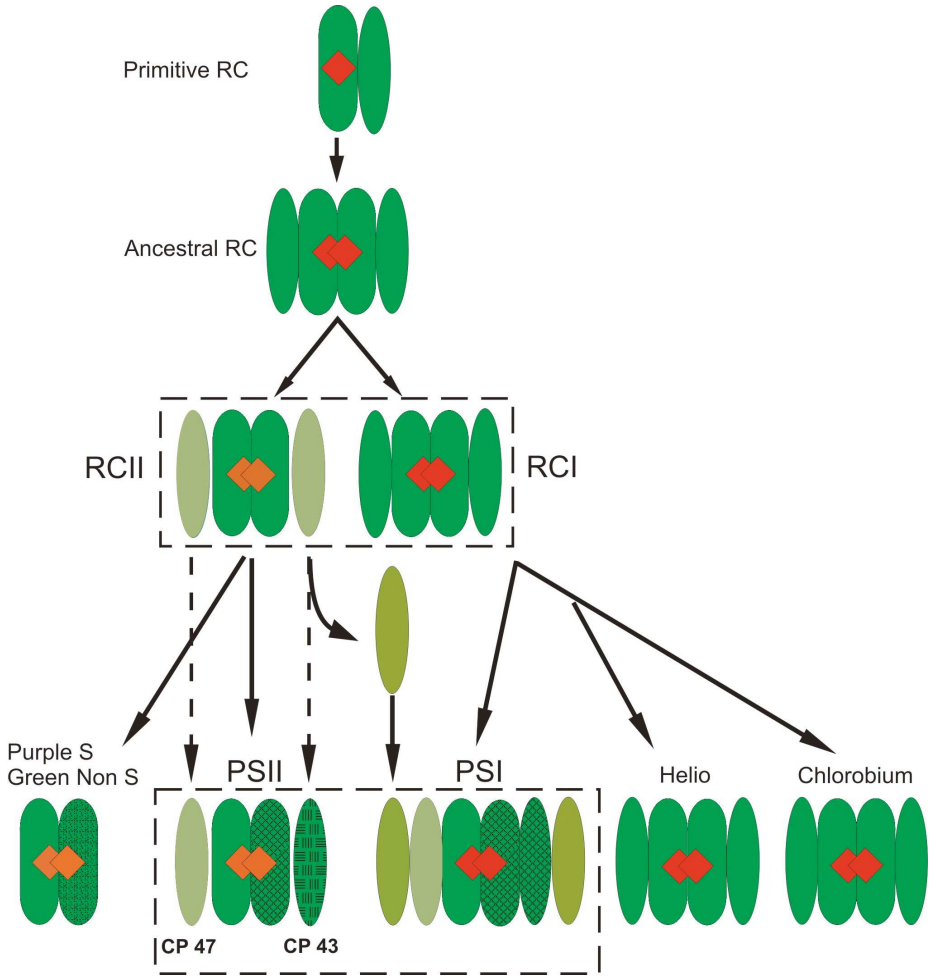


Fig. 1.2. Systemic arrangement of evolution of reaction centers (RC) in anoxygenic and oxygenic organism, adopted (Larkum et al. 2007). Green ovals denotes protein subunits, red rhombus denotes special pair of Chl molecules whereas RCI and RCII are two different types of RC which latter differentiated into two different photosystem, PSI and PSII. CP43 and CP47 are chlorophyll-binding (antenna) subunits of PSII.

It is hard to decide which type of RC preceded during evolution, although sequence similarity between FeS-type and Q-type does not indicate common origin. High similarity on structural data strongly suggests that all the RCs are derived from same

ancestor (Sadekar et al. 2006, Cardona et al. 2011). There are two models (Larkum et al. 2007) which explain the evolution of RC. The first fusion model suggests that there is differentiation of one RC into two different RC types in different organisms (Mathis 1990, Blankenship 1992), whereas the presence of both RC types in cyanobacteria and their relative might be due to the transfer of genetic material of one organism into another (Hartman 1998). The second fission model suggests that two types of RC originated from gene duplication in RC of individual organism by means of the advantage of using both photosystem in a row (Olson and Pierson 1987a, b). This is helpful in producing required amount of energy for oxidation of water, whereas the presence of single type of RC in bacteria is caused due to evolutionary loss. The accumulation of $^3O_2$ in atmosphere due to oxidation of water creates a favorable condition in nature to flourish the living being. The higher forms of organism used the advantages of autotrophic growth allowed with photosynthesis. It is supposed that single endosymbiotic event gave rise to the plastids of all eukaryotic prototroph (Cavalier-Smith 2000, Stiller and Hall 1997). It is expected that the chloroplasts of algae and higher plants evolved by the engulfment of precursor of cyanobacteria into non-photosynthetic eukaryotic hosts (Margulis 1996, Cavalier-Smith 2000, Stiller and Hall 1997).

1.2. Outline of photosynthesis

Photosynthetic prokaryotes (photosynthetic bacteria and cyanobacteria) and eukaryotes (algae and plants) are photoautotrophic organisms, which derive their energy with the help of sunlight and cellular carbon directly from atmospheric carbon dioxide (CO_2). In plants, photosynthetic reactions take place inside thylakoid membranes of chloroplasts (Fig. 1.3A). Inside chloroplasts, a continuum of thylakoid membranes forms appressed grana stacks and non-appressed stroma membranes. During photosynthesis, plants are able to synthesize energy-rich organic compounds (e.g. carbohydrates) with the help of simple inorganic substances by a chain of the enzymatically catalyzed redox reactions, where CO_2 is an electron acceptor and H_2O serves an electron donor (equation 1). The synthesis of carbohydrate from carbon and water requires a large input of light energy.

$$6CO_2 + 12H_2O + \text{Light Energy} \longrightarrow C_6H_{12}O_6 + 6O_2 + 6H_2O$$

Equation 1: Net equation of photosynthesis

The standard free energy required for the reduction of one mole of CO_2 to glucose is +478 kJ/mol. The sun light is absorbed by Chl and carotenoid molecules bound to thylakoid membrane protein. The absorption wavelength of Chl (Chl *a* and Chl *b*) are in blue and red region of light, while of carotenoids is in blue and green region of light (for review see, Ke 2001). The energy absorbed in the form of photon drives light reaction in thylakoid membrane and ultimately the physical form of energy of sunlight is captured and stored in the chemical form as NADPH and ATP. There are four main protein complexes which bound to thylakoid membranes are involved in photosynthesis process: PSII, cytochrome b_6/f complex ($cytb_6$/f), PSI and ATP synthase (Fig. 2.1). The major part of PSII is located in grana stacks whereas PSI and ATP synthase are located in stroma exposed region of thylakoid membranes (Fig. 1.3B). However, $cytb_6$/f is playing intermediate role between PSII and PSI (Anderson and Andersson 1982, Chow et al. 1991, Albertsson 2001, Danielsson et al. 2004, Chow et al. 2005). During the process of electron transport from PSII to PSI, a proton gradient is formed across the thylakoid membrane and used for the formation of ATP by ATP synthase. ATP and NADP formed in grana are further utilized in the stroma for carbon assimilation reaction during Calvin-Benson cycle.

1.3. Thylakoid structure and organization

Thylakoid is a membrane-bound compartment present in all photosynthetic organisms. It is associated into granal stacks, interconnected by a pair of membranes called stromal thylakoid which are in contact with the stroma on both sides (Fig. 1.3A, B). Thylakoid membranes are 5-7 nm thick and consist of a lipid bilayer (50% of dry weight) along with proteins, pigments and other major components, which are vital for photosynthesis. The thylakoid lipids contain high amount of trienoic acid and alfa-linolenic acid, which contribute to the fluidity of the membrane, necessary for the diffusion of lipophilic compounds such as plastoquinone and protein complexes. The fatty acid tails of the lipid

bilayer form a non-aqueous, hydrophobic, central core to the membrane, while the hydrophobic heads are at the surface.

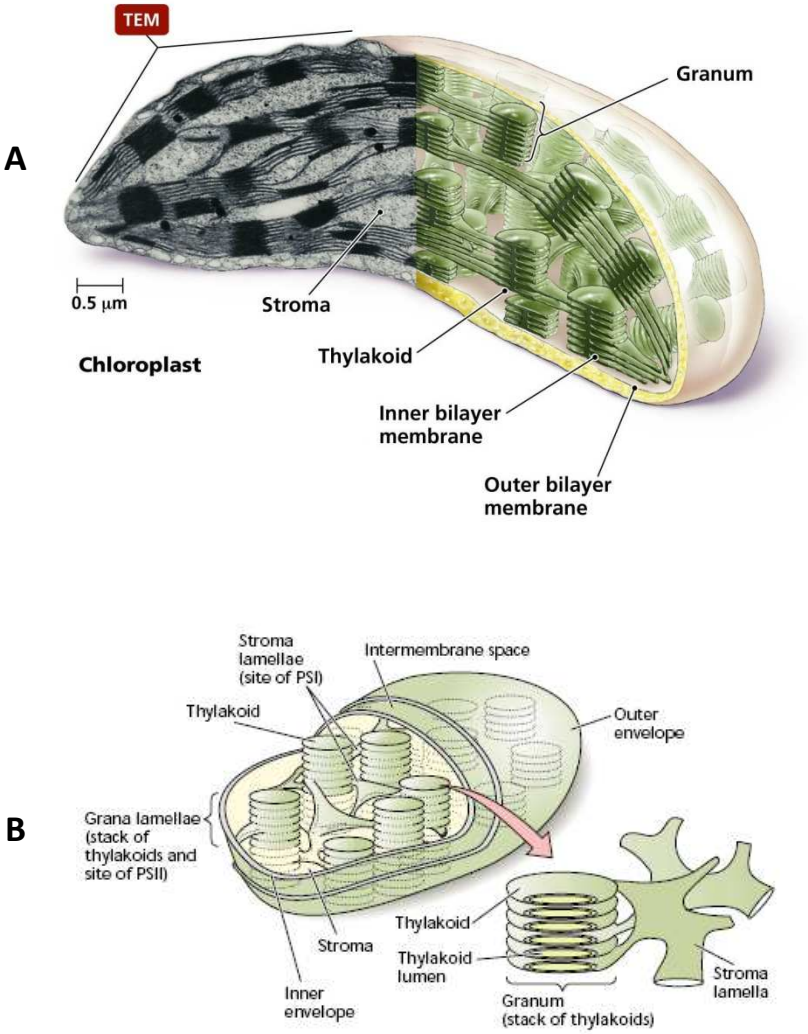


Fig. 1.3. Structure and organization of thylakoid membrane (A) Schematic picture of the overall organization of the membranes in the chloroplast. (B) Electron micrograph of a chloroplast from a leaf of higher plant at. 18 000× resolution. (Figure source: Plant physiology by Taiz and Zeiger 2006)

The outermost half of the membranes, which are located next to the stroma, are 3-5 nm thick, whereas the membrane next to the lumen is 2-3 nm thick. It contains most of the protein required for light reaction; whereas proteins required for CO_2 fixation and reduction are located outside the thylakoid membranes in surrounding aqueous phase. Several models of the three-dimensional structure of thylakoid system have been

proposed to explain the architecture of granum-stroma assemblies in chloroplasts of higher plants (Brangeon and Mustardy 1979, Arvidsson and Sundby 1999). The grana thylakoids are organized in the form of cylindrical stacks and are connected to the stroma thylakoids via tubular junctions (Staehelin and Arntzen 1983, Staehelin and van der Staay 1996). Thylakoid membranes join the stromal lamellae at different points around the periphery of a granum (Fig. 1.3) (Jotham and Staehelin 2011) and bound around the grana stacks in the form of multiple, right-handed helices at an angle of 20º to 25° as postulated by a helical thylakoid model (Paolillo, 1970; Musta´rdy and Garab, 2003). The stroma thylakoid also exhibit significant architectural variability, which is dependent in part on the number and the orientation of adjacent grana stacks to which they are connected. The structure derives from folding and joining of separate sheets of lamellae which are interconnected and probably originated from a single point, the prolamellar body, in the developing chloroplast (Staehelin and Arntzen 1979). The thylakoid system appears to be a single interconnected giant closed vesicle with continuous lumen, a feature of great importance in electron transport and ATP generation. Composition of lumen is complex but it is expected that the proteins of the water-splitting Mn-complex and light–harvesting complex may occupy a part of the volume. The ultrastructure of chloroplast is different for shade and sun plant. In the shade plants there is extensive granal stacking (Anderson et al. 1973) whereas in the sun plant granal stacking is reduced (Boardman 1977). This might be a type of adaptation for plants to provide more space for the enzyme used in carbon fixation reaction (Bjorkman and Holmgren 1963, Anderson et al. 1973, Rozak et al. 2002, Chuartzman et al. 2008).

1.4. Light absorption and energy delivery by antenna system

The first step in photosynthesis is to capture the energy from the sunlight by the pigments present in antenna system attached to proteins in the thylakoid membranes. When pigment molecules become excited, it moves from lower energy state to higher energy state. The unstable excited molecules immediately return back to the ground state by the mechanism of de-excitation. The energy released during de-excitation can be of different forms. The energy released may dissipate non-radiatively in the form of heat

and radiatively in the form of radiation. The energy of emitted photon is always less than that of photon absorbed due to loss of a part of absorbed energy in the form of heat.

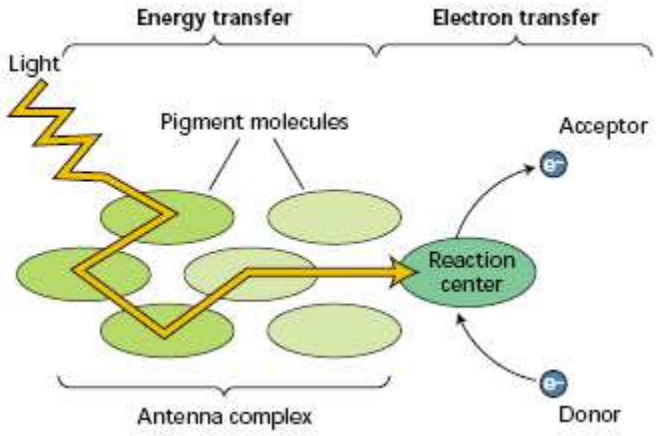


Fig. 1.4. Representation of light energy trapping and energy transduction (Figure source: Plant physiology by Taiz and Zeiger 2006)

The transfer of absorbed energy from one exited molecule to the other neighboring molecule by antenna system is a physical process but operates by intermolecular energy transfer of electronic excited state. This physical process is highly dependent on energetic coupling of antenna pigments which allows energy transfer over several nanometers on a picoseconds time scale. The unidirectional process of energy transfer takes place via a chain of pigments molecules from antenna complex to the RCs. In the RC, the excitation energy is utilized for the reduction of primary acceptor molecules through charge separation events called photochemistry.

One of the primary functions of antenna system is to absorb light and funnel the resulting excited-state energy rapidly and efficiently to the primary acceptor molecules. The efficiency of energy transfer depends upon the relative orientation of energy donors and acceptors, and the spectral overlap of the pigments. The study of ultrastructure of plant chloroplasts using single-molecule spectroscopy explores the spatial location of photosystem in plants (Vácha et al. 2007). These pigment binding proteins are prearranged in two supramolecular complexes i.e. PSII and PSI (Zouni et al. 2001, Kamiya and Shen 2003). Each photosystem is composed of two moieties i.e. core

complex and outer antenna complex. The former is responsible mainly for the co-ordination of all the co-factors of the electron transport chain along with several Chl molecules and the latter is located around the core and composed of light harvesting complex (LHC).

1.5. Outline of thesis

A brief summary of each chapter is presented here:

The second chapter explores the functional stability of PSII complex in relation to the light stress. This chapter begins with the understanding of PSII. It deals the photosynthetic electron transport chain with possible site of generation of ROS. Later dealt about the structural components of PSII along with the intrinsic and extrinsic proteins involve in PSII. We also focused in detail about the effects of light stress in PSII. This chapter also contains an overview of ROS and its production in PSII specific with singlet oxygen ($^1O_2$) and superoxide anion radicals ($O_2^{\bullet -}$).

The third chapter describes the methods used for growing cyanobacteria, isolation of thylakoid and PSII-enriched membranes. It also describes the singlet oxygen imaging by fluorescence probe (singlet oxygen sensor green) in the intact cells of cyanobacteria using laser scanning confocal microscopy. We also provide the details of EPR measurements in thylakoid as well as in PSII. Overall, this chapter gives an overview of the detailed methodology of all the techniques used in this work.

The fourth chapter describes the protective mechanism of ROS against photooxidative damage in PSII and thylakoid membranes. This begins with the study of redox changes of cyt b_{559} and the effect of water-splitting Mn complex on light-induced redox changes of cyt b_{559}. The scavenging activity of plastoquinol in PSII was studied using EPR spin-trapping technique. The role of plastoquinol as a $^1O_2$ scavenger in PSII of higher plant was dealt. The protective roles of small chlorophyll-binding proteins (SCPs) in thylakoid membranes were investigated. In this study, the effect of SCPs on the PSII membrane specific to light-induced damage and generation of $^1O_2$ was assessed in the strains of the cyanobacterium *Synechocystis sp.* PCC 6803 lacking PSI (PSI-less strain) or lacking PSI together with all SCPs. The damage caused by oxidative stress in the

presence and absence of light in PSI-less and PSI-less/*scp*ABCDE^{-} mutants has been discussed.

The fifth chapter is conclusion of all the above mentioned results about the overall effects of light treatment on PSII. Structural damage and changes in the biophysical characteristics of various components have been discussed in relation to the generation of ROS.

The sixth chapter is references.

The seventh chapter includes Curriculum-vitae

The eight chapter includes published manuscripts.

CHAPTER 2

2. Overview of photosystem II and reactive oxygen species

2.1 Structure and organization of PSII

Photosystem II is a multisubunit pigment-protein complex that has abilities of charge separation and water oxidation. After a decades of intense research, the first crystal structure of PSII at 3.8 Å resolutions was published in thermophilic cyanobacterium, *Thermosynechococcus elongatus* (Zouni et al. 2001). The structure and organisation of PSI and PSII isolated from red alga was determined using electron microscopy and single particle image analysis (Gardian et al. 2007). However, an improved crystal structure at 3.0 Å resolution (Loll et al. 2005, Barber 2008) followed by 2.9 Å (Guskov et al. 2009) and 1.9 Å (Umena et al. 2011) which unreveals the structural arrangement of protein subunits and cofactors of PSII. PSII is located in the thylakoid membranes as a dimer consisting of two PSII complexes. Each PSII consists of a heterodimer, which is originated probably from a homodimer (Nitschke and Rutherford 1991). The 350 kDa protein is composed of atleast 20 protein subunits and 92 bound cofactors (35 Chl molecules, two pheophytine and two heme molecules, 11-12 carotenoids, more than 20 lipids, more than 15 detergents molecules, three plastoquinones, a unique water-splitting Mn complex, one bicarbonate, three to four Ca^{2+}, two heme iron, one non heme iron and one Cl^{-} ion) in each monomer (Guskov et al. 2009, Umena et al 2011). However, each PSII monomer also contain 1,300 water molecules, five oxygen atoms, which served as oxo bridges connecting the five metal atoms and four water molecules bound to the water-splitting Mn complex.

The central core of PSII (Zouni et al. 2001, Kamiya and Shen 2003, Ferreira et al. 2004, Loll et al. 2005) is composed of the D1 and D2 proteins, the two Chl binding proteins CP43 and CP47, a small membrane spanning protein cyt b_{559}. Whereas, the lumenal side of PSII consists of four Mn atoms, a 33 kDa manganese stabilizing extrinsic proteins, a 24 kDa and 17 kDa extrinsic polypeptides.

Despite the pseudo-symmetrical arrangement of the cofactors the energy transfer takes place only on one side of PSII. D2 protein is bordered on the cyt b_{559}, a subunit consisting of two membrane-spanning helices; the two histidines and a heme coordinate (see review Stewart and Brudvig 1998). The water splitting is accomplished by one calcium and four manganese ion, with one associated chloride ion. This so-called water-splitting Mn complex ('oxygen evolving complex ' or OEC) is located on the lumen side of the PSII.

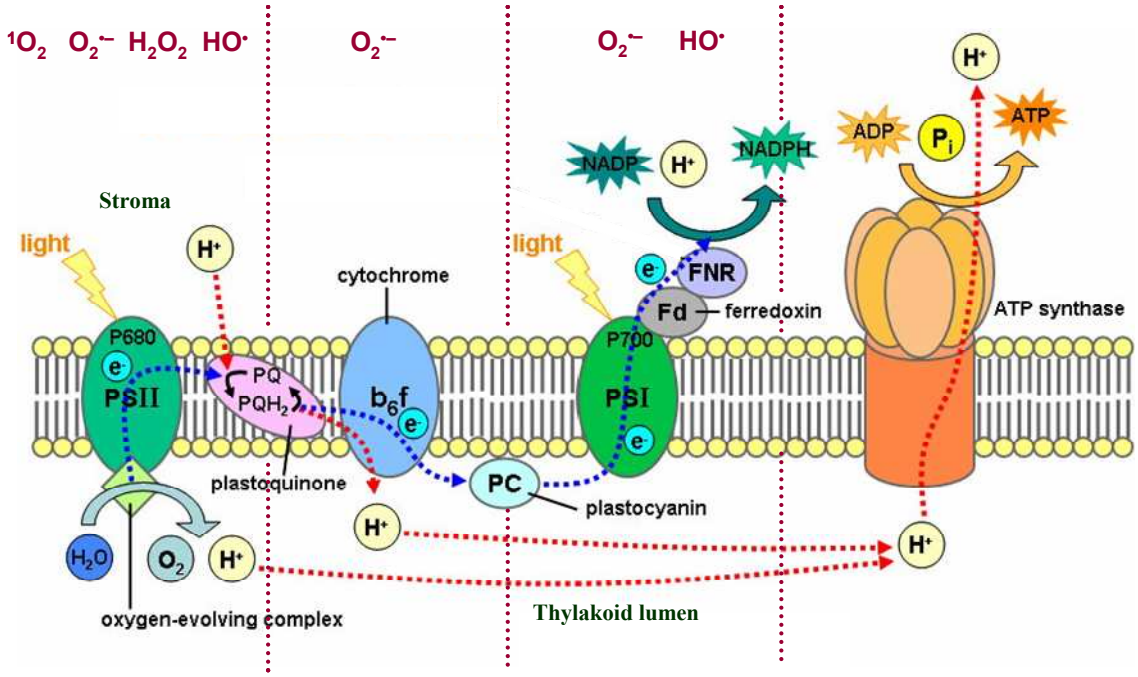


Fig. 2.1: Diagram of the photosynthetic electron transport chain and possible places of genration of ROS, diagram is an overview of the various ROS in PSII, cyt b_6f complex and PSI may be generated during photosynthesis. The photosynthetic electron transport begins at PSII, in which the central P680 Chls are excited by light energy and through various cofactors electrons transported to plastoquinone (PQ). Photolysis of water takes place at the donor side of PSII and provides the necessary electrons. Here, protons and $^3O_2$ are released into thylakoid lumen. Double reduced and protonated plastoquinol (PQH_2) leaves PSII and oxidized by cyt b_6f complex, the released protons pass into the lumen. The electrons are supplied through the one-electron carriers via plastocyanin (PC) to the PSI. Chls (P700) excited by light energy, and electrons are transmitted through different cofactors on ferredoxin (Fd). The ferredoxin-$NADP^+$ reductase (FNR) uses ferredoxin to $NADP^+$ to reduced to NADPH. The built up proton gradient over the thylakoid membrane is used by ATPase to synthesize ATP. NADPH and ATP are used in the fixation of CO_2 into carbohydrates. The formation of ROS takes place in PSII by one electron reduction of $^3O_2$, whereas, the formation of $^1O_2$ takes place via excitation energy transfer. While in PSI one electron reduction forms $O_2^{\bullet-}$ and HO$^\bullet$ forms via Fenton reaction. The formation of $O_2^{\bullet-}$ in cyt b_6f complex also proceed via one electron reduction of $^3O_2$ while the invovments of free metals and reduced ferredoxin in the light-induced production of HO$^\bullet$ in thylakoid membranes of chloroplasts was also reported (Šnyrychová et al. 2006) (Figure source: Plant physiology by Lincoln Taiz and Eduardo Zeiger, 2006).

The exact function of the cofactors Ca^{2+} and Cl^- is not yet clear, but for functioning of water splitting activity, both the ions are required. When Ca^{2+} is replace by Sr^{2+} and Cl^- by Br^-,

the rate of oxygen evolution was slowed down (Ghanotakis et al. 1984, Ono et al. 1987, Ishida et al. 2008, Murray and Barber 2006). The manganese cluster is stabilized by three extrinsic proteins and shielded from the lumen side (33, 23 and 17 kDa protein in plants, 33 kDa and 12 kDa protein and cyt c_{550} in cyanobacteria).

In order to split water, a high oxidation potential of 0.81 V is required. PSII is capable of generating this potential with the help of two central Chls (P680) molecules upto a high oxidation potential of *E*m 1.2 V. Chls (P680) molecules are excited by light quanta for charge separation in PSII (review Rappaport and Diner 2008). The electron transfer process between the cofactor is rapid, within few picoseconds after excitation $^3P680^*$ reduces the pheophytin ($Pheo^{\bullet-}$) and in 200 - 500 ps, $Pheo^{\bullet-}$ reduces plastoquinone (Q_A). In early steps, the oxidized $P680^{\bullet+}$ is reduced once in every 200 ns by tyrosine (Tyr_Z) which in turn with kinetics between 100 - 800 µs (dependent on the S-state). However, after accumulation of four oxidation equivalents water-splitting Mn complex abstracts electrons from water molecules. The last step evolves the transfer of electron from Q_A to Q_B (100 - 600 µs) and Q_B is further reduced twice and protonated. The protonated plastoquinol (PQH_2) enters the plastoquinone (PQ) pool. The PQ pool provides a link between PSII and PSI via cyt b_6f, where PQH_2 reoxidised to PQ and return to PSII.

The manganese cluster cycles have five different oxidation states, which are called S states (S_0, S_1, S_2, S_3 and S_4) (Kok et al. 1970). Among them, the S_1 and S_0 are stable states in the dark and 75% of the RCs are present in the S_1 state and 25% are in the S_0 state (Inoue 1996). After accumulation of four positive charges S_4 is converted into S_0 state by the oxidation of water. Four electrons are supplied together with release of four protons at the expense of two water molecules, while dioxygen is released. Using this mechanism, it is possible to use water as an electron donor.

2.2. Protein subunits of PSII

Each monomer of PSII consists of at least 20 protein subunits (Fig. 2.1., 2.2 and Table 2.2). These 20 proteins are divided into two major parts intrinsic and extrinsic on the basis of their location in PSII complex (Table 2.2). Intrinsic are embedded into the membrane and extrinsic are attached to the membrane.

Chain	Subunit name	Size (amino acids)	Location (in thylakoid)
A	D1, RC subunit	344	intrinsic
B	CP47, antenna subunit	510	intrinsic
C	CP43, antenna subunit	461	intrinsic
D	D2, reaction centre subunit	352	intrinsic
E	*Cytochrome* b_{559}, α-subunit	84	intrinsic
F	*Cytochrome* b_{559}, ß-subunit	45	intrinsic
H	PsbH	66	intrinsic
I	PsbI	38	intrinsic
J	PsbJ	40	intrinsic
K	PsbK	37	intrinsic
L	PsbL	37	intrinsic
M	PsbM	36	intrinsic
0	PsbO Mn-stabilizing protein	246	extrinsic
T	PsbT	32	intrinsic
U	12 kDa extrinsic protein	246	extrinsic
V	*Cytochrome c-550*		extrinsic
y	ycf12	46	intrinsic
X	PsbX	41	intrinsic
Y	PsbY	41	intrinsic
Z	PsbZ	62	intrinsic

Table 2.1. Protein subunits of PSII.

2.3. Membrane intrinsic protein subunits

2.3.1. Reaction Centre

The photosynthetic RC is formed by two large protein subunits D1 (*PsbA*) and D2 (*PsbD*) (Fig. 2.2) in analogy with RC of purple bacteria that is formed by L and M polypeptides (Deisenhofer et al. 1985, 1995). D1 and D2 polypeptides of RC are composed of five TMHs and both subunits are intercrossed each other. The core of the RC forms approximately two 30 kDa

interlocking proteins (D1 and D2) which contain all the cofactors for electron transport to the plastoquinone and for water-splitting Mn complex (Fig. 2.2).

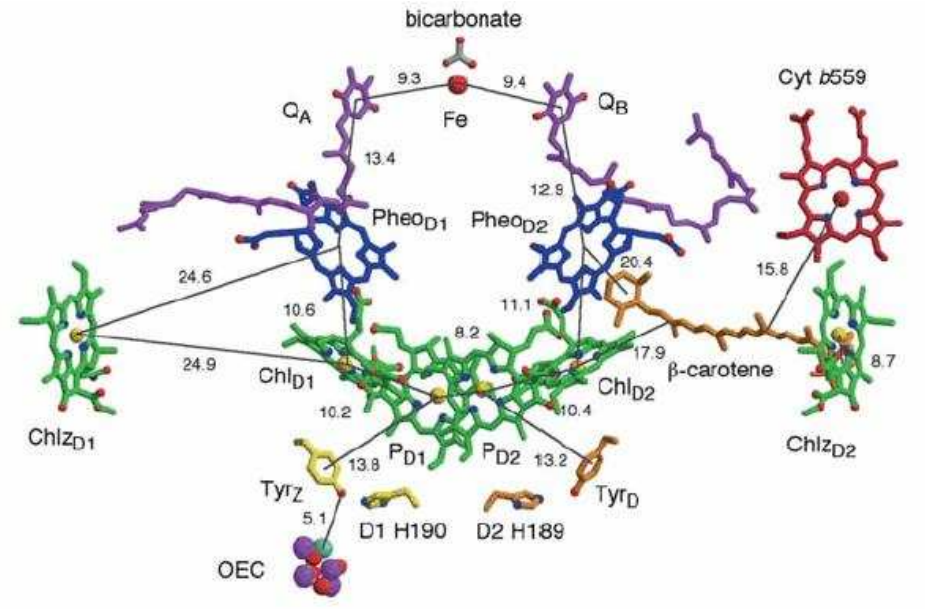


Fig. 2.2 Electron transfer cofactors of PSII RC (Ferreira et al. 2004).

The additional function of D1 protein is to provide the binding site for water-splitting Mn complex that makes it vulnerable to radiation damage caused by side reaction during charge separation. Therefore, for replacing the damage D1 protein, a special mechanism called D1-turnover (Baena-Gonzalez and Aro 2002, Vass and Aro 2008, Nishiyama et al. 2006) takes place in PSII. D1 and D2 proteins harbour all the cofactors of the electron transport chain (ETC), namely four Chl-*a* molecules, two pheophytin (PheoD1 and PheoD2) and two plastoquinone (Q_A and Q_B) along with a third plastoquinone Q_C (Guskov et al. 2009). D1 protein also provides most of the ligating amino acids for a unique water-splitting Mn complex located close to the electron donor site of RC (Fig. 2.2).

The PSII RC has a complicated three dimensional structure; various crystallographic investigations of cyanobacterial PSII have provided high resolution structures from 3.8 to 1.9 Å (Zouni et al. 2001, Kamiya and Shen 2003, Ferreira et al. 2004, Loll et al. 2005, Guskov et al. 2009, Umea et al. 2011) that explain the general arrangement of the protein matrix and cofactors. However, 25 integral lipid was also reported in each monomer in which 11 are monogalactosyl-diacylglycerol (MGDG), 7 digalactosyl-diacylglycerol (DGDG), 5 sulfoquinovosyl-

diacylglycerol (SQDG) and 2 phosphatidyl-glycerol (PG) molecules and are distributed asymmetrically in PSII membrane (Guskov et al. 2009). The head groups of negatively charged PG and SQDG is located exclusively on the cytoplasmic side, whereas the location of uncharged DGDG on the lumenal side and those of MGDG on both sides of membrane. The structural and functional aspects of PSII RC are interrelated. In order to maintain the stable photosynthetic activity of the RC, the structural integrity of the Dl, D2 proteins and cyt b_{559} complex is essential. The fact that an environmental stress such as drought, flood, high intensity light, or high temperature can cause a decrease in photosynthetic yield is primarily ascribed to damage to the PSII RC.

2.3.2. Peripheral antenna complex

The pigment-binding proteins in plant and cyanobacteria are arranged in two supramolecular complexes PSI and PSII. Both photosystem is composed of core complex, involved in charge separation and the peripheral antenna, which plays a important role in light harvesting and decipation of excitation energy to the RC. The LHC of PSII is composed of the core pigment protein complexes CP47, CP43 and the light-harvesting complex II (LHCII) (Green and Durnford 1996). The peripheral antenna is composed of different protein encoded by the Lhcb gene (Jansson 1994). There are six major proteins (Lhcb1-6) in peripheral antenna and are associated with pigment to form the complexes CP24, CP26, CP29 and LHCIIb. The protein CP24, CP26 and CP29 are monomeric, whereas LHCIIb is a trimetric complex (Nield et al. 2000, 2006, Peter and Thomber 1991) and its proper folding is important for the stabilization of PSII. The localization of CP47 is in close proximity to monomer interface in association with subunit D2 and CP43 is located close to D1 at the periphery of the complex. Both CP47 and CP43 are folded into six TMHs and bound to 29 Chl molecules, in which 16 are associated with CP47 and 13 are with CP43 (Fig. 2.2). Interestingly, each Chl-*a* molecules are in contact with the carotenoid molecules with van der waal force of attraction (Guskov et al. 2009) which makes the path for transfer of excitation energy from carotenoids to Chl-*a*. The transfer of excitation energy mediated by carotenoids is very efficient for photoprotection of PSII from excited triplet Chl ($^3Chl^*$) (Loll et al. 2005). Because carotenoid can quench the $^3Chl^*$ and save PSII from oxidative

damage, which may occur due to the formation of ROS (Mimuro and Katoh 1991, Telfer 2002, Krieger-Liszkay et al. 2008, Lepedus et al. 2011).

2.3.3. Cytochrome b_{559}

Cyt b_{559} is present in all the oxygenic photosynthetic organisms as an integral part of PSII. Cyt b_{559} is tightly bound to D1 and D2 homologous proteins in PSII. It is a heme-bridged heterodimer consisting of α and β subunits, encoded by *psbE* and *psbF* genes (Babcock et al. 1985, Tae et al. 1988). The heme iron of cyt b_{559} is coordinated by two histidine residues; His22 and His17 of the α and β subunits located near to the stromal side and oriented perpendicular to the membrane plane. The crystal structures of PSII from thermophilic cyanobacteria *Thermosynechococcus elongatus* and *Thermosynechococcus vulcanus* reveal that the heme iron is distanced at about 30Å from the head group of Q_B site and about 17 Å from the head group of the plastoquinone Q_C whereas 50–60Å from the water-splitting Mn complex (edge-to-edge distance) (Kamiya and Shen 2003, Ferreira et al. 2004, Loll et al. 2005, Guskov et al. 2009).

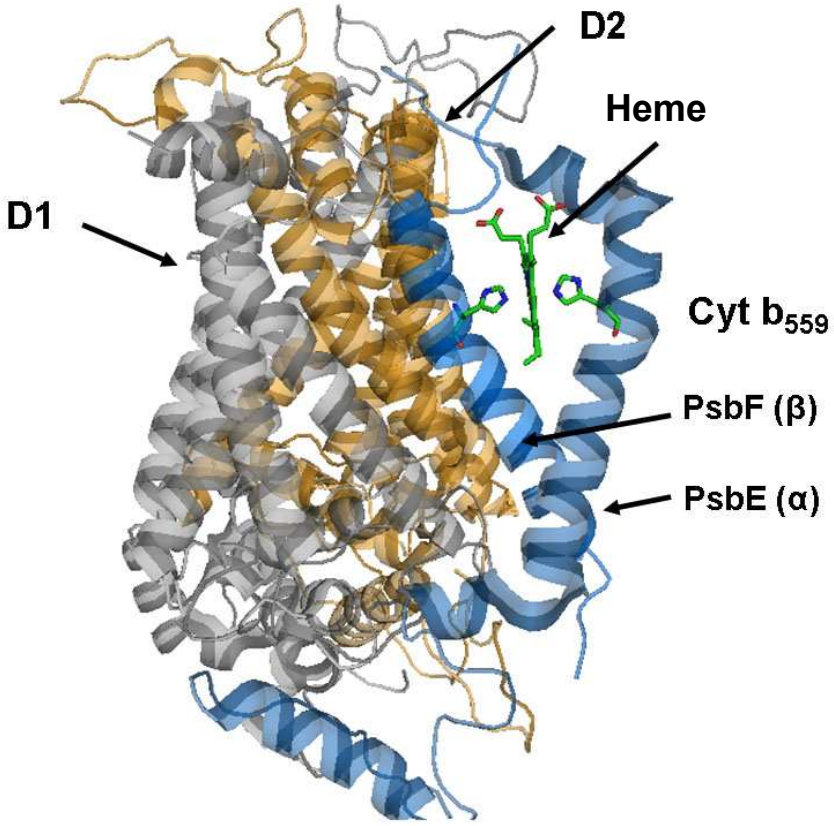


Figure 2.3: Schematic diagram of the Cyt b_{559} D1/D2-subunit with a view from the plane of the membrane to the core subunits D1 (gray), D2 (brown) and the two Cyt b_{559} subunits PsbE + F (blue). The iron core of the heme is co-ordinated with by two histidines (green) of PsbE and PsbF.

The heme iron of cyt b_{559} harbors three different redox properties (Kaminskaya 1999, Roncel et al. 2001, Ortega et al. 1988, Stewart and Brudvig 1998, Tiwari and Pospíšil 2009, Pospíšil and Tiwari 2010) and spreads over the redox potential range from 400 mV to 0 mV: (i) the dominant high potential form with midpoint potential (*E*m) about +400 mV; (ii) intermediate potential form with *E*m about +170 to +200 mV; (iii) low potential form, *E*m is about +50 to +100 mV and the very low potential (*E*m~0 mV). Several hypotheses have been proposed for the presence of different redox form of cyt b_{559} 1) Due to the changes in hydrogen-bonding pattern of the axial ligands (M. Roncel et al. 2001, Berthomieu et al. 1992); due to different changes in the orientation of ligating histidines (Babcock et al. 1985) and changes due to the electrostatic environment around heme (Krishtalik et al. 1993). The light-induced reduction and oxidation of the heme iron is one of the important properties of cyt b_{559}, which is possibly involved in the regulation of photochemical efficiency in PSII (Whitmarsh and Pakrasi 1996, Steward and Brudvig 1998, Faller et al. 2005, Shinopoulos and Brudvig 2012).

2.3.4. Low molecular weight intrinsic proteins

The intrinsic protein subunits have low molecular weight and contain only TMH (except PsbZ, which shows two). The function of intrinsic proteins is mostly unknown. The general overview of these proteins is given in table 2.1. There are several intrinsic small proteins reported like PsbH, PsbJ, PsbK, PsbL, PsbM, PsbT, PsbX, PsbY, ycf12, PsbZ along with some light-inducible proteins which are expressed in light stress.

PsbH, located next to TMH d of CP47, which might influence the electron transfer between Q_A and Q_B (Bumba et al. 2005, Komenda and Barber 1995, Mayes et al. 1993), plays an important role in the replacement of photodamaged D1 protein. It is also reported to be important for the stability and assembly of the PSII complex (Rokka et al. 2005, Komenda et al. 2002, 2005, Iwai et al. 2004).

PsbI is reported to be present close to D1 and CP47, which forms several H-bonds both with D1 and CP43 (Guskov et al. 2009). The function of PsbI is ambiguous but its role in the

activity of oxygen evolution has been shown (Ikeuchi et al. 1995, Schwenkert et al. 2006), whereas its role in stabilization of the PSII complex and in the D1-turnover was confirmed recently (Dobakova et al. 2007). On the other hand, the function of PsbJ is expected to be involved in channel formation and its absence cause hindrance in PQ/PQH_2 exchange (Regel et al. 2001).

The role of psbK is reported to be involved in the stabilization of PSII RC complex, (Takahashi 1994). However, PsbL might involve in restoration of electron transfer activity in RCs (Hoshida et al. 1997, Ozawa et al. 1997) and is essential for the normal PSII assembly and recovery from photodamage.

Similarly, PsbT subunit is reported to be involved in PSII recovery after photodamage (Ohnishi and Takahashi 2001), stabilization of the Q_A-binding site (Ohnishi et al. 2007) and stabilization of homodimer. The absence of PsbZ subunit can affect the rate of cyclic electron flow around PSI (Bishop et al. 2007).

2.4. The extrinsic proteins

The structure and role of extrinsic proteins in higher plants and cyanobacteria have been studied using various biophysical approaches (review, Bricker and Burnap 2005, Roose et al. 2007). In higher plants, three extrinsic proteins appear to be required for maximal rates of O_2 evolution at physiological inorganic cofactor concentrations. The three extrinsic proteins are 33 kDa (PsbO), 24 kDa (PsbP), and 18 kDa (PsbQ) and are located on the lumen side of thylakoid membrane, where the oxidation of water occurs.

The cyanobacterial and higher plant both contains 33 kDa extrinsic protein but the homologues of 16 and 24 kDa polypeptides are absent in cyanobacteria. However, two different small analogues proteins (9, 12 kDa and cyt C_{550}) were reported to be present in cyanobacteria but their role is still not clear. In the absence of the 12 kDa (PsbU) and cyt C_{550} (PsbV) the rate of oxygen-evolution was reported to be strongly reliant on both Ca^{2+} and Cl^-. It signifies that these proteins carry out a similar function like 18 and 23 kDa extrinsic subunits in higher plants (Enami et al. 1998). The function of 33 kDa protein has been studied extensively and it was shown that the PsbO protein of plants is very similar in structure to that of cyanobacterial PsbO protein. It appears that these three extrinsic proteins play a central role in the stabilization of

manganese cluster, and is essential for efficient O_2 evolution (Popelková et al. 2002, 2011). PsbO is also involved for photoautotrophic growth and in the assembly of PSII in higher plants along with PsbP (Bricker and Burnap 2005, 1996, Popelková et al. 2011). The absence of PsbO causes an imbalance in the levels of ion cofactors in OEC (Debus 1992, Ifuku et al. 2005), which enhances the chances of oxidative stress. By the damage of PsbO, PSII become more vulnerable to photoinhibition. This accelerates the turnover of the D1 protein and perturbation of electron transfer processes at the donor side of PSII (Komenda and Barber 1995). It is believed that these subunits play a significant role in the stabilization of the whole PSII complex and optimization of the oxygen evolution process (Shen et al. 1997, Enami et al. 1997).

2.5. Effects of high light on PSII.

When photosynthetic organisms are exposed to high light, the activity of PSII declines rapidly and this process is known as photoinhibition of PSII (Powles 1983, Aro et al. 1993, 2005). Photoinhibition is possible under a wide range of light intensities of visible (400-700 nm) and ultraviolet light (220-400 nm). The UV light is more efficient than visible light in inducing photoinhibition (Jones and Kok 1966, Renger et al. 1989, Jung and Kim 1990).

2.5.1. Photoinhibition: damage and repair of PSII

In natural conditions, light stress becomes obvious when the rate of photoinhibition exceeds the rate of repair (Prášil et al. 1992, for reviews see, Aro et al. 1993a, Melis 1999, Andersson and Aro 2001, Tyystjärvi 2008, Vass and Aro 2008). The concepts of photodamage and photoinactivation of PSII are essentially similar to photoinhibition (Nishiyama et al. 2006, Chow and Aro 2005). During the occurrence of photoinhibition process plant endures two distinct mechanisms the high light-induced photodamage of PSII and the repair of photodamaged PSII. PSII is the target of such photoinhibition and specific damage to the reaction center-forming D1 protein and OEC which results in the inhibition of electron transport in PSII (Ohad et al. 1985, Mattoo et al. 1989, Barbato et al. 1991a, Barber and Andersson 1992, Aro et al. 1993).

After photoinhibition the damaged PSII protein moves to the stroma thylakoid region and the damaged D1 protein of RC is replaced with a new copy via repair process of PSII as the degradation of the photoinactivated Dl protein is a prerequisite for the synthesis and stable integration of newly synthesized Dl (Zer et al. 1994). The new copy of D1 protein moved back to the grana membranes. In the grana membrane, PSII are reassembled and lastly a functional PSII dimer is formed. Plants have developed several strategies to keep the photoinhibition as low as possible, like continuous and rapid exchange of the D1 proteins are an important role to limit the production of ROS and maintain the PSII activity at high level (Ohad et al. 1984). It was earlier reported that the removal of 5 or 10 residues from N terminal blocked the D1 synthesis and removal of 20 residues restored to assemble oxygen-evolving complex proteins in dimeric PSII complexes but inhibited PSII repair at the level of D1 degradation (Komenda et al. 2007b, 2008), which says that the selectivity of degradation of PSII proteins. In order to localize the process of photoinhibition in PSII, the terms donor and acceptor sites of photoinhibition were introduced (review Vass and Aro 2008).

2.5.2. Donor-side photoinhibition

The donor side-induced photoinhibition results in the accumulation of highly oxidizing radicals $P680^{+\bullet}$ and $Tyr_Z^{+\bullet}$, when electrons transfer from water splitting is not sufficiently high to cope up with the electron need of $P680^{+\bullet}$ This may also be possible by incomplete oxidation of water which limits the electron supply to $P680^{+\bullet}$. Electron donation from OEC could be affected after partial release of Ca^{2+} under low pH of lumen (Krieger and Weis 1993). Donor side photoinhibition could be directly observed after chemical alteration of OEC in an active PSII (Callahan et al. 1986, Eckert et al. 1991, Chen et al. 1992). Highly oxidized, long lived $P680^{+\bullet}$ and/or $Tyr_Z^{+\bullet}$ can oxidize a wrong component of the RC (Callahan et al. 1986, Chen et al. 1992, Anderson et al. 1998) which enhances the formation of reactive $O_2^{\bullet-}$ and $HO^{\bullet}$ and increases the light sensitivity of PSII (Hideg et al. 1994a, Vass and Aro 2008). Furthermore, some time fully functional OEC unable to reduce $P680^{+\bullet}$ due to a miss in S-state cycle (Kok et al. 1970). The miss in S-state cycle slows down the decays of $P680^{+\bullet}$ (up to 200 μs), $P680^{+\bullet}$ may recombine with Q_A^- (Renger and Holzwarth 2005) or some other components of PSII and reduces $P680^{+\bullet}$, which may lead to the donor-side inactivation of PSII (Anderson et al. 1998).

2.5.3. Acceptor-side photoinhibition

The acceptor side photoinhibition is induced due to high light intensities in PSII with a functional donor side. The photoinhibition occurs in PSII acceptor site that contains functional P680, Pheo and other cofactors while electron transfer from Q_A^- to Q_B is hampered due to reduction of plastoquinone pool (Vass et al. 1992). In a fully reduced plastoquinole pool, there is a lack of electron acceptors on the PSII acceptor side, and consequently the lifetime of the charge pairs is enhanced. The probability of formation of triplet Chl ($^3Chl^*$) becomes high. A clear correlation has been demonstrated between the formation of $^1O_2$ and the degradation of D1 protein (Keren et al. 1997, Hideg et al. 1998). The $^1O_2$ produced in the presence of unstabilized Q_A^- (Vass and Aro 2008) might be the real damaging agent in photoinhibition. With the increasing light intensity the formation of $^3Chls^*$ and $^1O_2$ are expected to increase when the photosynthetic electron transfer is light-saturated (Vass and Cser 2009). Recently, formation of $O_2^{\bullet-}$ in PSII by one-electron reduction of $^3O_2$ was reported. (Cleland and Grace 1999, Zhang et al. 2003, Tiwari and Pospíšil 2009, Pospíšil and Tiwari 2010). It has been suggested that $O_2^{\bullet-}$ produced on the PSII acceptor side undergoes dismutation to form H_2O_2 (Klimov et al. 1993) and via Fenton chemistry with non-heme iron can be converted into $HO^{\bullet}$ (Miyao et al. 1995, Pospíšil et al. 2004), which shows the involvement of various ROS at the acceptor side-induced photoinhibition.

2.6. Reactive oxygen species

2.6.1. Overview of reactive oxygen species

Molecular oxygen in the ground state is the most stable and least reactive due to its electron configuration. The both unpaired electrons in $^3O_2$ have parallel spin. $^3O_2$ can act as an oxidizing agent, but only in the case it attempts to oxidize another atoms or molecules by accepting a pair of electrons from it and both electrons should have the same spin order to fit into vacant space in the antibonding orbitals. According to Pauli's exclusion principle, two electrons in same molecular orbital have different spin. This causes spin prohibition in limited

responsiveness. By reducing or spin reversal, the spin prohibition repealed and $^3O_2$ can be activated in different reactive intermediates. Some of the standard reduction potential of these activated oxygen species are summarized in table 2.2.

Singlet oxygen is the reactive form of $^3O_2$ and can be generated by an input of energy ($^1\Delta g\ O_2$ = 22.4 kcal and $^1\Sigma_g^+O_2$ = 37.5 kcal) to the $^3O_2$. In chloroplasts, generation of $^1O_2$ is possible by energy transfer to $^3O_2$ by the $^3Chl^*$ molecules (equation 2.1). In the electrons antibonding orbital $^1O_2$ have an antiparallel spin, so that the spin prohibition is lifted.

$$^3Chl^* + {}^3O_2 \longrightarrow {}^1Chl + {}^1O_2$$

Equation 2.1: The generation of $^1O_2$ by the Interaction of $^3Chl^*$ and molecular oxygen

Singlet oxygen has a high oxidation potential and mainly reacts with carbon double bonds, which may result in damage to lipids, amino acids or pigments (Caspi et al. 2000).

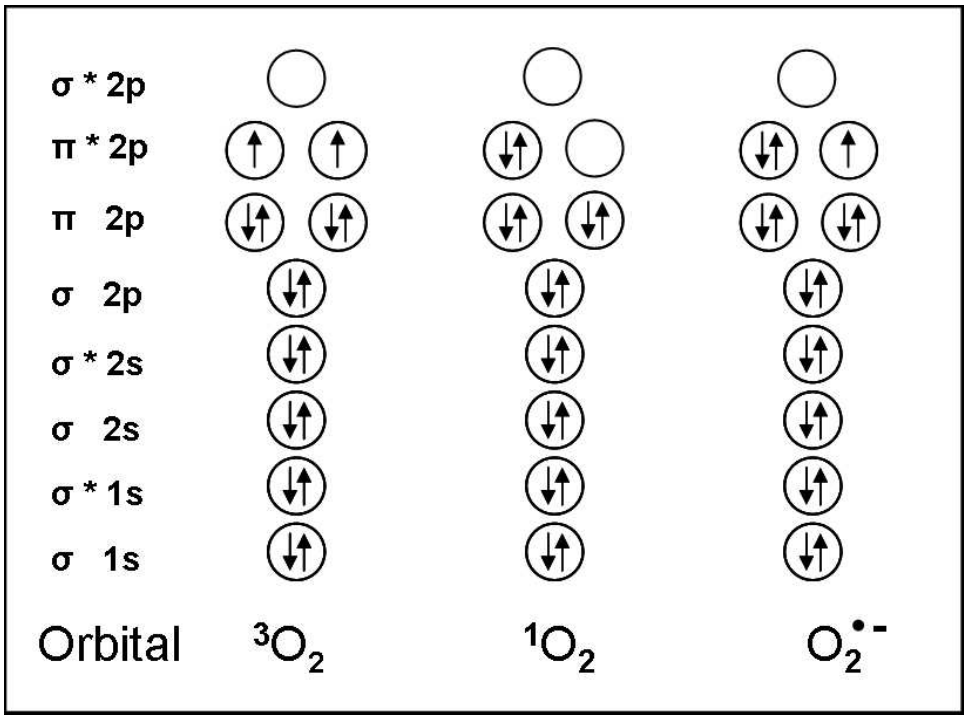
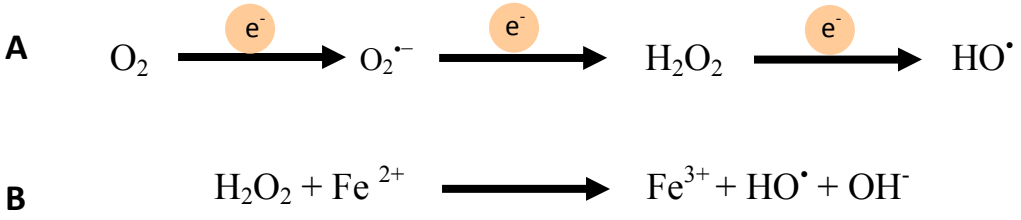


Figure 2.4. Simplified description of the electron distribution of $^3O_2$, $^1O_2$ and $O_2^{\bullet -}$ (Halliwell and Gutteridge 1999).

The formation of ROS in PSII is mainly due to leakage of electrons which reduces $^3O_2$ and forms ROS. $^3O_2$ is known for a long time to become reduced by components of the electron transport chain leading to formation of $O_2^{\bullet-}$. The high light-induced formation of $O_2^{\bullet-}$ in PSII RC is well-known (review Pospíšil 2009, Vass 2011). The one electron reduction of $^3O_2$ on the acceptor side of PSII can be possible by a number of potential reductant (e.g. $Pheo^{\bullet-}$, Q_A^-, Q_B^-) (review of Pospíšil 2009, 2012, Vass 2012) which generates $O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$ (Equation 2.2 A). On the other hand, the formation of ROS at donor side of PSII is favorable, when the oxidation of water is incomplete and ultimately causes formation of $O_2^{\bullet-}$ and $HO^{\bullet}$. Conversely, $O_2^{\bullet-}$ can undergo dismutation by the enzyme superoxide dismutase (SOD) and resuts into the formation of H_2O_2 (Halliwell and Gutteridge 1999). The formation of ROS in PSII is scavenged by different antioxidant under physiological steady state condition and often confined to the site of origin.

Redox couple	Standar redox potential	Reference
$O_2/HO_2^{\bullet}$	-37 mV	Ilan et al. (1976)
$O_2/O_2^{\bullet-}$	-160 mV	Halliwell and Gutteridge (1999)
$H_2O_2/H_2O,HO^{\bullet}$	320 mV	Halliwell and Gutteridge (1999)
O_2/H_2O_2	810 mV	Hauska et al. (1983)
$O_2^{\bullet-}/H_2O_2$	940 mV	Asada and Takahashi (1987)
$HO^{\bullet}/H_2O$	2.31 V	Halliwell and Gutteridge (1999)

Table- 2.2 Standard Reduction Potential of redox couple.



Equation. 2.2. One electron reduction of $^3O_2$ (A) and Fenton reaction (B)

However, the equilibrium between the formation and scavenging of ROS is disturbed during adverse environmental stresses such as high light, which enhance the generation of ROS. H_2O_2 can be converted into $HO^\bullet$ via so called Fenton reaction with transitional metals like Fe^{2+} or Cu^{2+} (equation 2.2 B). Addition of one electron to the $^3O_2$ forms $O_2^{\bullet-}$ with one unpaired electron. However, the further addition one electron to $O_2^{\bullet-}$ will form peroxide ion O_2^{2-} (a non-radical). Since the extra added electron in to $O_2^{\bullet-}$ and O_2^{2-} goes in antbonding orbitals, the strength of the oxygen-oxygen bond drops. However, the two electrons reduction product of $^3O_2$ is H_2O_2 (equation 2.2) and four electrons reduction forms two molecules of water and $HO^\bullet$.

2.6.2 Singlet oxygen

2.6.2.1. Lifetime of singlet oxygen

Molecular oxygen has two low-lying singlet excited states, $^1\Delta_gO_2$ and $^1\Sigma_g^+O_2$ ($^1\Delta gO_2$ = 22.4 kcal and $^1\Sigma_g^+O_2$ = 37.5 kcal) above the triplet state. The transition from the $^1\Delta_gO_2$ state to $^1\Sigma_g^+O_2$ is not possible due to the higher energy level of $^1\Sigma_g^+O_2$ state. Thus, $^1\Sigma_g^+O_2$ state is a relatively short-lived species, whereas the $^1\Delta_gO_2$ is relatively long-lived due to a spin-allowed transition of $^1O_2$ from high energy state to low energy state. Both type of $^1O_2$ are different in stability, which could be confirmed by the relative life time. The life time of $^1\Delta_gO_2$ and $^1\Sigma_g^+O_2$ is 5 min and 7-12 s in the gas phase (Arnold et al. 1968) and 10^{-6}-10^{-3} s and 10^{-11}-10^{-9} s in solution (Merkel et al. 1972), respectively. $^1\Delta_gO_2$ state is commonly referred as $^1O_2$. The life time of $^1O_2$ is enhanced by several folds when water is replaced by deuterium oxide (Halliwell and Gutteridge 1999). It is assumed that the life time of $^1O_2$ in cell is 200 ns (Gorman and Rodgers 1992). This

means that $^1O_2$ reacts immediately with surrounding environments. The diffusion limits of $^1O_2$ in the cells were indicated at 100 nm (Hatz et al. 2008.) However, it was shown that a small amount of $^1O_2$ (5-10%) produced may escape the biological membranes and diffuse into the extracellular space (Skovsen et al. 2005, Krieger-Liszkay 2004). With the help of a hydrophilic spin trap, the detection of $^1O_2$ produced in PSII, thylakoid membranes and even outside (Fischer et al. 2007, Hideg et al. 1994a,1994b, Krieger-Liszkay, 2008)has been already established.

2.6.2.2. Detection of singlet oxygen

There are various methods to detect $^1O_2$ in biological systems. In the RC of PSII, $^1O_2$ can be directly detected by chemical trapping methods (Telfer et al. 1994a) by fluorescence probe (Kalai et al. 1998), chemiluminescence (Macpherson et al. 1993) and by electron paramagnetic resonance (EPR) spectroscopy (Hideg et al. 1994b, Hoshida et al. 1997). Among them, the EPR spectroscopy is a one of the most preferred method of $^1O_2$ detection (Hideg et al. 1994b, Spetea et al. 1997, Fufezan et al. 2007). The EPR spectroscopy is the most frequently performed using spin trap, TEMP (2,2,6,6 - tetramethyl) which is a stable spin trap used for the detection of $^1O_2$.

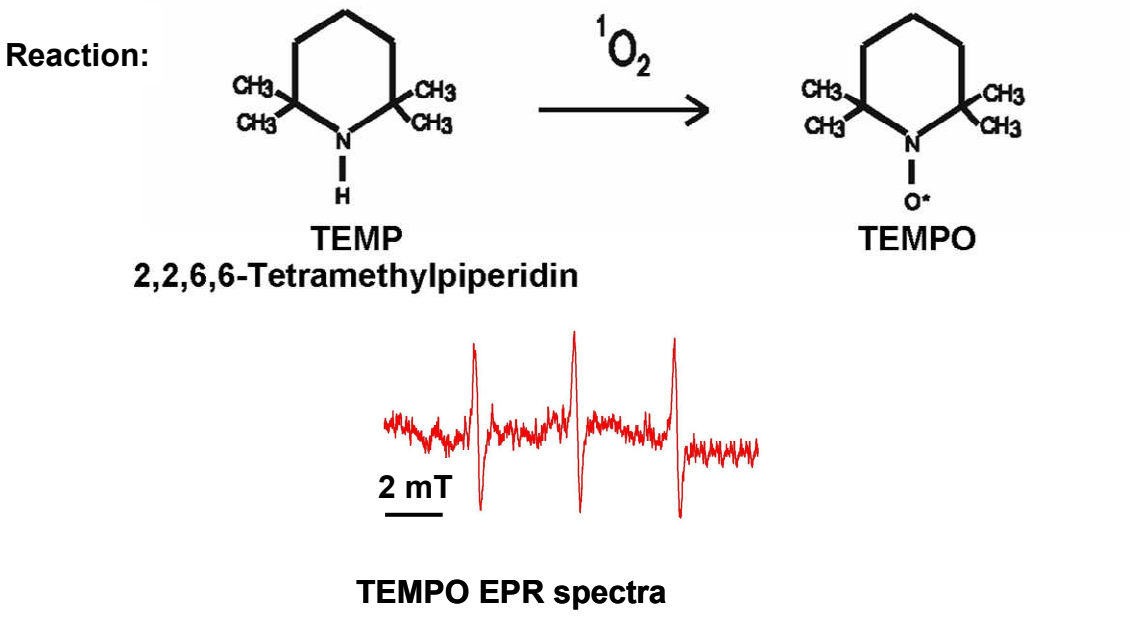


Figure 2.6: Reaction of $^1O_2$ with TEMP to TEMPO

2.6.2.3. Singlet oxygen formation in PSII via triplet energy transfer

In plants, algae and cyanobacteria, light energy is captured by means of pigments. The central light harvesting pigment Chl is a tetrapyrrole structure with a central magnesium ions and a phytol skeleton, which is responsible for the lipophylic character. The conjugated double bonds in tetrapyrrol ring system cause electrons to be displaced by light absorption in an excited state. This excited state can be maintained up to a few nanoseconds, which is sufficient time for an energy or electron transfer. In the excited singlet state of Chl, electrons change their spin orientation and slightly lower energy triplet state is formed via inter-system crossing. The energy of the $^3Chl^*$ is sufficient for $^3O_2$ to react and produce highly reactive $^1O_2$, which results in oxidative damage to biological systems, unless it is not quenched by carotenoids (Fig. 2.7). Despite the tight coupling of Chl, $^1O_2$ formation occurs in the antenna of PSII which contributes photodamage (Santabarbara et al. 2001, 2002, Rinalducci et al. 2004). $^3Chl*$ is formed either by intersystem crossing from singlet Chl in the antenna complex or by charge recombination of primary radical pair [$P680^{+\bullet}$ $Pheo^{\bullet-}$] in the PSII RC (Krieger-Liszkay et al. 2008, Triantaphylides and Havaux 2009, Vass and Cser 2009, Pospíšil 2012).

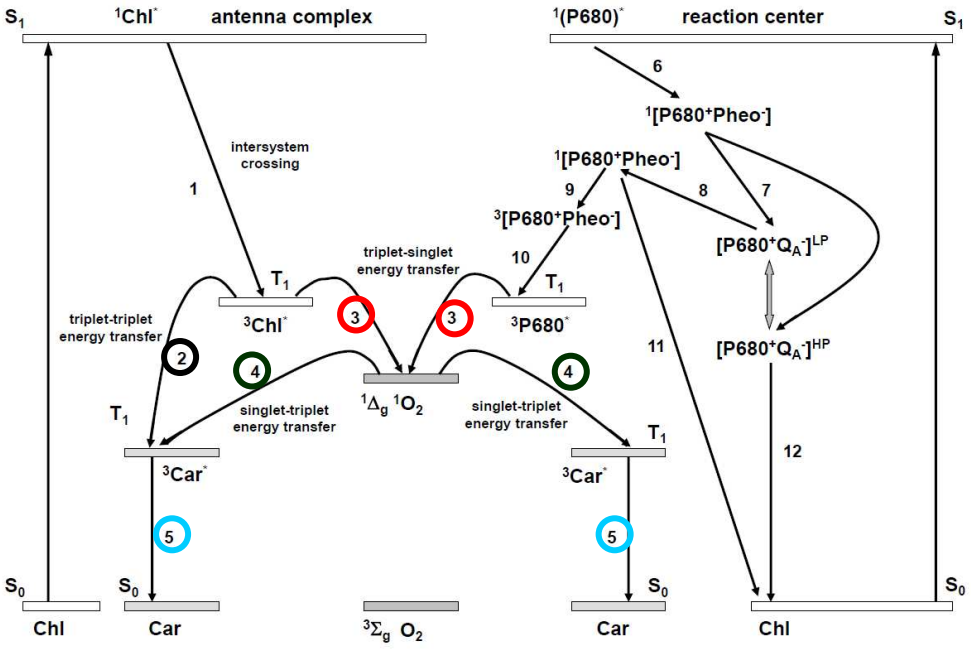


Fig. 2.7: Generation and quenching mechanism of $^1O_2$ in antenna complex and RC of PSII through excitation energy transfer (Pospíšil 2012). The excitation energy transfer from the triplet Chl to carotenoid forms the triplet carotenoid (^{3}Car*) (reaction 2). The triplet excitation energy transferred from ^{3}P680* to $^3O_2$ forms $^1O_2$ which takes place in RC and antenna complex of PSII (reaction 3). The singlet–triplet energy transfer from $^1\Delta_gO_2$ to carotenoid is possible by the interaction of $^1\Delta_gO_2$ and carotenoid which results in to the formation of ^{3}Car* and $^3O_2$ (reaction 4). The triplet excitation energy of carotenoid is released as heat (reaction 5).

2.6.3. Superoxide anion radicals

2.6.3.1. Lifetime of superoxide anion radicals

The average life time of $O_2^{\bullet-}$ in solutions in microsecond (10^{-6}). The rate of hydrogen atom abstraction by $O_2^{\bullet-}$ ($k \sim 0.3–1.2 \times 10^3$ M^{-1} s^{-1}) (Aikens and Dix 1991) is far lower than $HO^{\bullet}$ ($k \sim 10^9$ M^{-1} s^{-1}) (Sawyer and Valentine 1981), hence the reactivity of $O_2^{\bullet-}$ are less than $HO^{\bullet}$ radical.

2.6.3.2. Detection of superoxide anion radicals

Different methods are employed for the detection of $O_2^{\bullet-}$ formation like using chemiluminesce assay (Tarpey 2004), cyt c reduction in the presence of xanthine/xanthine oxidase (Ananyev 1994) and EPR spin-trapping spectroscopy (Pospíšil et al. 2004, 2006, Tiwari and Pospíšil 2009, Pospíšil and Tiwari 2010). Detection of $O_2^{\bullet-}$ using EPR spin trapping was improved due to the presence of various spin traps like DEPMPO and EMPO. Superoxide anion radicals react with DMPO and yields DMPO-OOH adducts (Goldstein et al. 2004). The average life of DMPO-OOH adducts at neutral pH is 60 s (Buettner and Oberley 1978,

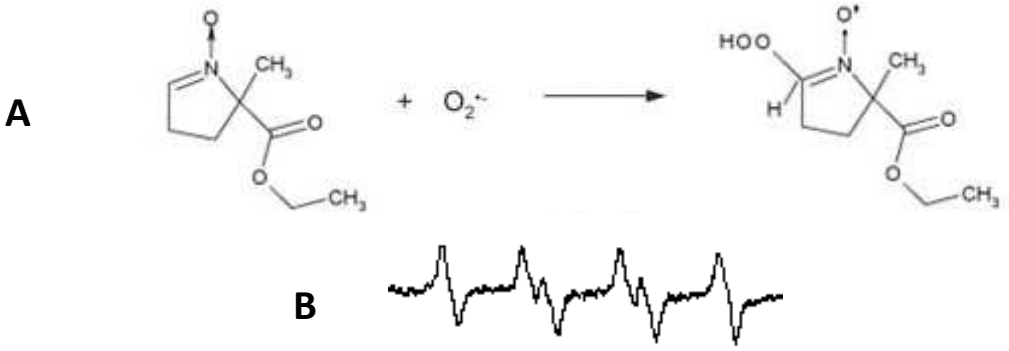


Fig. 2.8. Detection of $O_2^{\bullet-}$ using EMPO A) interaction of EMPO with $O_2^{\bullet-}$ forms EMPO-OOH adduct B) the representative EPR spectrum EMPO-OOH adduct.

Finkelstein et al. 1979, Yamazaki et al. 1990, Frejaville et al. 1995), of DEPMPO-OOH is about 15 min (Frejaville et al. 1994, 1995, Villamena and Zweier 2002), and EPMO-OOH is about 5-8 min (Olive et al. 2000, Zhang et al. 2000, Stolze et al. 2002). The rate of $O_2^{\bullet-}$ trapping by DMPO is strongly dependent on pH, whereas the DEPMPO-OOH stability is different with biological system (Dikalov et al. 2002). In last few years, this problem was partially overcome by use of EMPO spin trap for $O_2^{\bullet-}$.

2.6.3.3. Formation of superoxide anion radicals in PSII

Among the different ROS, $O_2^{\bullet-}$ is one of the destructive ROS in living cells causing oxidative damage and producing more reactive radicals. Superoxide anion radicals can be formed at the acceptor side of PSII but its molecular mechanism of formation is not clearly understood. Molecular oxygen is known to get reduced by components of the electron transport chain leading to the formation of $O_2^{\bullet-}$ and H_2O_2. It has been suggested that primary electron acceptor $Pheo^{\bullet-}$ (Ananyev et al. 1994), primary quinone electron acceptor Q_A^- (Cleland and Grace 1999), secondry quinone electron acceptor Q_B^- (Zhang et al. 2003), plastoquinol and the 'low potential' form of the PSII subunit of cyt b_{559} (Kruk and Strzałka 1999, 2001, Khorobrykh and Ivanov 2002, Pospíšil et al. 2006) have served as the reductants of $^3O_2$ (see review Pospíšil 2009). When the redox potentials of pheophytin cofactors (-610 mV. Rutherford et al. 1981), Q_A (-80 mV, Krieger et al, 1995), Q_B (≈ 0 mV Krieger et al, 1995), cyt b_{559} (0 -80 mV, Stewart and Brudvig 1998) and the PQ pool (100 mV, Golbeck and Kok, 1979) is not obvious, than an electron transfer to $^3O_2$ is thermodynamically possible. The potentials, however, depend on several factors such as pH, concentration of the molecules. For example, the $O_2/O_2^{\bullet-}$ potential is positive when the O_2 concentration is low. As in PSII membrane the concentration of $^3O_2$ (250 μM) is very high in comparision to $O_2^{\bullet-}$. In redox terms, the Q_A^- is most favorable for reduction of $^3O_2$ as the life time of Q_A^- is short under normal circumstances however, under certain conditions (e.g. under high light, during the assembly of PSII etc.) they may have lifetimes long enough to reduce $^3O_2$ (see review Pospíšil 2009).

From a thermodynamical point of view, $Pheo^{\bullet-}$ is the strongest reductant located on PSII acceptor side. However, under normal conditions $Pheo^{\bullet-}$ is either very quickly reoxidized by Q_A or interacts with $P680^{\bullet+}$ via the recombination pathway. Therefore, the life-time of $Pheo^{\bullet-}$ is very low and restricts its eventual reduction of $^3O_2$. Conversly, the reduction of $^3O_2$ by less reducing Q_A^- (Em = –80 mV pH 7) is more likely, because Q_A to Q_B forward electron transfer is slower. It is possible that the particular electron donor to $^3O_2$ can change, depending on the intactness of the photosynthetic preparation (Zastrizhnaya et al. 1997) or the phase of photoinhibition. This makes the possibilities that in the early phase of photoinhibition Q_A^- serves as a reductant of $^3O_2$, whereas in later $Pheo^{\bullet-}$ donates electron to $^3O_2$ (Pospíšil et al. 2004).

CHAPTER 3

3. Methodology

This chapter describes the methods used for the isolation and storage of thylakoid membranes and PSII-enriched membranes from plant and cyanobacteria. This is followed by the specific details of the methods that have been used in this study.

3.1. Culture and growth condition of Cyanobacteria:

PSI-less and PSI-less/*scp*ABCDE$^-$ strains of *Synechocystis* sp. PCC 6803 were grown in a continuous white light (30 μmol m^{-2} s^{-1}) at 25 °C in BG-11 medium supplemented with 5 mM glucose and 5 mM sodium bicarbonate as the main carbon source (Rippka *et al.* 1979). The cell culture was grown in sterile glass bottle placed on a multi-position magnetic stirrer RT 5 power (*IKA* Werke GmbH**,** Staufen*,* Germany) and permanently stirred to obtain constant CO_2 concentration in the growing medium. The cells were studied during the end of log phase at a density of approximately 10^8-10^9 cells ml^{-1}. Cell density was determined by a manual microscopic cell count. For the determination of Chl concentration, the cell were pelleted at 10 000 x g for 3 min and extracted by 80% methanol. The Chl concentration was determined by the measuring the absorbance at 646 and 663 nm according to Lichtenthaler (1987).

3.2. Membranes preparation from spinach and cyanobacteria

3.2.1 Preparation of PSII-enriched membranes from spinach

Highly active PSII-enriched membranes were prepared from fresh spinach leaves purchased from a local market using the method of Berthold et al. (1981) with the modifications described in Ford and Evans (1983) and stored at -80 °C until use. All the procedures of isolation and washing were carried out under dim green light at 4°C. The buffers used for the various steps of membrane isolation were buffer A, 400 mM sucrose, 40 mM HEPES, 15 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$ (pH 6.5), 5 mM Na-ascorbate and 2 gm/l BSA. BSA and Na-ascorbate were added immediately before use; buffer B, 400 mM sucrose, 40 mM MES, 15 mM NaCl and 5 mM $MgCl_2$ (pH 7.5).

Overnight dark adapted, deveined, cold leaves were homogenized with buffer A, 500 ml of buffer A was used per 400 g leaves. Homogenate was then filtered through 2 layers of nylon bolting cloth. Filtrate was kept in cold in 6 x 400 ml tube and centrifuged at 9950 x g for 10 min at 4ºC. The pellet was mixed gently with paint brush and re-suspended in 600 ml of buffer B in 2 x 400 tubes. Suspension was again centrifuged at 9950 x g for 10 min at 4ºC. Supernatant was discarded; Chl concentration was measured and pellet is resuspended in 50-150 ml buffer B to final concentration of 2.7 mg Chl ml^{-1} and it was homogenized in ice bath. An aqueous solution of Triton X-100 (20% v/v in buffer B) was added to the suspension with continuous stirring until the final Chl concentration became 2 mg Chl ml^{-1} (final 5 % triton X-100). After incubation for 17 min on ice with continuous stirring, the suspension was centrifuged at 7000 x g for 7 min at 4ºC. The pellet was discarded and supernatant centrifuged at 48400 x g for 20 min at 4ºC. The supernatant was discarded; pellet resuspended in buffer B, and last step was repeated (1-3 times) until the clear supernatant appears. The final pellet was dissolved in 5-15 ml buffer B to make final Chl concentration to 3-4 mg Chl ml^{-1}, aliquot in 500 µl tube and stored in -80 until use.

3.2.2. Preparation of PSII membranes deprived of Mn complex

PSII membranes deprived of water splitting Mn complex and 17, 23 and 33 kDa extrinsic proteins were prepared by Tris treatment. Prior to the experiment, the membranes were slowly thawed at 0-4 °C. The thawed PSII membrane was resuspended in a cold buffer containing 0.8 M Tris-HCl (pH 8) with final Chl concentration of 1 mg Chl ml^{-1}. The resuspended membranes were incubated for 30 min at 4 °C in the darkness with continuous gentle stirring. After treatment, PSII membranes were washed twice in resuspension buffer containing 400 mM sucrose, 10 mM NaCl, 5 mM $CaCl_2$ and 40 mM MES-NaOH (pH 6.5). The pellet was dissolved in minimum amount of resuspension buffer. Chl concentration was determined as described in (Vernon, 1960) and the final Chl concentration in all the samples was adjusted to 3 mg Chl ml^{-1}. All the steps were carried out in dim green light in ice.

3.2.3. Preparation of PSII membranes deprived of Plastoquinone

PQ-depleted PSII membranes were performed in two steps phase preparation and phase separation using the methods of Wydrzynski and Inoue (1987). During phase prepration, thylakoid membrane were dilute to 300 μg Chl ml^{-1} in a plastic tube and shaken with heptane (the ratio of heptane/sample is 3:1) and isobutanol (the ratio of isobutanol/sample is 1:30) for 40 min at room temperature in dark. After formation of two phases, the upper organic phase was discarded without touching the lower phase and transfers the lower phase (PSII membranes) in separate tube. Bubble the collected PSII membranes with air (Helium) for 10 minutes to remove the residual organic phase. PQ-depleted PSII membranes were stored at -80 °C at the final concentration of 3 mg Chl ml^{-1} until further use.

3.2.4. Preparation of thylakoid membranes from *Synechocystis*

Thylakoid membranes were isolated from *Synechocystis sp.* PCC 6803 cells using the method described by Komenda and Barber (1995) with the following modifications. The cell culture was spun down for 5 min at 8000 x g and the pellet was resuspended in KPN buffer (50 mM K-phosphate, pH 8, 100 mM NaCl). After several washing steps, the cells were resuspended in KPN buffer containing a mini EDTA-free ROCHE protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany). Prior to the cell breaking, the cell culture with the same volume of glass beads was put in 15 ml tubes and kept in ice for 30 min. The cells were broken using a bead beater (BioSpec Products Inc., Bartlesville, Oklahoma, USA) with glass beads of 150-200 mm diameter over 6 cycles, each cycles comprising of one minutes of beating and 3 minutes of cooling. The unbroken cells, cell debris and thylakoid membranes were decanted off by repeated washing of the glass beads with KPN buffer. The decanted material was centrifuged at 3000 x g for 3 min to remove unbroken cells and cell debris. The supernatant was centrifuged at 40 000 x g for 20 min. The pellet was resuspended in 4 ml of KPN buffer along with an addition of 1 ml of glycerol and stored in -80 °C till use. The extraction procedure was carried out on ice in a dark room.

3.3. Measurments of oxygen evolution

The oxygen evolution activity of PSII enriched membranes and the suspension of *Synechocystis* cells (10 μg Chl ml^{-1}) was measured using a Clark type electrode (oxygen electrode chamber DW1, Hansatech, King's Lynn, Norfolk, UK) connected to an Oxyrecorder 3015 and supplied with Oxywin software 3.0 (Photon System Instruments, Brno, Czech Republic) at room temperature (25 ºC) with illumination of 2,500 μmol photons m^{-2} s^{-1} light intensity. The oxygen evolution in intact, and tris-treated PSII membrane (10 μg Chl ml^{-1}) was measured in the presence of artificial electron acceptors 0.4 mM 2,5-dimethyl-*p*-benzoquinone (DMBQ) and 2 mM potassium ferricyanide was placed in an oxygen electrode chamber, in the case of *Synechocystis* cells (10 μg Chl ml^{-1}) the photoinhibitory treatment was given before the measurements.

3.4. Photoinhibitory treatment

For assessment of the oxygen evolution and $^1O_2$ generation, the cell suspension or the thylakoid membranes were placed in the spectroscopic cuvette (2 ml) and exposed to continuous white light (1000 μmol photons m^{-2} s^{-1}). For assessment of Chl fluorescence spectra and protein damage, the cell suspension (50 ml, 1 μg Chl ml^{-1}) was transferred to plane-parallel cuvettes in which the cultures were bubbled with air and exposed to high light intensity illumination (2500 μmol photons m^{-2} s^{-1}) for 20 min. For assessment of oxygen evolution, Chl fluorescence spectra and protein damage, the high light intensity illumination was performed in the presence of 100 μg ml^{-1} tetracycline, an inhibitor of protein synthesis. The illumination was performed using a halogen lamp with a light guide (Schott KL 1500, Schott AG, Mainz, Germany) under slow continuous stirring with a tiny bar magnet. The light intensity was measured by quantum radiometer LI-189 (LI-COR Inc., Lincoln, Nebraska, USA).

3.5. Spectroscopic measurements

3.5.1. Measurement of redox form of cyt b_{559}

Optical absorption spectroscopy was used to study the amount of different redox states and redox properties of cyt b_{559} using Olis RSM 1000 spectrometer (Olis Inc., Bogart, Georgia, USA). The redox state and content of cyt b_{559} were determined from the absorbance changes measured at 559 nm by additions of 20 μM potassium ferricyanide (reference cuvette), 8 mM hydroquinone, 5 mM sodium ascorbate, sodium dithionite (test cuvette) to PSII membranes (150 μg Chl ml^{-1}) in a 3 ml quartz cuvette at room temperature. After addition of redox mediators, PSII membranes were slowly stirred for 5 min in the dark inside the spectrophotometer using a tiny bar magnet unless stated otherwise. After switching off the stirring, absorption spectra were recorded from 530 nm to 580 nm. The spectral slit width, the total band pass and the scan speed was 0.12 µm, 0.5 nm and 50 nm per min, respectively. The amount of different states of cyt b_{559} was calculated from the average spectra of five measurements. Different redox potential forms of cyt b_{559} were determined by treatment minus control spectrum. Total high potential (HP) form of cyt b_{559} was determined by difference spectra of hydroquinone-reduced minus ferricyanide-oxidized cyt b_{559}. The intermediate potential (IP) form of cyt b_{559} was determined by difference spectra of ascorbate-reduced minus hydroquinone-reduced cyt b_{559}, whereas the low potential (LP) form of cyt b_{559} was obtained by difference spectra of dithionite-reduced minus ascorbate-reduced cyt b_{559}. The light-induced formation of the HP form of cyt b_{559} was obtained by difference of the absorbance spectrum measured after illumination for 100 s and the dark-adapted ferricyanide oxidized spectrum. Illumination was performed with continuous white light (1000 μmol photons m^{-2} s^{-1}) by 90° rotating the cuvette at each 15 s interval using a halogen lamp with a light guide (Schott KL 1500, Schott AG, Mainz, Germany).

3.5.2. EPR measurements with PSII-enriched membranes

The detection of $O_2^{\bullet -}$ was accomplished by EMPO, 5-(ethoxycarbonyl)-5-methyl-1-pyrroline N-oxide (Alexis Biochemicals, Lausen, Switzerland). PSII membrane particles (150 µg Chl ml^{-1}) were illuminated in a glass capillary tube (Blaubrand® intraMARK, Brand, Germany) in the presence of 25 mM EMPO, 100 µM desferal, 40 mM MES (pH 6.5). Illumination was performed with continuous white light (1000 μmol photons m^{-2} s^{-1})

using a halogen lamp with a light guide (Schott KL 1500, Schott AG, Mainz, Germany). EPR spectra were recorded using an EPR spectrometer MiniScope MS200 (Magnettech GmbH, Berlin, Germany). Signal intensity was evaluated as a height of the central peak of EPR spectrum. EPR conditions were as follows: microwave power, 10 mW; modulation amplitude, 1 G; modulation frequency, 100 kHz; sweep width, 100 G; scan rate, 1.62 G s^{-1}.

3.5.3. EPR measurements with thylakoids membranes

For detection of $^1O_2$, the spin-trapping was accomplished by 2, 2, 6, 6-tetramethyl-4-piperidone (TEMPD) (Sigma, St Louis,MO, USA). Thylakoid membranes (150 µg Chl ml^{-1}) were illuminated on the ice with continuous stirring in the presence of 50 mM TEMPD and 40 mM MES (pH 6.5). In some measurements, 10 mM histidine (Sigma, St. Louis, MO, USA) or 50% D_2O (Sigma) was added to the sample prior to the illumination as indicated. After illumination, the sample was centrifuged at 5000 x g for 4 min to separate 2, 2, 6, 6-tetramethyl-4-piperidone-1-oxyl (TEMPONE) from the thylakoid membranes. The separation of the two phases was carried out to prevent the reduction of TEMPONE by non-specific reducing component in the thylakoid membranes. After centrifugation, the upper phase was immediately transferred into the glass capillary tube (Blaubrand® intraMARK, Brand, Hamburg, Germany) and frozen in the liquid nitrogen until its use. Prior to data collection, the capillary tube was taken away from the liquid nitrogen and EPR spin-trapping data were collected at room temperature. For detection of HO• the spin-trapping was accomplished by a 4-pyridyl-1-oxide-Ntert- butylnitrone (POBN)/ethanol spin trapping system (Pou et al. 1994). Thylakoid membranes (150 µg Chl ml^{-1}) were illuminated on the ice with continuous stirring in the presence of 50 mM POBN, 170 mM ethanol and 40 mM MES (pH 6.5). After illumination, the sample was immediately transferred into the glass capillary tube and EPR spectra were measured. Other EPR conditions were same as above (3.5.2.).

3.6. Singlet oxygen imaging by confocal laser scanning microscopy

Singlet oxygen imaging was performed by a confocal laser scanning microscope, Olympus Fluorview 1000 confocal unit, combined with inverted microscope IX 80 (Olympus Czech Group, Prague). Singlet Oxygen Sensor Green (SOSG) reagent® (Molecular Probes Inc., Eugene, Oregon, USA) was chosen to visualize $^1O_2$ production in the intact PSI-less and PSI-less/*scp*ABCDE$^-$ *Synechocystis* cells (Flors et al. 2006). The cells of both *Synechocystis* strains were harvested in the middle of the log-phase to facilitate SOSG uptake along with nutrients. The suspensions were diluted to a cell density of approximately 10^8-10^{10} cells ml^{-1}, stained with 250 μM SOSG and kept at 37 °C for 15 min to enhance the efficiency of fluorochrome uptake through mucoid sheath surrounding the cyanobacterial cell wall. Subsequently, the samples were incubated for 3 h at room temperature, either in dark or with exposure to the photoinhibitory treatment. Cell suspensions in 50% deuterium oxide or 10 mM histidine served as a positive or a negative control, respectively.

The transmission images were obtained by a transmitted light detection module with 405 nm excitation using a near-UV LD laser and Nomarski DIC channel. Concurrently, visualized fluorescence channels result from excitation by a 488 nm line of argon laser, representing the signals for SOSG signal detected by 505-525 nm emission filter and Chl fluorescence with 650-750 nm emission filter set. The proper intensity of lasers was set according to unstained samples at the beginning of each experiment.

3.7. Gel electrophoresis and immunoblotting

The thylakoid membranes were prepared as mention above (3.2.4). The isolated protein complexes from the thylakoid membrane is solubilized in 1% β-n-dodecyl maltoside (DM) and analyzed using a clear variant of blue-native electrophoresis at 4 ºC in a 5-14 % gradient polyacrylamide gel according to Schägger and von Jagow (1991) . In this variant, Coomassie Blue was remoced from all the solutions and the upper electrophoretic buffer contained 0.05% sodium deoxycholate and 0.02% DM. Samples with the same Chl content (1 μg) were loaded onto the gel.

The protein composition of the complexes was analysed using electrophoresis in a denaturing 12-20 % linear gradient polyacrylamide gel containing 7 M urea (Komenda et al. 2002). Complete lanes from the native gel were excised, incubated for 30 min in 25 mM Tris (pH 7.5) containing 1 % SDS (w/v) and fit into the top of the denaturing gel in such a way that no bubbles come in between the gel; two lanes were analyzed in a single denaturing gel. The separated proteins were stained using Sypro Orange and than transferred onto a polyvinylidene difluoride (PVDF) membrane. The primary antibody raised in rabbit against residues 58-86 of D1 polypeptide were used for the incubation of membranes subsequently with West Pico and later incubated with secondary antibody-horseradish peroxidase conjugate and finally with chemiluminescent substrate. The chemiluminescence of the blot as well as the Sypro Orange fluorescence of proteins in the gel was recorded by LAS-4000 imaging system (Fuji, Tokyo, Japan) whereas; the quantification was performed using ImageQuant TL software (GE Healthcare, Uppsala, Sweden).

CHAPTER 4

4. Results and Discussion

4.1. Effect of water-splitting Mn complex on light-induced redox changes of cyt b_{559}

4.1.1. Characterisation of redox form of cyt b_{559} in PSII membranes (Paper I)

Characterization of different redox state of cyt b_{559} was performed by measuring the absorption changes at 559 nm after oxidation of cyt b_{559} with potassium ferricyanide and reduction by hydroquinone (HP form), sodium ascorbate (IP form) and sodium dithionite (LP form). This confirms that the removal of water-splitting Mn complex caused conversion of the HP form into the IP and the LP form of cyt b_{559}.

In agreement with previous reports, we have demonstrated that the exposure of PSII membranes to continuous white light caused reduction and oxidation of the heme iron (Buser et al. 1990, Buser et al. 1992, Barber and De Las Rivas 1993, Tiwari and Pospíšil 2009, Pospíšil and Tiwari 2010). We present here the convincing evidence that the mechanism of light-induced redox changes of cyt b_{559} depends upon the integrity of PSII electron donor side. We demonstrated that photoreduction of cyt b_{559} in the PSII membranes containing water-splitting Mn complex was suppressed by DCMU (Fig. 4.1.2 A), whereas in the PSII membranes lacking water-splitting Mn complex photoreduction and photooxidation of cyt b_{559} were abolished by exogenous SOD (Fig. 4.1.3). Based on these observations it is proposed that in PSII membranes with the intact water-splitting Mn complex photoreduction of the heme iron is mediated by plastoquinol, whereas in the PSII membranes deprived of water-splitting Mn complex, photoreduction and photooxidation are mediated by $O_2^{\bullet -}$ in PSII.

Illumination of the PSII membranes with the intact water-splitting Mn complex caused reduction of cyt b_{559} (Fig. 4.1.1A, trace a). Photoreduction of cyt b_{559} has been reached within 100 s of continuous illumination and reduced form of cyt b_{559} was found stable within the whole period of illumination upto 300 s. The addition of hydroquinone in the sample after illumination for 100 s did not cause any further reduction of the heme iron (Fig. 4.1.1A, trace b), whereas sodium ascorbate and sodium dithionite resulted in reduction of cyt b_{559} (Fig. 4.1.1A, traces c and d). These results confirm that illumination

of the PSII membranes with the intact water-splitting Mn complex caused photoreduction of the HP form of cyt b_{559}, whereas the IP and the LP forms of cyt b_{559} were unaffected.

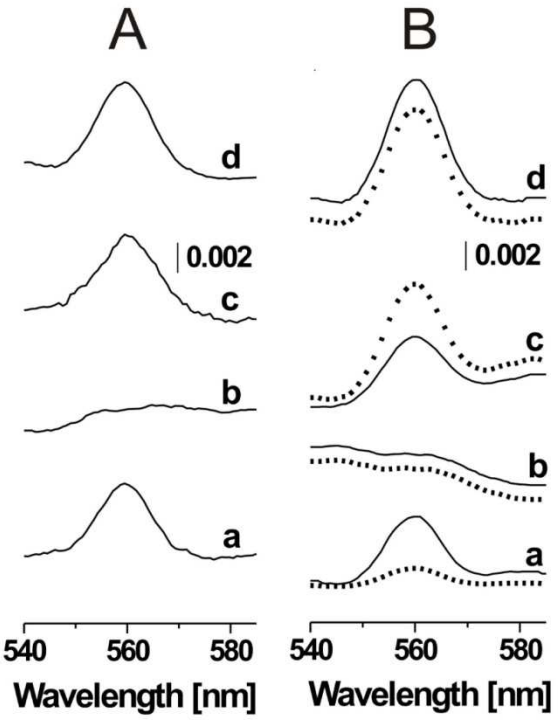


Fig 4.1.1. Light-induced redox changes of cyt b_{559} measured in spinach PSII membranes with the intact water-splitting Mn complex (A) and deprived of water-splitting Mn complex (B). PSII membranes (100 µg Chl ml^{-1}) were illuminated with continuous white light (1000 μmol photons m^{-2} s^{-1}) for 100 s (solid line) and 300 s (dotted line).

However, illumination of the PSII membranes with the deprived of water-splitting Mn complex caused reduction of cyt b_{559} (Fig. 4.1.1B, trace a, solid line) and cause no further reduction after addition of hydroquinone in 100 s illuminated sample (Fig. 4.1.1B, trace b solid line) and it was reduced by sodium ascorbate and sodium dithionate (Fig. 4.1.1B, trace c and d solid line). The ascorbate reducible form increased after 300 s of illumination 4.1.2B, trace c and d dotted line), whereas no further reduction was observed after addition of hydroquinone (Fig. 4.1.1B, trace b dotted line), and by addition of sodium ascorbate and sodium dithionite resulted in the reduction of cyt b_{559} (Fig. 4.1.1B, traces c and d, dotted lines). These results confirm that illumination of the PSII

membranes deprived of water-splitting Mn complex caused reduction and oxidation of the IP and the HP form of cyt b_{559}, respectively.

4.1.2. Role of cytochrome b_{559} in PSII (Paper I)

We have shown in previous chapter about the form and function of cyt b_{559}. Here we will discuss about the important role of cyt b_{559} in $O_2^{\bullet-}$ production in PSII. Cyt b_{559} is an essential component of purified PSII, which is a functional subunit of D1, D2 and cyt b_{559} complex (Nanba and Satoh 1987).

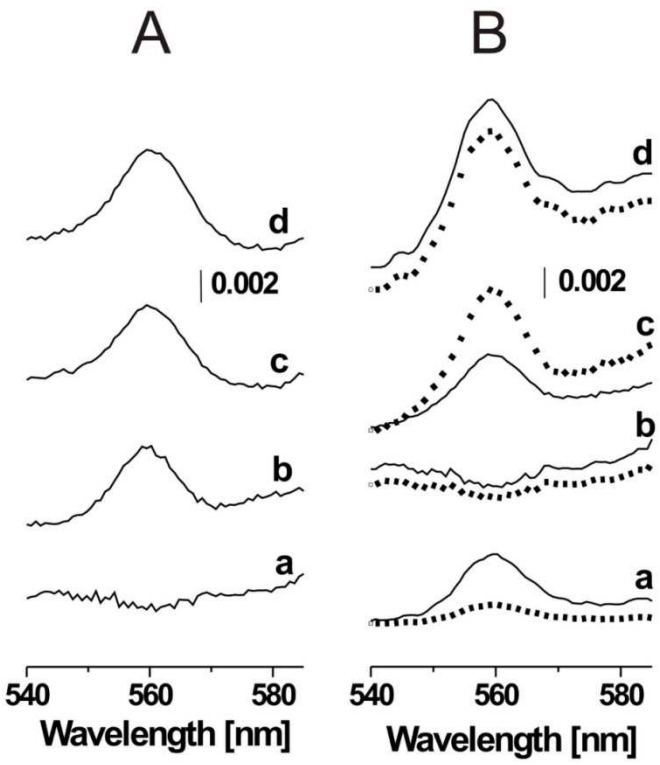


Fig. 4.1.2. Effect of DCMU on light-induced redox changes of cyt b_{559} measured in spinach PSII membranes with the intact water-splitting Mn complex (A) and deprived of water-splitting Mn complex (B). PSII membranes (100 μg Chl $m1^{-1}$) were illuminated with continuous white light (1000 μmol photons $m^{-2}s^{-1}$) for 100 s (solid line) and 300 s (dotted line). Prior to illumination, 10 μM DCMU was added to PSII membranes. The spectra represent the difference of light minus ferricyanide-oxidized spectra (a), hydroquinone-reduced minus light spectra (b), ascorbate-reduced minus hydroquinone-reduced spectra (c) and dithionite-reduced minus ascorbate-reduced spectra (d)

It is oriented perpendicular to the membrane and located more on the stromal side of thylakoid membrane (see Fig. 2.3). PSII membrane is not functional in a *Synechocystis*-deletion mutant lacking cyt b_{559} subunits (Pakrasi et al. 1990). However, the deletion of cyt b_{559} subunits lead to loss of heme and in most cases fatal consequences as no PSII is assembled. This complicates the functional study of the cyt b_{559} and remained unclear the role of cyt b_{559} in PSII electron transport to date. Illumination of PSII membranes with the intact water-splitting Mn complex caused photoreduction of the HP form of cyt b_{559} which is in correlation with earlier work (Buser et al. 1990, Buser et al. 1992, Barber and De Las Rivas 1993, Tiwari and Pospíšil 2009, Pospíšil and Tiwari 2010).

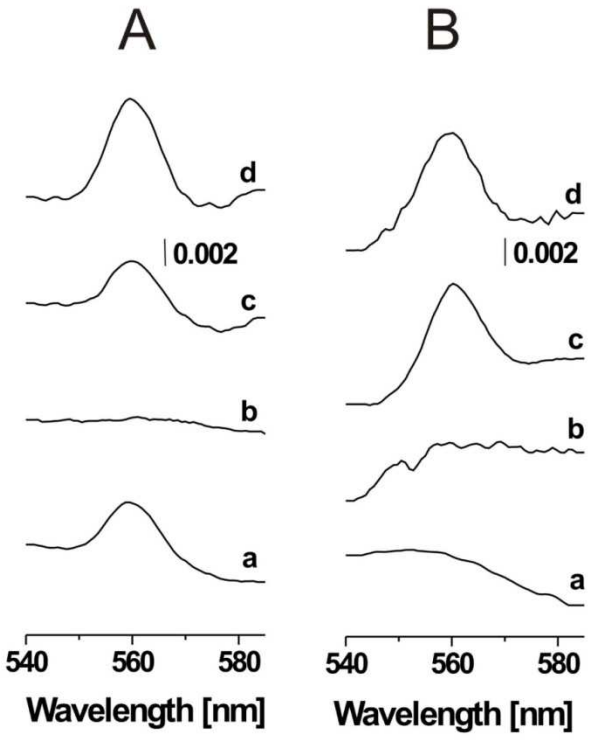


Fig. 4.1.3. Effect of SOD on light-induced redox changes of cyt b_{559} measured in spinach PSII membranes with the intact water-splitting Mn complex (A) and deprived of water-splitting Mn complex (B). PSII membranes (100 μg Chl ml^{-1}) were illuminated with continuous white light (1000 μmol photons $m^{-2}s^{-1}$) for 100 s. prior to illumination 400 U ml^{-1} SOD was added to PSII membranes. The spectra represent the difference of light minus ferricyanide-oxidized spectra (a), hydroquinone-reduced minus light spectra (b), ascorbate-reduced minus hydroquinone-reduced spectra (c) and dithionite-reduced minus ascorbate-reduced spectra (d)

In the presence of DCMU illumination of PSII membranes deprived of water-splitting Mn complex caused photoreduction of cyt b_{559} (Fig. 4.1.2, trace a, solid line), whereas further illumination upto 300 s resulted in photooxidation of cyt b_{559} (Fig. 4.1.2 B, trace a, dotted line). Further addition of hydroquinone did not cause any reduction of cyt b_{559}, whereas the addition of sodium ascorbate and sodium dithionite caused reduction of cyt b_{559} (Fig. 4.1.2B, traces b-d). This observation reveals that the photoreduction of heme iron in PSII membranes with the intact water-splitting Mn complex is mediated by plastoquinol and in the PSII membranes deprived of water-splitting Mn complex mediated through $O_2^{\bullet-}$. The recent crystal structure by Guskov et al. (2009) shows the presence of Q_C site, which contains additional plastoquinone. The distance between the head group of plastoquinol bound at the Q_C site and the heme iron is 17 Å. Thus it is proposed that possible rebinding of plastoquinol molecule at Q_C site might provide an appropriate condition for electron donation to the heme iron of cyt b_{559}.

4.2. Singlet oxygen scavenging activity of plastoquinol in photosystem II (Paper II)

4.2.1. Singlet oxygen scavenging activity in photosystem II

Plastoquinone is present in PSII and known to work as electron carrier. One electron photoreduction of Q_A form semiquinone ($PQ^{\bullet-}$) whereas, two electrons photoreduction along with protonation of Q_B form plastoquinol which is released into the membrane and replaced by an additional plastoquinone molecule (Petrouleas and Crofts, 2005). Under high-light intensity degradation of D1 protein is increased by consequence of formation of $^3P680^*$, when the redox state of plastoquinone pool and plastoquinone acceptors Q_A and Q_B is over reduced. In the RC of PSII, two carotenoid molecules are located. The distance between carotenoid molecules and P680-Chl from D1 protein is approximately 20 Å and 30 Å consequently. The distance between D2 from the carotenoid molecules and accessory Chl molecules is 13.2 Å and 23.5 Å, respectively (Guskov et al. 2009). The effective quenching of $^3P680^*$ by carotenoids is less likely due to large distances.

The appropriate distance between the carotenoids and Chl is necessary in the charge

separation to ensure the transfer of electrons from water-splitting to tyrosine Z to P680. After the charge separation $P_{680}^{+\bullet}$ is formed, with extremely high oxidizing potential (1.12 V) (Rappaport et al. 2002) and carotenoids are far away enough to be immediately reduced them. However, it was shown that the carotenoids may contribute to the quenching of $^1O_2$ in PSII (Telfer et al. 1994b, Telfer 2002, Juan et al. 2011). Other important $^1O_2$ scavengers in biological membranes are α-tocopherol (Trebst 2003, Krieger-Liszkay and Trebst 2006, Juan et al. 2011). If the formation of $^1O_2$ will be in immediate surrounding of D1 and there will be less efficient quenching, than $^1O_2$ cause damage to D1 protein. During exposure of PSII to single flash, a correlation between the D1 degradation and the formation of $^3P680^*$ was found (Keren et al. 1995, 1997). The degradation of the D1 protein is also coupled with the formation of $^1O_2$ (Hideg et al. 1994b). In RC of PSII, when $^3Chl^*$ is not properly quenched by carotenoids, the interaction of $^3O_2$ with $^3Chl^*$ results in the formation of $^1O_2$ (see Fig. 2.7).

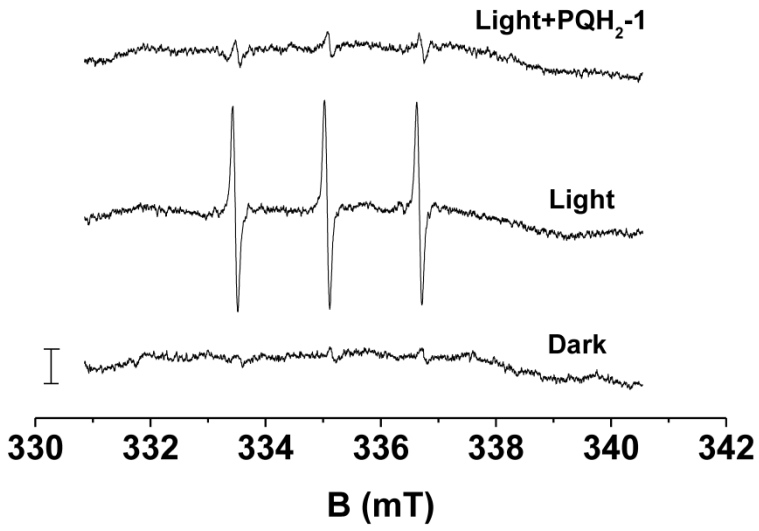


Fig. 4.2.1. Effect of exogenous plastoquinol (PQH_2-1) on the rose bengal-induced TEMPONE EPR spectra**.** Rose bengal (5 μM) was illuminated with white light (1000 μmol m^{-2} s^{-1}) for 10 min in the presence of 50 mM TMPD and 25 mM phosphate buffer (pH 7.0). Prior to illumination, 100 μM PQH_2-1 was added to the reaction mixture. Vertical bar represents 1000 relative units.

Carotenoids and α-tocopherol plays important role in the prevention of the D1 protein degradation (Telfer et al. 1994a, Telfer 2002, 2005, Krieger-Liszkay 2005, Trebst et al. 1997, 2002, 2003) and in photoprotection (Durchan et al. 2010). If the formation of $^1O_2$ takes place in the vicinity of carotenoid the scavenging of $^1O_2$ by carotenoid molecules is most likely due to singlet-trplet energy transfer as the energy level of carotenoids is below the energy level of $^1O_2(^1\Delta_g)$. Carotenoids loose the received triplet excitation energy into heat and thus help in maintenance of PSII structure and function by scavenging the $^1O_2$. Scavenging of $^1O_2$ by carotenoids is known to occur primarily through energy transfer mechanism (Damjanovic et al. 1999, Cantrell et al. 2003, Krieger-Liszkay and Trebst 2006, Gruszka et al. 2008, Triantaphylides and Havaux 2009). In this process, the energy of the excited $^1O_2$ is transferred to the carotenoid molecule. In scavenging of $^1O_2$ by α-tocopherol, the interaction of $^1O_2$ with α-tocopherol form intermediate 8-hydroperoxy-tocopherone via oxidation. This intermediate hydrolyzed irreversibly to α-tocopherolquinone (Trebst et al. 2002, Kruk et al. 2005). Recently, the photoprotection of D1 protein by trolox, a water soluble form of alpha-tocopherol was shown in PSII RC (Arellano et al. 2011). However, it has been proposed that not only α-tocopherol analogues are active in preserving the photosynthetic activity and D1 protein degradation in the presence of inhibitor, but also short chain of plastoquinones are active (PQ-1 and PQ-2).

4.2.2. Effect of plastoquinol on $^1O_2$ formation (Paper II)

Singlet oxygen was generated by photosensitization of rose bengal. Illumination of rose bengal results in the formation of excited triplet rose bengal which reacts with triplet $^3O_2$ to produce $^1O_2$ (Baroli et al. 2004, Fischer et al. 2004). Addition of TMPD spin trap compound to rose bengal in the dark did not result in the appearance TEMPONE EPR spectra (Fig. 4.2.1). When rose bengal was illuminated in the presence of plastoquinol (PQH_2-1), TEMPONE EPR signal was completely suppressed (4.2.1). On the other hand,

when PQ-depleted PSII membranes exposed to continuous white light (1000 μmol photons $m^{-2}s^{-1}$) resulted in the generation of TEMPONE EPR spectra (Fig. 4.2.2A).

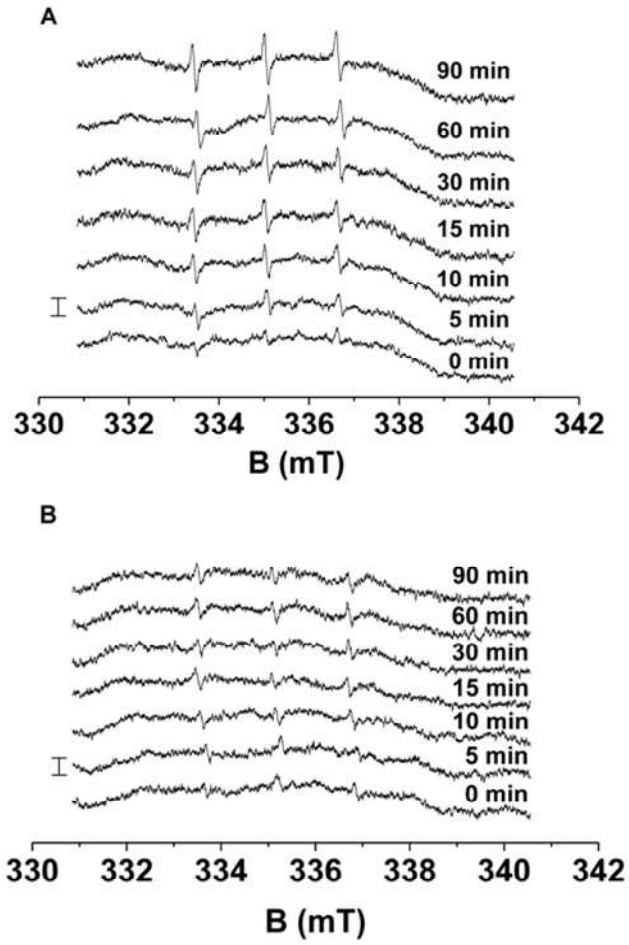


Fig. 4.2.2 Light-induced TEMPONE EPR spectra measured in spinach PQ-depleted PSII membranes in the absence (A) and in the presence (B) of exogenous plastoquinol (PQH_2-1). PQ-depleted PSII membranes (150 μg Chl. $m1^{-1}$) were illuminated with white light (1000 μmol m^{-2} s^{-1}) in the presence of 50 mM TMPD and 40 mM MES-NaOH (pH 6.5) for the time indicated in (B), 100 μM PQH_2-1 was added to the sample prior to illumination. Vertical bar represents 500 relative units.

However, addition of exogenous plastoquinol (PQH_2-1) to PSII membranes prior to illumination diminished TEMPONE EPR signals (Fig 4.2.2B). The suppression of

TEMPONE EPR signals after addition of exogenous plastoquinol (PQH_2-1) was noticeable with the whole period of illumination (Fig 4.2.2B).

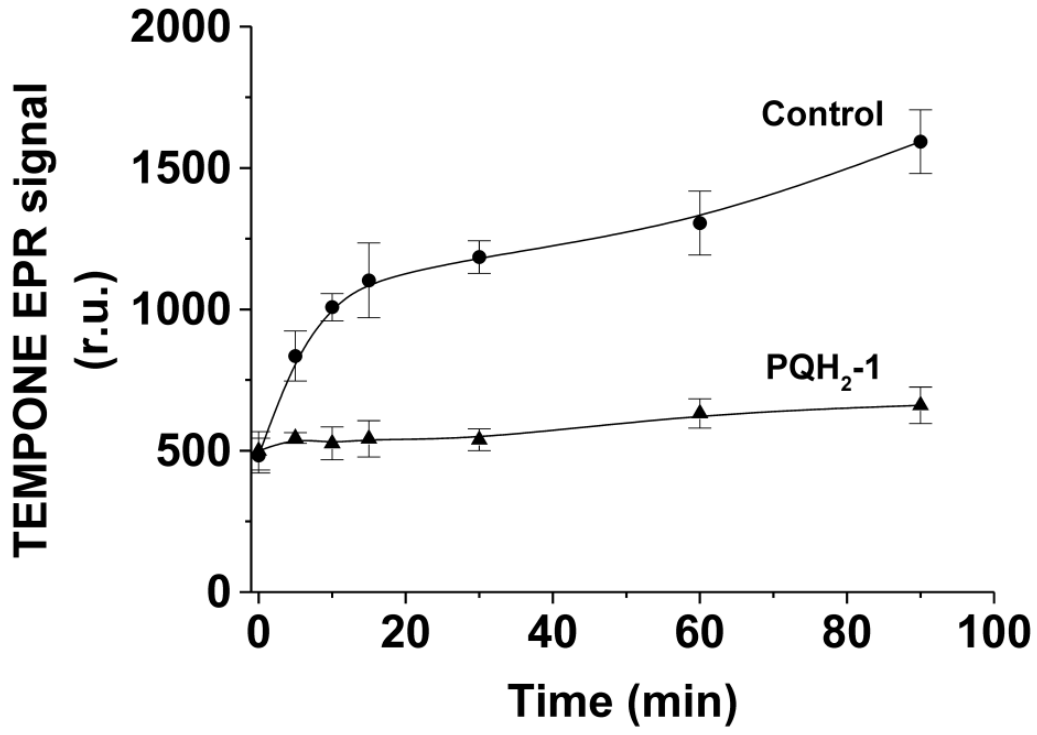


Figure 4.2.3. Effect of exogenous plastoquinol (PQH_2-1) on the time dependence of TEMPONE EPR signal measured in spinach PQ-depleted PSII membranes. PQ-depleted PSII membranes were illuminated in the absence (control) and in the presence (PQH_2-1) of 100 μM exogenous plastoquinol added to the sample prior to illumination.

It is well known that $^1O_2$ produce at photoinhibitory condition in PSII (Macpherson et al. 1993, Telfer et al. 1994a, Hideg 1994a, 1998, 2002, 2007). In agreement with previous reports, the present study reveals that $^1O_2$ formed in PQ depleted PSII by exposure of continuous white light is due to photoinhibition. Addition of exogenous plastoquinol (PQH_2-1) to PSII membranes suppresses the light-induced TEMPONE EPR signals (Fig 4.2.2B). The suppression of TEMPONE EPR signals by exogenous PQH_2-1 confirms the $^1O_2$ activity of plastoquinol in PSII.

4.3. Role of small chlorophyll-binding proteins in thylakoid membrane. (Paper III)

4.3.1. Chlorophyll-binding proteins (Paper III)

Light-harvesting complexes (LHC) are found in a wide variety among the different photosynthetic species. The most abundant light harvesting membrane proteins are chlorophyll a/b binding proteins (CABs) which contains three transmembrane helices (MSHs). MSHs are capable of binding a minimum of 12 Chls and two xanthophylls (Thornber et al. 1994). However, the early light inducible protein (ELIPs) and small CAB-like proteins (SCPs) is other group of nuclear-encoded high light inducible (HLIPs) proteins, which are homologous to CABs (Grimm and Kloppstech 1987, Funk and Vermaas 1999). The *Scp* genes were first reported in cyanobacterium *Synechococcus sp.* Strain PCC 7942, predicted as single transmembrane α-helices and present in all sequenced cyanobacteria (Funk and Vermaas, 1999). Till now five different *Scp* genes have been reported *Scp*A, *Scp*B, *Scp*C, *Scp*D and *Scp*E, in which four of them show similarity to the first and third transmembrane region of CAB protein. Where as *ScpA* the largest (384 residues) showing least similarity with the CAB protein (Funk and Vermaas, 1999).

4.3.2. Role of SCPs (Paper III)

Although the exact function of SCPs is still not apparent, different roles of SCPs have been proposed 1) assembly/repair during the turnover of pigment- binding protein, (Promnares et al. 2006), 2) binding of pigment molecules (Funk and Vermaas 1999), 3) acclimation of plants to high light (Adamaska et al. 1992, Potter and Kloppstech 1993), 4) inhibit degradation of PSII- assiciated Chl (Vavilin et al. 2007) and 5) protection of plant from photooxidative damage and accumulation of ROS (He et al. 2001).

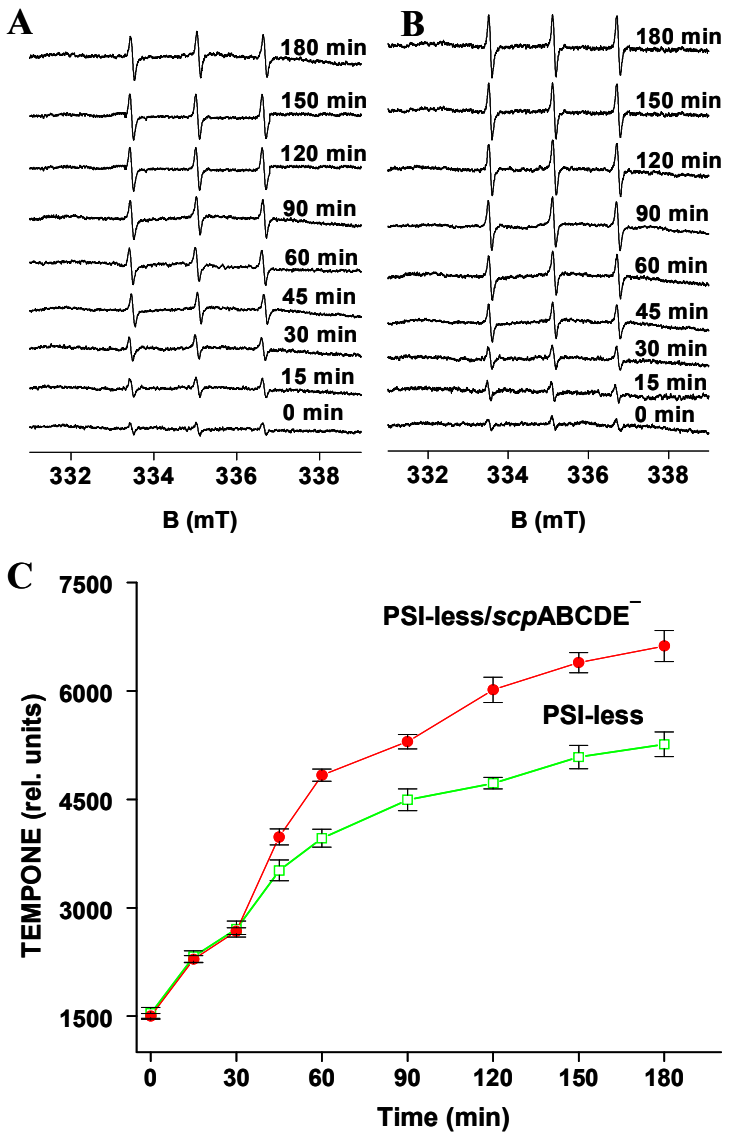


Figure 4.3.1. Light-induced production of $^1O_2$ in the thylakoid membrane of *Synechocystis* PCC 6803**.** Light-induced TEMPONE EPR spectra were measured in the thylakoid membranes isolated from PSI-less (A), PSI-less/*PsbH*$^-$ (B) and PSI-less/*scp*ABCDE$^-$ strains of *Synechocystis* PCC 6803. (C) Time profile of $^1O_2$ production in PSI-less and PSI less/*scp*ABCDE$^-$**.**

4.3.3. Involvements of SCPs in $^1O_2$ formation (Paper III)

The production of $^1O_2$ in thylakoid membrane has been studied from long time and the role of $^1O_2$ in photo-damage of D1 protein has been already established (Komenda and Barber 1995, Komenda et al. 2005, Vass 2011, Andersson and Aro 2001, Krieger-

Liszkay 2005). Neverthless no direct evidence on the involvement of SCPs in the production of $^1O_2$ have been reported so far.

In this study, we have reported that the production of $^1O_2$ is affected by the presence and absence of SCPs protein with the exposure of high light (Fig. 4.3.1. A, B). $^3Chl^*$ is the major source of $^1O_2$ in RC as well as antenna system. The production of $^1O_2$ in RC occurs due to charge recombination where as in the antenna system by ex-citation energy transfer from $^3Chl^*$ to $^3O_2$. However the formation of production of $^1O_2$ is enhanced in the presence of high light (Krieger-Liszkay 2005, Fischer BB, 2010).

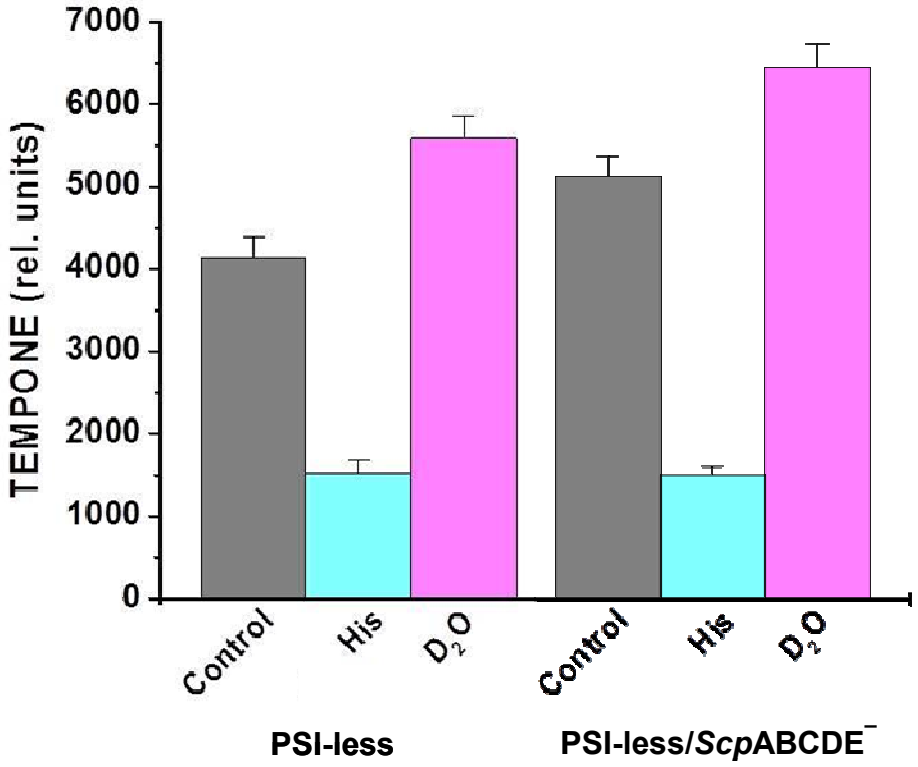


Figure 4.3.2. Effects of scavengers and D_2O on $^1O_2$ production in thylakoid membranes of *Synechocystis* PCC 6803. TEMPONE EPR signal was measured in the thylakoid membranes isolated from PSI-less and PSI-less/*Scp*ABCDE⁻ strains of *Synechocystis* PCC 6803. The TEMPONE EPR signals observed in all samples also included the TEMPONE EPR signal caused by impurity of the spin trap compound.

However, we observed the significant increase in the production through out the illumination period (Fig. 4.3.1.C) in both PSI-less and PSI-less/*Scp*ABCDE⁻ strains of

Synechocystis sp. PCC 6803 used for these studies. These results reveal that the absence of SCPs results in the increase in $^1O_2$ formation during the later phase of illumination, whereas $^1O_2$ formation was unaffected during the early phase (Fig. 4.3.1). Moreover, when histidine was used as a scavenger of $^1O_2$, it shows complete suppression of $^1O_2$ formation (Fig. 4.3.2), Similarly, upon addition of 50% D_2O, $^1O_2$ production increased by 30% in PSI-less strain, whereas 28% in PSI-less/S*cp*ABCDE$^-$ strain (Fig. 4.3.2) since the life time of $^1O_2$ in D_2O might be prolonged by several folds compared to that in H_2O (Egorov et al. 1989).

4.3.4. Singlet oxygen imaging in *Synechocystis* cells (Paper III)

Singlet oxygen sensor green is a unique fluorescence probe for $^1O_2$ detection which reacts selectively with $^1O_2$ without any adverse interaction with $O_2^{\bullet-}$ and $HO^{\bullet}$ (Molecular Probes 2004, Flors et al. 2006, Regas et al. 2009).

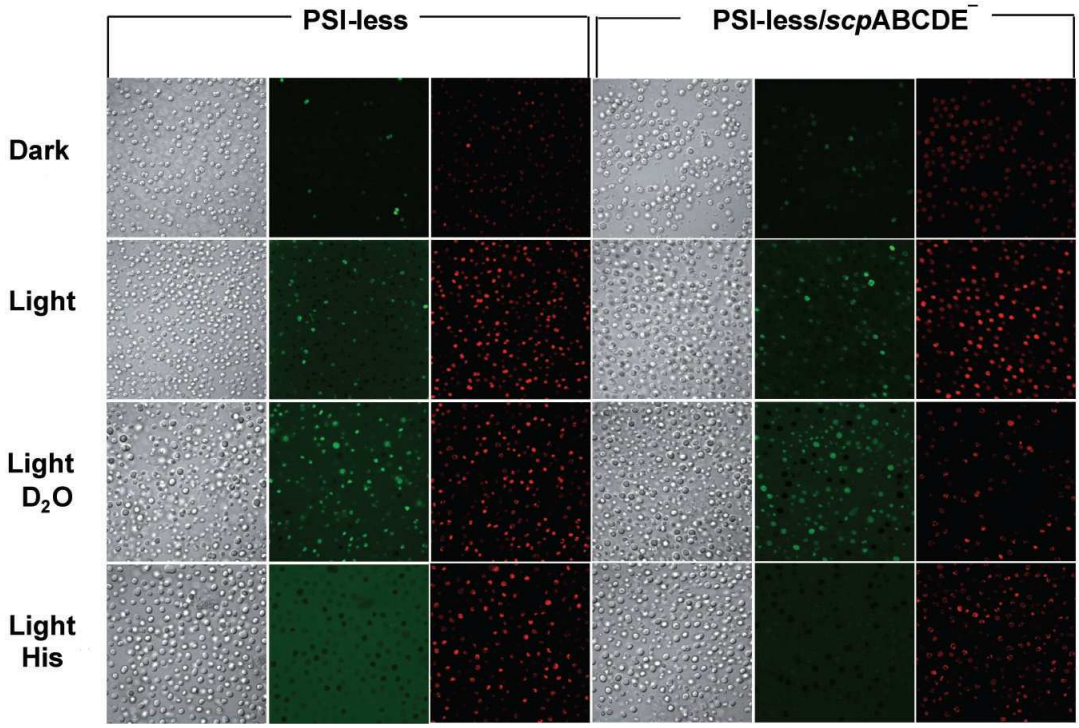


Figure 4.3.3 *In-vivo* detection of $^1O_2$ production using singlet oxygen sensor green (SOSG) in *Synechocystis* sp. PCC 6803 cells, shown under confocal microscopy in PSI-less and PSI-less/*scp*ABCDE$^-$ mutants.

Singlet oxygen imaging in the intact cells of both strains was performed by green fluorescence of SOSG using confocal laser scanning microscope (Fig. 4.3.3). In the absence of $^1O_2$, SOSG exhibits blue fluorescence with excitation at 372 and 393 nm and emission at 395 and 416 nm. In the presence of $^1O_2$, SOSG fluorescence is green shifted with excitation and emission at 504 and 525 nm, respectively.

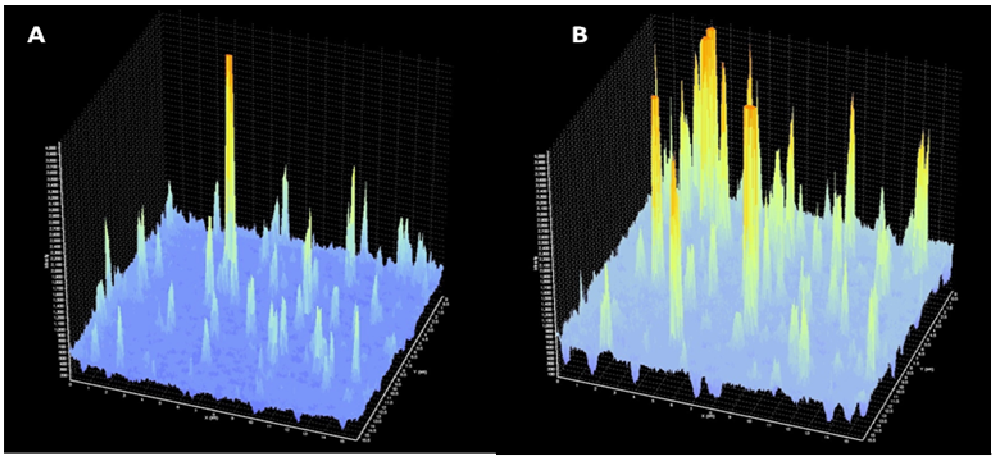


Fig. 4.3.4. Signal intensities of SOSG fluorescence measured using confocal laser scanning microscopy in PSI-less (A), PSI less/*scp*ABCDE$^-$ (B) cells.

The detection of green fluorescence (Fig. 4.3.3) was performed by confocal laser scanning microscopy with excitation and emission at 504 and 525 nm, consequently. In the dark, the intact cells of both strains show very low green fluorescence emission (Fig. 4.3.3). Exposure of the intact cells of both strains to the continuous white light enhances the number of cells showing green fluorescence emission (Fig. 4.3.3). The signals of green fluorescence were pronounced, when water in the media was replaced by deuterium oxide solution. The intensity of florescence was significantly higher in the case of PSI-less/*scp*ABCDE$^-$ (Fig 4.3.4B) than PSI-less (Fig 4.3.4A). These observations reveal that exposure of cell to high intensity illumination results in $^1O_2$ formation under in *vivo*. To verify that SOSG green fluorescence is exclusively linked to $^1O_2$ formation, the effect of $^1O_2$ scavenger on SOSG green fluorescence was tested (Fig. 4.3.2). The addition of hydrophylic $^1O_2$ scavenger histidine in the intact cells of PSI-less and PSI-

less/*Scp*ABCDE$^-$ strains completely suppressed SOSG fluorescence emission (Fig. 4.3.2).

4.3.5. Role of SCPs in oxidative damage of PSII (Paper III)

In order to correlate the functional inactivation and generation of $^1O_2$ in PSII with oxidative damage of the PSII proteins, we carried out one direction SDS denaturing PAGE and second direction 2D CN/SDS-PAGE, in the presence and absence of illumination. In the control non-illuminated cells of both strains the large PSII subunits D1, D2, CP43 and CP47 were almost exclusively detected in dimeric (RCC2) and monomeric (RCC1) PSII core complexes (Fig. 4.3.5).

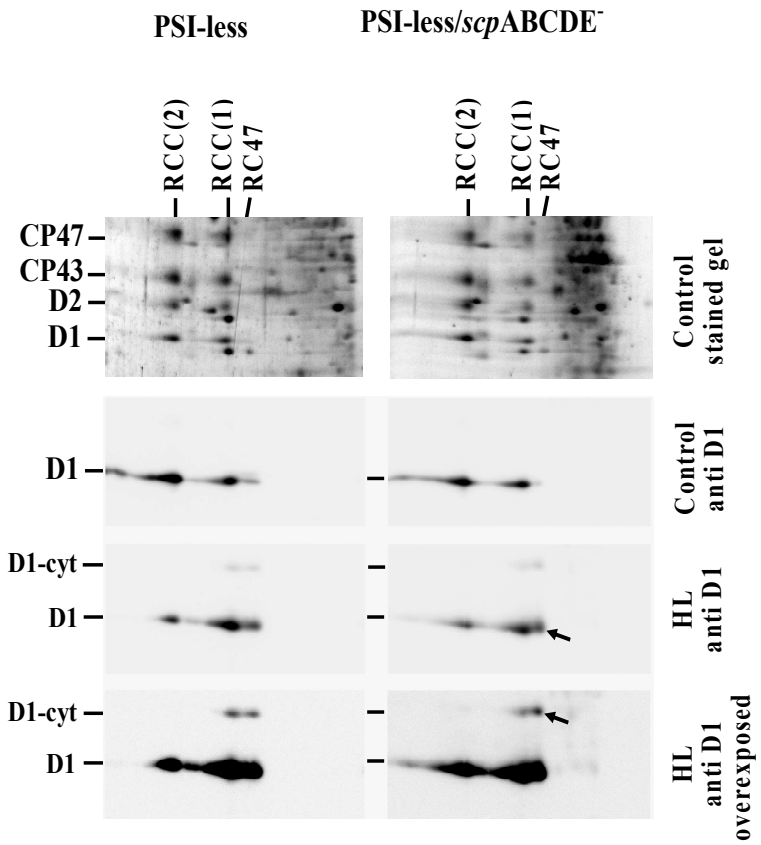


Figure 4.3.5. 2D analysis of the D1 protein in the cells of the PS I-less and PS I-less/*scp*ABCDE$^-$ *Synechocystis* strains before (control cells) and after their high light (HL) treatment. RCC(2) and RCC(1) designate the dimeric and monomeric PSII core complex, respectively; RC47 indicate the monomeric PSII core complex lacking CP43. Arrowheads represent D1 adduct and D1 mobility shift in the RC47 complex of the PSI-less/*scp*ABCDE$^-$ strain.

The exposure of cells of both strains with continuous white light for 20 min in the presence of antibiotic tetracycline caused disappearance of more than 50% of the RCC(2) with simultaneous increase in the level of RCC(1) and appearance of the RC47 complex (Fig. 4.3.5., HL anti D1). The appearance of the D1-cytochrom b_{559} adduct (Fig. 4.3.5, D1-cyt) in the gel region corresponding to RCC(1) and RC47 complexes is associated with the symptom of oxidative stress (Komenda and Masojídek 1995). Furthermore, the migration of the D1 band in RC47 complex was slower than the D1 band in RCC(2) and RCC(1) in PSI-less/*Scp*ABCDE$^-$ and in all PSII complexes of the PSI-less strain (Fig. 4.3.5, arrowhead). The light induced shift in D1 mobility occurs due to oxidative stress (Komenda et al. 2002, Lupínková and Komenda 2004) which was caused by $^1O_2$. SCPs are associated to CP47 antenna of the PSII complex in the vicinity of the PsbH protein (Promnares et al. 2006) and thus it might be possible that Chl molecules released from the antenna during high light treatment will be capture by SCPs and help to maintain the CP47 antenna against light-induced damage.

CHAPTER 5

5. Conclusions

This thesis examines the generation of potentially damaging ROS during light stress in the PSII membrane *in vivo* and *in vitro.* Thus, we investigated the role of cyt b_{559}, plastoquinol and small CAB-like proteins (SCPs) in generation of reactive oxygen species. We attempt to answer following main aspects of these phenomenons:

i) Understand the participation of water-splitting Mn complex on light induced redox changes of cyt b_{559}

ii) Clarify the scavenging activity of plastoquinol against singlet oxygen in PSII of higher plants.

iii) Elucidate the protective role of SCPs in cyanobacterial PSII membrane.

When photosynthetic organisms are exposed to high light, the activity of PSII declines rapidly and this phenomenon is referred as photoinhibition of PSII (Powles 1983, Aro et al. 2005). During the occurrence of photoinhibition process plant endures two distinct mechanism, the high light-induced photo inactivation of PSII and the repair of photodamaged PSII. PSII is the target of such photoinhibition and specific damage to the reaction center-forming D1 protein and oxygen evolving complex (OEC). This results in the inhibition of electron transport in PSII, in isolated thylakoids, and PSII reaction center complexes (Barber and Andersson 1992, Aro et al. 1993).

The donor side-induced photoinhibition occurs with the accumulation of highly oxidizing radicals ($P_{680}^{+\bullet}$ and $Tyr_Z^{+\bullet}$). When electron transfer from water-splitting Mn complex is not sufficiently high to cope up with the electron needs of $P_{680}^{+\bullet}$, which may also possible by incomplete oxidation of water molecules that limits the electron supply to $P_{680}^{+\bullet}$. The electron donation from OEC could be affected after partial release of Ca^{2+} under low pH of lumen (Krieger and Weis 1993). The donor side photoinhibition could be directly observed after chemical alteration of OEC in an active PSII. (Chen et al. 1992, Tiwari and Pospíšil 2009). However, it enhances the formation of reactive $O_2^{\bullet-}$ and $HO^{\bullet}$ radicals and increasing the light sensitivity of PSII (Hideg et al. 1994a, Vass and Aro. 2008). Our study demonstrates that the exposure of PSII membranes to continuous white light caused reduction and oxidation of the heme iron as also reported previously (Buser et al. 1990, Buser et al. 1992, Barber and De Las Rivas 1993, Tiwari and Pospíšil 2009,

Pospíšil and Tiwari 2010). The mechanism of light-induced redox changes of cyt b_{559} depends upon the integrity of PSII electron donor side. We demonstrated that photoreduction of cyt b_{559} in the PSII membranes containing water-splitting Mn complex was suppressed by DCMU, whereas, in the PSII membranes lacking water-splitting Mn complex, photoreduction and photooxidation of cyt b_{559} were abolished by exogenous SOD. Based upon these studies, it is proposed that, in the PSII membranes with the intact water-splitting Mn complex, photoreduction of the heme iron is mediated by plastoquinol. Whereas, in the PSII membrane deprived of water-splitting Mn complex, photoreduction and photooxidation of heme iron is mediated by $O_2^{\bullet-}$.

The acceptor side photoinhibition is induced due to high light-intensity in PSII with a functional donor side. The photoinhibition occurs in PSII acceptor site that contain functional P680, Pheo and D1 protein but in which electron transfer from Q_A^- to Q_B is hampered due to reduction of plastoquinone pool (Vass et al. 1992). In a fully reduced plastoquinol pool, there is a lack of electron acceptors on the acceptor side; consequently the lifetime of charge pairs is enhanced. The charge pairs (eg. $P_{680}^{\bullet+}$ $Pheo^{\bullet-}$) recombines to the triplet state of chlorophyll ($^3Chl^*$) and can react with $^3O_2$ to form $^1O_2$ (Krieger-Liszkay et al. 2008). The $^1O_2$ produced in the presence of unstabilized Q_A^- (Vass and Aro 2008) might be the real damaging agent during photoinhibition. The formation of ^{3}Chls and $^1O_2$ are expected to increase with the increasing light intensity (Vass and Cser 2009). Carotenoids and α-tocopherol are known to play an important role in the quenching of $^1O_2$ in PSII (Telfer et al. 1994b, Telfer 2002, Trebst 2003, Krieger-Liszkay and Trebst 2006). However, the effective quenching of $^3P680^*$ by carotenoids is less likely due to large distances. We have demonstrated here by the use of short chain plastoquinol molecules that these may also contribute efficiently for the quenching of $^1O_2$. We observed formation of $^1O_2$ in PQ depleted PSII by exposure of continuous white light during the process of photoinhibition. However, this light-induced formation of TEMPONE EPR signals of $^1O_2$ was suppressed by the addition of short chain plastoquinol (PQH_2-1) to the PSII membranes. The suppression of $^1O_2$ by exogenously added PQH_2-1 confirms the role of plastoquinol in the quenching of $^1O_2$ in PSII.

Under stress conditions, cyanobacteria accumulate single-helix CAB-related proteins called small CAB-like proteins (SCPs) or high light-induced-proteins (HLIPs).

During degradation of D1 protein, Chls are released from the protein and thus the risk of formation of $^3Chl^*$ is enhanced. The $^3Chl^*$ can react with $^3O_2$ to produce the very reactive $^1O_2$ if no efficient quenchers are present in close proximity. SCPs bind to the released Chls and eliminate their photodynamic potential to cause $^1O_2$ by interacting with carotenoids that are also hypothetically bound to SCPs (Xu et al. 2004). In this study, the effect of SCPs on the PSII-specific light-induced damage and generation of $^1O_2$ was assessed in the mutants strains of the cyanobacterium *Synechocystis sp*. PCC 6803 lacking PSI (PSI-less strain) or lacking PSI together with all SCPs (PSI-less/*scp*ABCDE$^-$ strain).

The light-induced oxidative modifications of D1 protein reflected by a mobility shift of the D1 protein and by generation of a D1-cytochrome b_{559} adduct were more pronounced in the PSI-less/*scp*ABCDE$^-$ strain. This increase in protein oxidation was correlated with a faster formation of $^1O_2$ as detected by the green fluorescence of singlet oxygen sensor green and by electron paramagnetic resonance. Our results provide direct evidence on the protective role of SCPs against formation of $^1O_2$ and related protein oxidation in the photo inactivated PSII both *in vivo* and *in vitro.* It might be possible that Chl molecules released from the antenna during high light treatment will be capture by SCPs and help to maintain the CP47 internal antenna against light-induced damage.

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CURRICULUM-VITAE

Curriculum-vitae

Personal Information:

Name	Rakesh Kumar Sinha
Date of Birth	01/02/1982
Permanent address	Professor Colony, Gaya, Bihar, India- 823001
Nationality	India
Residing Country	Czech Republic
Passport No.	G0251870
Email	life.dbt@gmail.com

Academic Vignette

2000-2004	Assam Agricultural University, Assam, India Degree: B. Sc. (Bachelor of Agricultural science) Specialization: Plant Breeding
2004- 2007	Indira Gandhi Agricultural University, Raipur, Chhattisgarh, India Degree: M. Sc. (Master of Biotechnology) Field of Study: Molecular Biotechnology for Insect pest.
2008 onwards	Department of Biophysica, Palacky University, Olomouc, Czech Republic Degree: Ph.D. Field of Study: Biophysics. Ph.D. research topics: Generation of reactive oxygen species in PSII

National Scholarship and Scientific Awards

2000 - 2004	Indian council of Agricultural Research, ICAR Fellowship
2000 – 2004	University Merit Scholarship, AAU, Jorhat, Assam, India
2004 - 2007	Jawaharlal Nehru University fellowship
2007-2008	Department of Biotechnology awards, Gogernment of India, New Delhi, India
2010	Travel grants for 6th SPPS PhD Student Conference in plant Biology, Hanasaari, Finland by Physiologia Plantarum awards
2012	Awarded “Dean Prize” for scientifiv publication, by Faculty of Science Placky University, Olomouc, Czech Republic.

Practical Acquaintance and experience:

- 190 days research experience as part of Ph.D. at University of Alberta, Faculty of Agricultural, Life & Environmental Science, , Edmonton, Canada.
- One year and six month experience at Department of biotechnology, NRCPB, IARI, New Delhi, India from 6th march, (2007) to 31 September 2008. (Transcriptional profiling of carotenoid biosynthesis genes in tomato).
- Directorate of Rice Research, Hyderabad, India
- Project: Fine mapping of gall-midge resistance gene *Gm-5*
- Six months teaching experience in college of Agriculture, Kawardha, Raipur (C.G.).

Published full length article

- **Sinha RK**, Komenda J, Knoppová J, Sedlářová M and Pospíšil P: Small CAB-like proteins prevent formation of singlet oxygen in the damaged Photosystem II complex of the cyanobacterium *Synechocystis sp. Plant, Cell and Environment* (2012), **35:** 806–818**.**
- **Sinha RK**, Tiwari A and Pospíšil P: Water-splitting manganese complex controls light-induced redox changes of cytochrome b_{559} in Photosystem II, *Journal of Bioenergetics and Biomembranes*. (2010), **42(4):** 337-344.
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Symposium and poster presentation

Sinha RK, Komenda J, Knoppová J, Sedlářová M and Pospíšil P: Role of PsbH and small chlorophyll-binding proteins in singlet oxygen production in the thylakoid membrane of *Synechocystis sp.* PCC 6803". 6th SPPS PhD Student Conference in plant Biology, Hanasaari, Finland. 2010**,** (Oral).
Sinha RK, Tiwari A and Pospíšil P (2009) Photoreduction of cytochrome b_{559} in control and donor side inhibited photosystem II. Mendel University of Agriculture and Forestry, Brno, Czech Republic, (2009), (Oral).

Genes & Promoters Cloned (GenBank submissions)

1. **Sinha R. K,** and Bansal K.C. 2008. Tomato genotype EC521086, Chromoplast specific DXP reductoisomerase (DXR) gene, promoter region. GenBank Accession No. FJ169864
2. **Sinha R.K,** Chinnusamy V, Bansal KC. 2008. Tomato genotype VRT-32-1 Chromoplast specific DXP reductoisomerase (DXR) gene, 5' partial. GenBank Accession No. FJ171691
3. **Sinha R.K,** Dalal M, Chinnusamy V, Bansal KC. 2008. Tomato genotype EC521086 Chromoplast specific DXP Synthase (*DXS*) gene promoter region, 5' partial. GenBank Accession No. FJ171692

4. **Sinha R.K,** Chinnusamy V, Bansal KC. 2008. Tomato genotype VRT-32-1 Chromoplast specific DXP Synthase (*DXS*) gene promoter region, 5' partial. GenBank Accession No. FJ171693
5. **Sinha R.K,** Dalal M, Chinnusamy V, Bansal KC. 2008. Tomato genotype VRT-32-1 Chromoplast specific geranylgeranyl dophosphate (*GGPP*) gene promoter region, 5' partial. GenBank Accession No. FJ171694
6. **Sinha R.K,** Bansal KC. 2008. Tomato genotype EC521086 Chromoplast specific geranylgeranyl dophosphate (*GGPP*) gene promoter region, 5' partial. GenBank Accession No. FJ171695
7. **Sinha R.K,** Dalal M, Chinnusamy V, Bansal KC. 2008. Tomato genotype VRT-32-1 Chromoplast specific isopentenyl diphosphate isomerase (*IPI*) gene promoter region, 5' partial. GenBank Accession No. FJ171696
8. **Sinha R.K,** Chinnusamy V, Bansal KC. 2008. Tomato genotype EC521086 Chromoplast specific isopentenyl diphosphate isomerase (*IPI*) gene promoter region, 5' partial. GenBank Accession No. FJ171697
9. **Sinha R.K,** Dalal M, Chinnusamy V, Bansal KC. 2008. Tomato genotype EC521086 Chromoplast specific phytoene desaturase (PDS) gene promoter region, 5' partial. GenBank Accession No. FJ171698.

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Water-splitting manganese complex controls light-induced redox changes of cytochrome b_{559} in Photosystem II

Rakesh Kumar Sinha · Arjun Tiwari · Pavel Pospíšil

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Abstract The effect of water-splitting Mn complex on light-induced redox changes of cytochrome b_{559} (cyt b_{559}) was studied in spinach photosystem II (PSII) membranes. Photoreduction of the heme iron in the intact PSII membranes was completely suppressed by DCMU, whereas photoreduction and photooxidation of the heme iron in the Mn-depleted PSII membranes were unaffected by DCMU. Interesingly, photoreduction and photooxidation of the heme iron in the Mn-depleted PSII membranes were completely diminished by exogenous superoxide dismutase (SOD), whereas no effect of SOD on photoreduction of the heme iron was observed in the intact PSII membranes. The current work shows that the light-induced redox changes of cyt b_{559} proceed via a different mechanism in the both types of PSII membranes. In the intact PSII membranes, photoreduction of the heme iron is mediated by plastoquinol. However, in the Mn-depleted PSII membranes, photoreduction and photooxidation of the heme iron are mediated by superoxide anion radical formed in PSII.

Keywords Cytochorome b_{559} · Photosystem II · Redox potential · Water-splitting manganese complex

Abbreviations

cyt b_{559}	cytochorome b_{559}
*E*m	midpoint redox potential
HP	high-potential form of cyt b_{559}
IP	intermediate potential form of cyt b_{559}
LP	low-potential form of cyt b_{559}
MES	2-[N-Morpholino]ethanesulfonic acid
PSII	photosystem II
SOD	superoxide dismutase
SOO	superoxide oxidase
SOR	superoxide reductase
Q_A	primary quinone electron acceptor of PSII
Q_B	secondry quinone electron acceptor of PSII
Pheo	pheophytin - primary electron acceptor of PSII
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
EMPO	5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide

R. K. Sinha · A. Tiwari · P. Pospíšil (✉)
Laboratory of Biophysics, Department of Experimental Physics,
Faculty of Science, Palacký University,
tř. 17. listopadu 1192/12,
771 46 Olomouc, Czech Republic
e-mail: pospip@prfnw.upol.cz

Introduction

Photosystem II (PSII) is a multisubunit enzyme known to catalyze oxidation of water and reduction of plastoquinone in the thylakoid membranes of higher plants, algae, and cyanobacteria (Renger and Holzwarth 2005; Rappaport and Diner 2008). Light-driven oxidation of water takes place at the catalytic site of the water-splitting Mn complex via a consecutive series of four oxidation steps with concomitant release of protons (Messinger and Renger 2008; Dau and Haumann 2008). Reduction of plastoquinone occurs at the Q_B site in the PQ/PQH_2 cavity via two-electron reduction from Q_A^- with a concomitant proton uptake (Petrouleas and Crofts 2005).

Cytochrome b_{559} (cyt b_{559}) is an intrinsic component of PSII tightly bound to D1 and D2 homologous proteins. It is a heme-bridged heterodimer consisting of α and β subunits, encoded by *psbE* and *psbF* genes (Babcock et al. 1985; Tae et al. 1988). The heme iron of cyt b_{559} is coordinated by two histidine residues; His^{22} and His^{17} of the α and β subunits located near to the stromal side and

oriented perpendicular to the membrane plane. The crystal structures of PSII from thermophilic cyanobacteria *Thermosynechococcus elongatus* and *Thermosynechococcus vulcanus* reveal that the heme iron is distanced at about 30 Å from the Q_B site and 50–60 Å from the water-splitting Mn complex (edge-to-edge distance) (Kamiya and Shen 2003; Ferreira et al. 2004; Loll et al. 2005; Guskov et al. 2009).

The light-induced reduction and oxidation of the heme iron is one of the important properties of cyt b_{559}, which is possibly involved in the regulation of photochemical efficiency in PSII (Whitmarsh and Pakrasi 1996; Stewart and Brudvig 1998; Faller et al. 2005). Within last two decades, a large effort has been made to characterize the actual reductant and oxidant of the heme iron in cyt b_{559} (Buser et al. 1992; Barber and De Las Rivas 1993; Faller et al. 2001; Tracewell et al. 2001). Supply of reducing and oxidizing equivalents on the electron acceptor and donor side of PSII has been shown as a major requirement for photoreduction and photooxidation of cyt b_{559}, respectively. Based on the observation that DCMU diminished photoreduction of cyt b_{559}, it has been proposed that the bound plastosemiquinone Q_B^-, bound plastoquinol (Q_BH_2) or mobile plastoquinol (PQH_2) molecules provide an electron to cyt b_{559} (Buser et al. 1992). The finding that photoreduction of cyt b_{559} was observed in PSII reaction centers, which lacks PQ molecules, reveals that $Pheo^-$ is another potent candidate for the reduction of cyt b_{559} (Nedbal et al. 1992; Barber and De Las Rivas 1993; Ortega et al. 1995). Photooxidation of cyt b_{559} by $P680^+$ was demonstrated at cryogenic temperature, when electron donation form water-splitting Mn complex to $P680^+$ is inhibited (De Paula et al. 1985). Later, it has been specified that cyt b_{559} is oxidized by $P680^+$ with β-carotene (β-car) and monomeric chlorophyll z (Chlz) as an intermediate in a linear or a branched pathway (Hanley et al. 1999; Tracewell et al. 2001; Faller et al. 2001).

Recently, it has been demonstrated that in addition to intrinsic cofactors in PSII, superoxide anion radical ($O_2^{\bullet-}$) and its protonated form, perhydroxyl radical ($HO_2^{\bullet}$), serve as an exogenous reductant and oxidant of cyt b_{559}, respectively (Tiwari and Pospíšil 2009). The authors demonstrated that the reduction of ferric heme iron by $O_2^{\bullet-}$ occurs as an outer-sphere reaction, whereas the oxidation of ferrous heme iron by $HO_2^{\bullet}$ proceeds via the inner-sphere reaction. It was proposed that the IP form of cyt b_{559} serves as superoxide oxidase (SOO), whereas the HP form of cyt b_{559} acts as superoxide reductase (SOR).

The current work provides evidence that photoreduction and photooxidation of cyt b_{559} are brought about by the different mechanisms depending upon the integrity of PSII electron donor side. In PSII membranes with the intact water-splitting Mn complex, photoreduction of the heme iron was abolished by DCMU. On contrary, in the PSII membranes deprived of water-splitting Mn complex photoreduction and photooxidation of cyt b_{559} were prevented by exogenous SOD. Thus, it is evidenced here that in the PSII containing water-splitting Mn complex photoreduction of cyt b_{559} occurs via plastoquinol; however, it is mediated via $O_2^{\bullet-}$ in the PSII deprived of water-splitting Mn complex.

Materials and methods

PSII membranes preparation

PSII membranes were prepared from fresh spinach leaves purchased from a local market using the method of Berthold et al. (1981) with the modifications described in Ford and Evans (1983). PSII membrane were suspended in a buffer solution containing 400 mM sucrose, 10 mM NaCl, 5 mM $CaCl_2$ and 40 mM Mes-NaOH (pH 6.5) and stored at −80 °C at the final concentration of 3 mg Chl ml^{-1} until further use. PSII membranes deprived of water-splitting Mn complex and 17, 23 and 33 kDa extrinsic proteins were prepared by Tris treatment. PSII membranes (1 mg Chl ml^{-1}) were incubated in a buffer containing 0.8 M Tris-HCl (pH 8) for 30 min at 4 °C, in the darkness with continuous gentle stirring. After treatment, PSII membranes were washed twice in a medium containing 400 mM sucrose, 10 mM NaCl, 5 mM $CaCl_2$ and 40 mM Mes-NaOH (pH 6.5).

Optical measurements

Optical absorption spectroscopy was used to study redox properties of cyt b_{559} using Olis RSM 1000 spectrometer (Olis Inc., Bogart, Georgia, USA). The redox state and content of cyt b_{559} were determined form absorbance changes measured at 559 nm by additions of 20 μM potassium ferricyanide (reference cuvette), 8 mM hydroquinone, 5 mM sodium ascorbate, sodium dithionite (test cuvette) to PSII membranes (150 μg Chl ml^{-1}) in a 3 ml quartz cuvette at 20 °C. After addition of redox mediators, PSII membranes were slowly stirred for 5 min in the dark inside the spectrophotometer using a tiny bar magnet unless stated otherwise. After switching off the stirring, absorption spectra were recorded from 530 nm to 580 nm. The spectral slit width, the total band pass and the scan speed was 0.12 μm, 0.5 nm and 50 nm per min, respectively. The amount of different states of cyt b_{559} was calculated from the average spectra of five measurements. Different redox potential forms of cyt b_{559} were determined by treatment minus control spectrum. Total HP form of cyt b_{559} was determined by difference spectra of hydroquinone-reduced minus ferricyanide-oxidized cyt

b_{559}. The IP form of cyt b_{559} was determined by difference spectra of ascorbate-reduced minus hydroquinone-reduced cyt b_{559}, whereas the LP form of cyt b_{559} was obtained by difference spectra of dithionite-reduced minus ascorbate-reduced cyt b_{559}. The light-induced formation of the HP form of cyt b_{559} was obtained by difference of the absorbance spectrum measured after illumination for 100 s and the dark-adapted ferricyanide oxidized spectrum. Illumination was performed with continuous white light (1,000 μmol photons $m^{-2}s^{-1}$) by 90° rotating the cuvette at each 15 s interval using a halogen lamp with a light guide (Schott KL 1500, Schott AG, Mainz, Germany).

EPR spin-trapping spectroscopy

The spin-trapping was accomplished by EMPO, 5-(ethoxycarbonyl)-5-methyl-1-pyrroline N-oxide (Alexis Biochemicals, Lausen, Switzerland). PSII membrane particles (150 µg Chl ml^{-1}) were illuminated in a glass capillary tube (Blaubrand® intraMARK, Brand, Germany) in the presence of 25 mM EMPO, 100 µM desferal, 40 mM Mes (pH 6.5). Illumination was performed with continuous white light (1,000 μmol photons $m^{-2}s^{-1}$) using a halogen lamp with a light guide (Schott KL 1500, Schott AG, Mainz, Germany). Spectra were recorded at room temperature using EPR spectrometer MiniScope MS200 (Magnettech GmbH, Germany). EPR conditions were as follows: microwave power, 10 mW; modulation amplitude, 1 G; modulation frequency, 100 kHz; sweep width, 100 G; scan rate, 1.62 G s^{-1}.

Results

Characterization of redox form of cyt b_{559} in PSII membranes

In this study, spinach PSII membranes with a different integrity of PSII electron donor side were used to study the effect of illumination on redox properties of cyt b_{559}. Characterization of redox state of cyt b_{559} was performed by measuring the absorption changes at 559 nm after oxidation of cyt b_{559} with potassium ferricyanide and reduction by hydroquinone (HP form), sodium ascorbate (IP form) and sodium dithionite (LP form). In the PSII membranes with the intact water-splitting Mn complex, the composition of cyt b_{559} was determined as 38% of hydroquinone-reducible HP form, 26% of sodium ascorbate-reducible IP form and 36% sodium dithionite-reducible LP form (Fig. 1a, traces a, b, c, respectively). The PSII membranes deprived of water-splitting Mn complex by Tris treatment exhibited 47% IP and 53% LP form of cyt b_{559} (Fig. 1b).

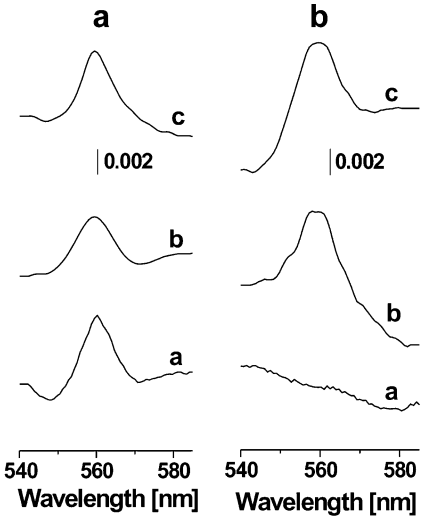

Fig. 1 Redox difference spectra of cyt b_{559} measured in spinach PSII membranes with the intact water-splitting Mn complex (**a**) and deprived of water-splitting Mn complex (**b**). The spectra represent the difference of hydroquinone-reduced minus ferricyanide-oxidized spectra (HP form of cyt b_{559}) (*a*), ascorbate-reduced minus hydroquinone-reduced spectra (IP form of cyt b_{559}) (*b*) and dithionite-reduced minus ascorbate-reduced spectra (LP form of cyt b_{559}) (*c*). The spectra were smoothed by using five points averaging of the experimental data by the Origin v4.1 software

These results confirm that removal of water-splitting Mn complex caused conversion of the HP form into the IP and the LP form of cyt b_{559}.

Light-induced redox changes of cyt b_{559} in the intact PSII membranes

Illumination of the PSII membranes with the intact water-splitting Mn complex caused reduction of cyt b_{559} (Fig. 2a, trace a). Figure 3a shows that photoreduction of cyt b_{559} has been reached within 100 s of continuous illumination and reduced form of cyt b_{559} was found stable within the whole period of illumination upto 300 s. The addition of hydroquinone in the sample after illumination for 100 s did not cause any further reduction of the heme iron (Fig. 2a, trace b), whereas sodium ascorbate and sodium dithionite resulted in reduction of cyt b_{559} (Fig. 2a, traces c and d). These results confirm that illumination of the PSII membranes with the intact water-splitting Mn complex caused photoreduction of the HP form of cyt b_{559}, whereas the IP and the LP forms of cyt b_{559} were unaffected.

Light-induced redox changes of cyt b_{559} in the Mn-depleted PSII membranes

Illumination of PSII membranes deprived of water-splitting Mn complex caused reduction of cyt b_{559} (Fig. 2b, trace a, solid line). Figure 3b shows that photoreduction of cyt b_{559} observed within 100 s of continuous illumination was followed by photooxidation of the heme iron. It has been recently

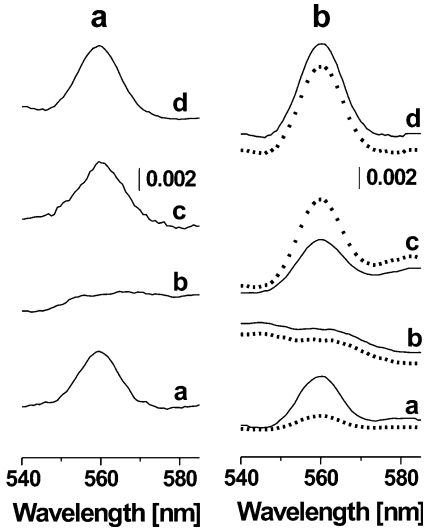

Fig. 2 Light-induced redox changes of cyt b_{559} measured in spinach PSII membranes with the intact water-splitting Mn complex (**a**) and deprived of water-splitting Mn complex (**b**). PSII membranes (100 μg Chl ml^{-1}) were illuminated with continuous white light (1,000 μmol photons $m^{-2}s^{-1}$) for 100 s (*solid line*) and 300 s (*dotted line*). The spectra represent the difference of light minus ferricyanide-oxidized spectra (*a*), hydroquinone-reduced minus light spectra (*b*), ascorbate-reduced minus hydroquinone-reduced spectra (*c*) and dithionite-reduced minus ascorbate-reduced spectra (*d*)

demonstrated that photoreduction and photooxidation of heme iron were followed by an upshift of *E*m to +310 mV and downshift of *E*m to +125 mV, respectively (Tiwari and Pospíšil 2009; Pospíšil and Tiwari 2010). The addition of hydroquinone in the sample after illumination for 100 s did not cause any further reduction of the heme iron (Fig. 2b, trace b, solid line), whereas it was reduced by sodium ascorbate and sodium dithionite (Fig. 2b, traces c and d, solid lines). After illumination for 100 s, the ascorbate-reducible form (Fig. 2b, traces c, solid lines) showed lowered value than the ascorbate-reducible form observed before illumination (Fig. 1b, traces c). The addition of hydroquinone in the sample after illumination for 300 s did not cause further reduction (Fig. 2b, trace b, dotted line), whereas sodium ascorbate and sodium dithionite resulted in reduction of cyt b_{559} (Fig. 2b, traces c and d, dotted lines). These results confirm that illumination of the PSII membranes deprived of water-splitting Mn complex caused reduction and oxidation of the IP and HP form of cyt b_{559}, respectively.

Effect of DCMU on photoreduction and photooxidation of cyt b_{559}

Further, the effect of DCMU on light-induced reduction and oxidation of cyt b_{559} was studied in the PSII membranes with the different integrity of PSII electron donor side. In the presence of DCMU, illumination of the PSII membranes with the intact water-splitting Mn complex for 100 s did not cause any photoreduction of the HP form of cyt b_{559} (Fig. 4a, trace a, Fig. 6a), whereas it was reduced by hydroquinone, sodium ascorbate and sodium dithionite (Fig. 4a, traces b-d). These results reveal that in the PSII membranes with the intact water-splitting Mn complex

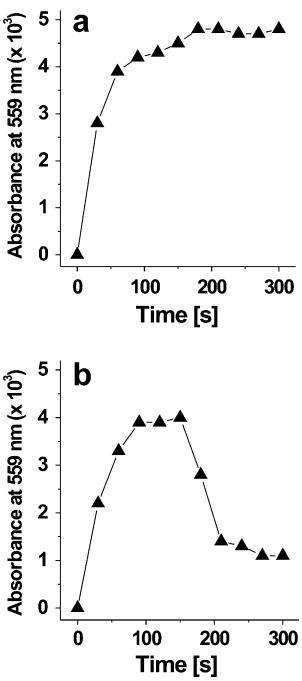

Fig. 3 Time course of light-induced redox changes of cyt b_{559} measured in spinach PSII membranes with the intact water-splitting Mn complex (**a**) and deprived of water-splitting Mn complex (**b**). The intensity of the absorption signal was calculated as the height of peak at 559 nm from the reference line connecting the lowest points near 545 and 575 nm. Each data point represents the mean value of at least three experiments

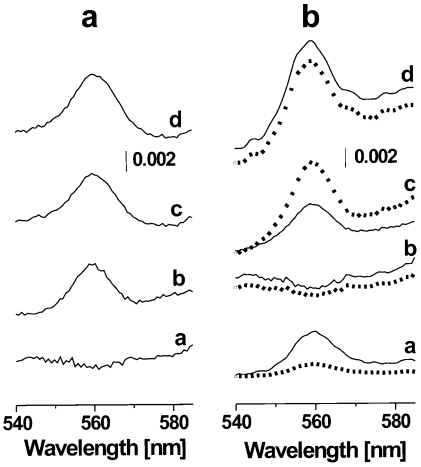

Fig. 4 Effect of DCMU on light-induced redox changes of cyt b_{559} measured in spinach PSII membranes with the intact water-splitting Mn complex (**a**) and deprived of water-splitting Mn complex (**b**). PSII membranes (100 μg Chl ml^{-1}) were illuminated with continuous white light (1,000 μmol photons $m^{-2}s^{-1}$) for 100 s (solid line) and 300 s (dotted line). Prior to illumination, 10 μM DCMU was added to PSII membranes. The spectra represent the difference of light minus ferricyanide-oxidized spectra (*a*), hydroquinone-reduced minus light spectra (*b*), ascorbate-reduced minus hydroquinone-reduced spectra (*c*) and dithionite-reduced minus ascorbate-reduced spectra (*d*)

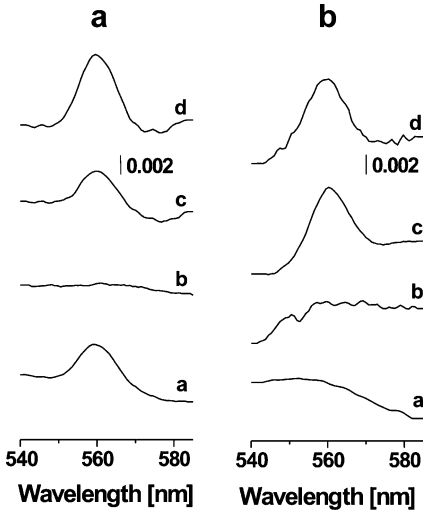

Fig. 5 Effect of SOD on light-induced redox changes of cyt b_{559} measured in spinach PSII membranes with the intact water-splitting Mn complex (**a**) and deprived of water-splitting Mn complex (**b**). PSII membranes (100 μg Chl ml^{-1}) were illuminated with continuous white light (1,000 μmol photons $m^{-2}s^{-1}$) for 100 s. Prior to illumination 400 U ml^{-1} SOD was added to PSII membranes. The spectra represent the difference of light minus ferricyanide-oxidized spectra (*a*), hydroquinone-reduced minus light spectra (*b*), ascorbate-reduced minus hydroquinone-reduced spectra (*c*) and dithionite-reduced minus ascorbate-reduced spectra (*d*)

photoreduction of the heme iron was completely suppressed by DCMU. Contradictory, in the presence of DCMU illumination of PSII membranes deprived of water-splitting Mn complex caused photoreduction of cyt b_{559} (Fig. 4b, trace a, solid line, Fig. 6b), whereas further illumination upto 300 s resulted in photooxidation of cyt b_{559} (Fig. 4b, trace a, dotted line, Fig. 6b). Further addition of hydroquinone did not cause any reduction of cyt b_{559}, whereas the addition of sodium ascorbate and sodium dithionite caused reduction of cyt b_{559} (Fig. 4b, traces b-d). These results suggest that photoreduction and photooxidation of the heme iron in the Mn-depleted PSII membranes were unaffected by DCMU. Based on these observations, it is concluded that in the PSII membranes with the intact water-splitting Mn complex photoreduction of the heme iron is mediated by reducing compound formed at the Q_B site, whereas in the PSII membranes deprived of water-splitting Mn complex the heme iron is reduced by the compound formed prior to the Q_B site.

Effect of SOD on photoreduction and photooxidation of cyt b_{559}

To study the involvement of $O_2^{\bullet-}$ in light-induced redox changes of cyt b_{559}, the effect of exogenous SOD on photoreduction and photooxidation of the heme iron was measured in the PSII membranes with the different integrity of PSII electron donor side. It was observed that in the PSII membranes with the intact water-splitting Mn complex, photoreduction of the heme iron was unaffected by

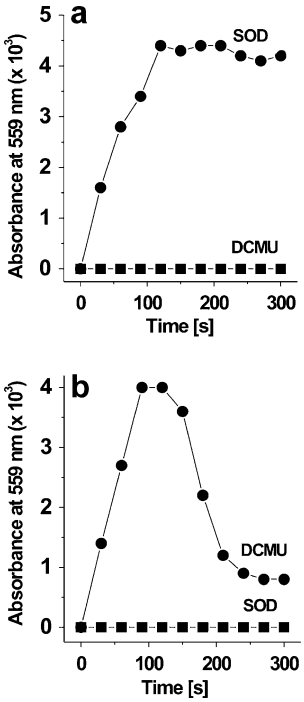

Fig. 6 Effect of DCMU and SOD on time course of light-induced redox changes of cyt b_{559} measured in spinach PSII membranes with the intact water-splitting Mn complex (**a**) and deprived of water-splitting Mn complex (**b**). Other experimental conditions were as described in Fig. 3

scavenging of $O_2^{\bullet-}$ using exogenous SOD (Fig. 5a, trace a, Fig. 6a). When hydroquinone was added, no further reduction of cyt b_{559} was observed (Fig. 5a, trace b), whereas the addition of sodium ascorbate and sodium dithionite caused reduction of cyt b_{559} (Fig. 5a, traces c and d). Contradictory, photoreduction and photooxidation of the heme iron of cyt b_{559} in the PSII membranes deprived of water-splitting Mn complex were completely diminished by exogenous SOD (Fig. 5b, trace a, Fig. 6b). Further addition of hydroquinone did not cause any reduction of cyt b_{559}, whereas the addition of sodium ascorbate and sodium dithionite causes reduction of cyt b_{559} (Fig. 5b, traces b-d). The effect of an exogenous SOD on photoreduction and photooxidation of the heme iron suggests that in the PSII membranes with the intact water-splitting Mn complex photoreduction of the heme iron is unaffected by $O_2^{\bullet-}$, whereas in the PSII membranes deprived of water-splitting Mn complex $O_2^{\bullet-}$ is involved in photoreduction and photooxidation of the heme iron. Based on these considerations it seems likely that in the PSII membranes deprived of water-splitting Mn complex, the heme iron of cyt b_{559} is reduced by $O_2^{\bullet-}$ formed prior to the Q_B site.

Light-induced production of $O_2^{\bullet-}$ in PSII membranes

Light-induced production of $O_2^{\bullet-}$ in PSII membranes was monitored using EPR spin-trapping spectroscopy. The spin-trapping was accomplished by spin trap compound EMPO known to react with $O_2^{\bullet-}$, forming the EMPO-OOH adduct. Exposure of the PS II membranes with the intact water-splitting Mn complex to continuous white light resulted in

the generation of EMPO-OOH adduct EPR spectra (Fig. 7a). Similarly, illumination of PSII membranes deprived of water-splitting Mn complex showed formation of EMPO-OOH adduct EPR spectra (Fig. 7b). The addition of exogenous SOD completely prevented the formation of EMPO-OOH adduct EPR spectra. Model EMPO-OOH adduct EPR spectrum was generated using xanthine/xanthine oxidase system. These results show that exposure of the both PSII membranes with the intact water-splitting Mn complex and PSII membranes deprived of water-splitting Mn complex caused $O_2^{\bullet-}$ production.

Discussion

Light-induced redox changes of cyt b_{559} were studied in the PSII membranes with the different integrity of PSII electron donor side. In agreement with previous reports, we have demonstrated that the exposure of PSII membranes to continuous white light caused reduction and oxidation of the heme iron (Buser et al. 1990; Buser et al. 1992; Barber and De Las Rivas 1993; Tiwari and Pospíšil 2009; Pospíšil and Tiwari 2010). We present here the convincing evidence that the mechanism of light-induced redox changes of cyt b_{559} depends upon the integrity of PSII electron donor side. We demonstrated that photoreduction of cyt b_{559} in the PSII membranes containing water-splitting Mn complex was supressed by DCMU (Fig. 4a), whereas in the PSII membranes lacking water-splitting Mn complex photoreduction and photooxidation of cyt b_{559} were abolished by exogenous SOD (Fig. 5b). Based on these observations, it is proposed that in PSII membranes with the intact water-splitting Mn complex photoreduction of the heme iron is mediated by plastoquinol, whereas in the PSII membranes deprived of water-splitting Mn complex, photoreduction and photooxidation are mediated by $O_2^{\bullet-}$ formed in PSII.

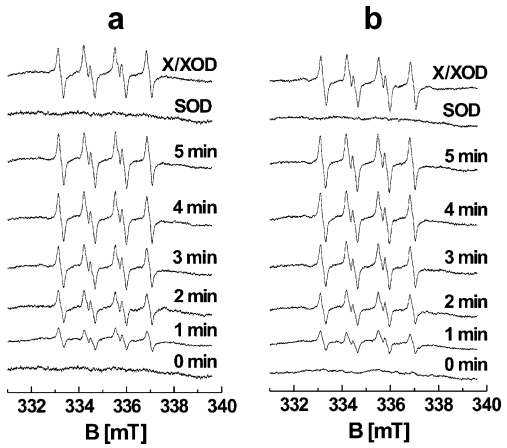

Fig. 7 Light-induced EPR spectra of the EMPO-OOH adduct measured in spinach PSII membranes with the intact water-splitting Mn complex (**a**) and deprived of water-splitting Mn complex (**b**). EPR spin-trapping spectra were obtained after the illumination of the PSII membranes (150 μg Chl ml^{-1}) with white light (1,000 μmol photons m^{-2}s^{-1}) in the presence of 25 mM EMPO, 100 μM desferal and 40 mM MES (pH 6.5). Superoxide anion radical production was completely prevented, when 500 U ml^{-1} SOD was added to the sample prior to the illumination. The upmost trace labeled X/XOD shows the EPR signal of the EMPO–OOH adduct generated by 1 mM xanthine and 50 U ml^{-1} xanthine oxidase

Photoreduction of cyt b_{559} by plastoquinol

Illumination of PSII membranes with the intact water-splitting Mn complex caused photoreduction of the HP form of cyt b_{559} (Fig. 2a). The observation that photoreduction of the heme iron of cyt b_{559} was completely suppressed by DCMU reveals that an electron carrier, which provides electron to the heme iron, is reduced at Q_B site. Based on the fact that the midpoint redox potential of the HP form of cyt b_{559} is 310–400 mV (pH 7) (Cramer and Whitmarsh 1977; Mizusawa et al. 1999; Roncel et al. 2003; Kaminskaya et al. 2007), the reduction of the HP form of cyt b_{559} by both plastosemiqunone (Q_B^-) and plastoquinol (Q_BH_2) is thermodynamically feasible. Due to the fact that midpoint redox potential of Q_B/Q_B^- redox couple is −45 mV (pH 7) (Hauska et al. 1983; Crofts and Wraight 1983), plastosemiquinone has redox power high enough for providing an electron to the HP form of cyt b_{559}. However, recent X-ray crystal structural analysis of PSII complexes in thermophilic cyanobacteria *Thermosynechococcus elongatus* showed that the heme iron is distanced at about 30 Å from the head group of plastoquinone bound at the Q_B site (Loll et al. 2005). On the opposite side, the fact that the midpoint redox potential of Q_B^-/Q_BH_2 redox couple is 290 mV (pH 7) (Hauska et al. 1983; Crofts and Wraight 1983) reveals that plastoquinol has less redox power for reduction of the HP form of cyt b_{559}. However, its ability to diffuse to the heme iron makes the plastoquinol more likely candidate for electron donation to the heme iron. Based on these considerations it seems likely that prior to the reduction of the heme iron, plastoquinol liberates from the Q_B site and diffuses to the vicinity of the heme iron. It has been recently proposed that exchange of plastoquinol molecule by plastoquinone at the Q_B site might occur via three possible mechanisms (Guskov et al. 2009). In the so-called altering and wriggling mechanisms, plastoquinol passes through the Q_C site, where the additional plastoquinone was proposed to be bound. Based on the X-ray PSII structural data (Guskov et al. 2009) the heme iron is distanced at 17 Å from the head group of plastoquinol bound at the Q_C site. It is proposed here that possible rebinding of plastoquinol molecule at Q_C site might provide an appropriate condition for electron donation to the heme iron.

Photoreduction and photooxidation of cyt b_{559} superoxide anion radical

Illumination of PSII membranes deprived of water-splitting Mn complex caused photoreduction and photooxidation of the IP and the HP form of cyt b_{559}, respectively (Fig. 2b). Based on the fact that the midpoint redox potential of the IP form of cyt b_{559} is 125–240 mV (pH 7) (Mizusawa et al. 1999; Roncel et al. 2003; Kaminskaya et al. 2007; Tiwari and Pospíšil 2009; Pospíšil and Tiwari 2010), the reduction of the IP form of cyt b_{559} by plastoquinol is not thermodynamically feasible and thus it is likely that another reductant might provide an electron to the heme iron. The observation that photoreduction and photooxidation of the heme iron of cyt b_{559} were completely diminished by exogenous SOD indicates that $O_2^{\bullet-}$ serves as reductant and oxidant of the heme iron. In the agreement with this proposal, we have recently demonstrated that $O_2^{\bullet-}$ interacts with the heme iron (Tiwari and Pospíšil 2009). It has been proposed that the IP form of cyt b_{559} serves as SOO known to catalyze the oxidation of $O_2^{\bullet-}$ to O_2. Based on the fact that the midpoint redox potential of $O_2/O_2^{\bullet-}$ redox couple is –160 mV (pH 7) (Wood 1987), the reduction of the IP form of cyt b_{559} by $O_2^{\bullet-}$ is thermodynamically feasible. On the other hand, the HP form of cyt b_{559} has been demonstrated to act as SOR known to catalyze the reduction of $O_2^{\bullet-}$ to H_2O_2. As the midpoint redox potential of $O_2^{\bullet-}/H_2O_2$ redox couple is 890 mV (pH 7) (Wood 1987), the oxidation of the HP form of cyt b_{559} by $O_2^{\bullet-}$ is favored from thermodynamic point of view. X-ray crystal structural analysis of PSII complexes showed that the heme iron is located in the vicinity of two channels, *i.e.* channel I and II (Guskov et al. 2009). The authors demonstrated that the channel I passes through the Q_C site, whereas both channels circumvent the Q_B site. It is proposed here that these channels facilitate diffusion of $O_2^{\bullet-}$ toward the heme iron and thus maintain reduction of the heme iron by $O_2^{\bullet-}$.

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Singlet oxygen scavenging activity of plastoquinol in photosystem II of higher plants: Electron paramagnetic resonance spin-trapping study

Deepak Kumar Yadav [a], Jerzy Kruk [b], Rakesh Kumar Sinha [a], Pavel Pospíšil [a,*]

[a] Laboratory of Biophysics, Department of Experimental Physics, Faculty of Science, Palacký University, tř. 17. Listopadu 12, 771 46 Olomouc, Czech Republic
[b] Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland

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ABSTRACT

Singlet oxygen ($^1O_2$) scavenging activity of plastoquinol in photosystem II (PSII) of higher plants was studied by electron paramagnetic resonance (EPR) spin-trapping technique. It is demonstrated here that illumination of spinach PSII membranes deprived of intrinsic plastoquinone results in $^1O_2$ formation, as monitored by TEMPONE EPR signal. Interestingly, the addition of exogenous plastoquinol (PQH_2-1) to PQ-depleted PSII membranes significantly suppressed TEMPONE EPR signal. The presence of exogenous plastoquinols with a different side-chain length (PQH_2-n, n isoprenoid units in the side chain) caused a similar extent of $^1O_2$ scavenging activity. These observations reveal that plastoquinol exogenously added to PQ-depleted PSII membranes serves as efficient scavenger of $^1O_2$.

1. Introduction

Photosystem II (PSII) is a pigment–protein complex embedded in the thylakoid membrane of higher plants, algae and cyanobacteria. It is involved in the conversion of light energy into chemical energy by electron transfer from water to plastoquinone [1,2]. When higher plants, algae and cyanobacteria are exposed to high-light intensity illumination, PSII activity is inhibited in a process called photoinhibition [3–5]. Photoinhibition is considered to be caused by damage to the D1 protein, which forms a heterodimer with the D2 protein along with the subsequent rapid degradation of the D1 protein [6–9]. Damage to PSII is thought to be due to the action of reactive oxygen species (ROS) formed on the electron acceptor side of PSII, when the plastoquinones Q_A and Q_B are highly reduced [10–13]. Singlet oxygen ($^1O_2$) generated from triplet chlorophyll species is considered as the main ROS responsible for PSII damage [14–16]. Recently, formation of hydroxyl radical as another ROS responsible for PSII damage has been demonstrated on PSII electron acceptor site [17,18].

Singlet oxygen is generated by the interaction of molecular oxygen with the excited triplet state of chlorophyll formed via charge recombination pathway [10–12]. The charge recombination of radical pair $^3[P680^+Pheo^-]$ results in the formation of triplet excited state $^3P680^*$ [19,20]. When $^3P680^*$ is not efficiently scavenged by carotenoids or α-tocopherol, the interaction of $^3P680^*$ with triplet molecular oxygen $^3O_2$ results in the formation of $^1O_2$.

Singlet oxygen scavenging by carotenoids and α-tocopherols occurs either by excitation energy transfer (physical scavenging) or by electron transport reaction (chemical scavenging) [21–23]. In the physical type of $^1O_2$ scavenging, denoted as $^1O_2$ quenching, the excitation energy transfer from $^1O_2$ to quencher (carotenoid and α-tocopherol) results in the formation of the triplet excited state of the quencher, while $^3O_2$ is formed. Subsequently, the triplet excitation energy of the quencher is lost as heat. Typically, one molecule of quencher can deactivate several hundreds of $^1O_2$ molecules [24]. In the chemical type of $^1O_2$ scavenging, the interaction of $^1O_2$ with scavenger (α-tocopherol) was shown to form intermediate 8-hydroperoxy-tocopherone known to hydrolyze irreversibly to α-tocopherolquinone [25]. On the contrary, α-tocopherol is destroyed and thus a continuous re-synthesis of α-tocopherol is required to keep its level sufficient for the photoprotection [26,27].

Singlet oxygen scavenging activity of plastoquinol molecules in thylakoid membrane, as well as in the liposomes, has been proposed in the previous studies [28–30]. Later, we have demonstrated that the addition of plastoquinone homologues to *Chlamydomonas reinhardtii* grown in the presence of a plastoquinone inhibitor prevented damage of D1 protein [27]. Recently, plastoquinol has been shown to decompose under illumination in *Chlamydomonas reinhardtii* cells as a consequence of reaction with $^1O_2$ generated in PSII [31]. The authors demonstrated that due to the irreversible oxidation of plastoquinol,

Abbreviations: PSII, photosystem II; MES, 4-morpholineethanesulfonic acid; TMPD, 2, 2, 6, 6-tetramethyl-4-piperidone; TEMPONE, 2, 2, 6, 6-tetramethyl-4-piperidone-1-oxyl; PQH_2, plastoquinol; DanePy, (3-[N-(β-diethylaminoethyl)-N-dansyl]amino-methyl-2,2,5,5-tetra-methyl-2,5-dihydro-1H-pyrrole)

* Corresponding author. Tel.: +420 585634174; fax: +420 585225737.
E-mail address: pospip@prfnw.upol.cz (P. Pospíšil).

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the continuous re-synthesis of plastoquinol is required to keep its level sufficient in PSII. Recently, we have demonstrated that plastoquinol is an active $^1O_2$ scavenger in organic solvents of different polarity [25]. As far as we know, no evidence has been provided on the $^1O_2$ scavenging activity of plastoquinol in higher plant PSII.

In the present work, we provide evidence on the $^1O_2$ scavenging activity of plastoquinol using electron paramagnetic resonance (EPR) spin-trapping spectroscopy. It is demonstrated here that the addition of exogenous plastoquinols to PQ-depleted PSII membranes scavenged $^1O_2$. On the basis of these observations, we conclude that plastoquinol serves as an efficient $^1O_2$ scavenger in PSII of higher plants.

2. Materials and methods

2.1. PSII membranes preparation

PSII membranes were isolated from fresh spinach leaves purchased from a local market using the method of Berthold et al. [32] with the modifications described in Ford and Evans [33]. PSII membranes suspended in a buffer solution containing 400 mM sucrose, 10 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$ and 50 mM MES-NaOH (pH 6.5) were stored at −80 °C at the final concentration of 3 mg Chl ml^{-1} until further use. PQ-depleted PSII membranes were prepared by plastoquinone extraction in a heptane-isobutanol solvent system using the methods of Wydrzynski and Inoue [34]. PSII membranes (0.3 mg Chl ml^{-1}) in plastic tube were shaken with heptane (the ratio of heptane/sample 3:1) and isobutanol (the ratio of isobutanol/sample 1:30) for 40 min. After formation of two phases, the lower phase (PSII membranes) was separated from the upper organic phase and bubbled with air for 10 minutes to remove the residual organic phase. PQ-depleted PSII membranes were stored at −80 °C at the final concentration of 3 mg Chl ml^{-1} until further use.

2.2. Singlet oxygen generation by rose bengal photosensitization

For the photosensitized generation of $^1O_2$, 5 μM rose bengal (Sigma) in 25 mM phosphate buffer (pH 7.0) was illuminated for 10 min with a continuous white light (1000 μmol photons m^{-2} s^{-1}) using a halogen lamp with a light guide (Schott KL 1500, Schott AG, Mainz, Germany). The absorption of light by rose bengal leads to a singlet excited state of the sensitizer, which is subsequently converted into its triplet excited state by intersystem crossing. In the presence of molecular oxygen, the triplet excited state of the rose bengal reacts with the ground triplet state of molecular oxygen. This process returns the sensitizer to its singlet ground state, while $^1O_2$ is formed [35,36].

2.3. EPR spin-trapping spectroscopy

The spin-trapping was accomplished by hydrophilic spin trap compound TMPD, (2, 2, 6, 6-Tetramethyl-4-piperidone) (Sigma). PQ-depleted PSII membranes (150 μg Chl ml^{-1}) were illuminated in the presence of 50 mM TMPD and 40 mM MES-NaOH (pH 6.5). Illumination was performed with continuous white light (1000 μmol photons m^{-2} s^{-1}) using a halogen lamp with a light guide (Schott KL 1500, Schott AG, Mainz, Germany). After illumination the sample was centrifuged at 5000*g* for 3 min to separate TEMPONE from the PQ-depleted PSII membranes. The separation of two phases was carried out to prevent the reduction of TEMPONE by non-specific reducing component in the PQ-depleted PSII membranes. After centrifugation, the upper phase was immediately transferred into the glass capillary tube (Blaubrand® intraMARK, Brand, Germany) and frozen in liquid nitrogen until the use. Prior to data collection, the capillary tube was taken away from the liquid nitrogen and EPR spin-trapping data were collected at room temperature. Spectra were recorded using EPR spectrometer MiniScope MS200 (Magnettech GmbH, Germany). Signal intensity was evaluated as a height of the central peak of EPR spectrum. EPR conditions were as follows: microwave power, 10 mW; modulation amplitude, 1 G; modulation frequency, 100 kHz; sweep width, 100 G; scan rate, 1.62 G s^{-1}.

3. Results

3.1. Effect of PQH_2-1 on rose bengal-induced $^1O_2$ formation

Effect of short-chain plastoquinol (PQH_2-1) on $^1O_2$ generation by photosensitization of rose bengal was studied using EPR spin-trapping technique. Spin trapping was accomplished by utilizing the oxidation of diamagnetic 2, 2, 6, 6-tetramethyl-4-piperidone (TMPD) by $^1O_2$ to yield paramagnetic 2, 2, 6, 6-tetramethyl-4-piperidone-1-oxyl (TEMPONE) [37]. Addition of TMPD spin trap compound to rose bengal in the dark did not result in the appearance of TEMPONE EPR spectra (Fig. 1). Small TEMPONE EPR signal observed in the dark was due to impurity of the spin trap. Illumination of rose bengal in the presence of molecular oxygen resulted in the formation of TEMPONE EPR signal (Fig 1). When rose bengal was illuminated in the presence of PQH_2-1, TEMPONE EPR signal was significantly suppressed. The observation that the addition of PQH_2-1 to paramagnetic TEMPONE does not affect TEMPONE EPR signal reveals that PQH_2-1 was not able to reduce TEMPONE to diamagnetic hydroxylamine (data is not shown). These observations indicate that plastoquinol serves as an efficient scavenger of $^1O_2$ generated from the photoactivation of rose bengal.

3.2. Singlet oxygen formation in PQ-depleted PSII membranes

In order to study selectively $^1O_2$ scavenging activity of the exogenous plastoquinol in spinach PSII membranes, $^1O_2$ formation was measured in PSII membranes deprived of the intrinsic plastoquinone and other prenyllipid (e.g. α-tocopherol) by the heptane/isobutanol extraction procedure. Fig. 2A shows TEMPONE EPR spectra measured in PQ-depleted PSII membranes. The addition of TMPD to PQ-depleted PSII membranes in the dark did not result in the appearance of TEMPONE EPR signal. Small TEMPONE EPR signal observed in non-illuminated PQ-depleted PSII membranes was due to impurity of the spin trap compound. Exposure of PQ-depleted PSII membranes to continuous white light resulted in the generation of TEMPONE EPR spectra. Due to the fact that TMPD is a hydrophilic nitroxide spin trap, the formation of TEMPONE EPR signal reflects $^1O_2$ production predominantly in the polar phase. When the height of the TEMPONE EPR signal was plotted against the illumination time, the remarkable enhancement in the TEMPONE EPR signal was observed in the initial period of illumination, whereas moderate increase in the TEMPONE EPR signal was detected during the later period of

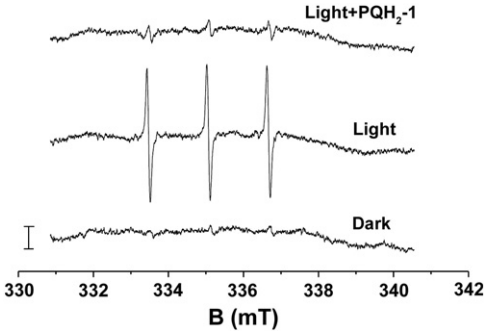

Fig. 1. Effect of exogenous plastoquinol (PQH_2-1) on the rose bengal-induced TEMPONE EPR spectra. Rose bengal (5 μM) was illuminated with white light (1000 μmol m^{-2} s^{-1}) for 10 min in the presence of 50 mM TMPD and 25 mM phosphate buffer (pH 7.0). Prior to illumination, 100 μM PQH_2-1 was added to the reaction mixture. Vertical bar represents 1000 relative units.

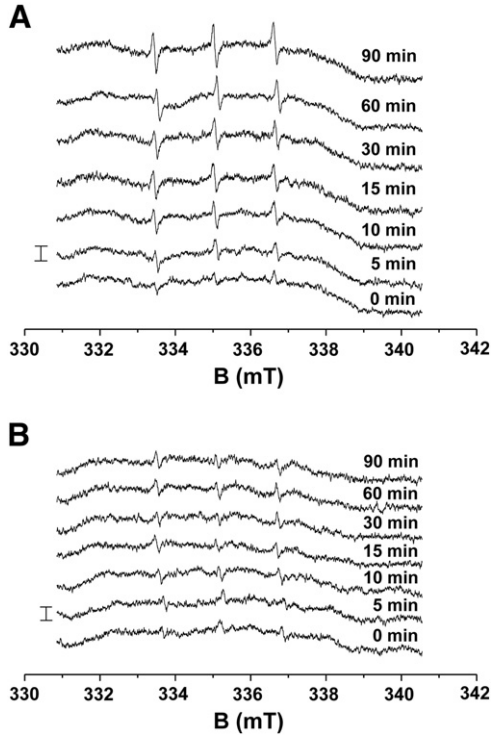

Fig. 2. Light-induced TEMPONE EPR spectra measured in spinach PQ-depleted PSII membranes in the absence (A) and in the presence (B) of exogenous plastoquinol (PQH_2-1). PQ-depleted PSII membranes (150 μg Chl ml^{-1}) were illuminated with white light (1000 μmol m^{-2} s^{-1}) in the presence of 50 mM TMPD and 40 mM MES-NaOH (pH 6.5) for the time indicated. In (B), 100 μM PQH_2-1 was added to the sample prior to illumination. Vertical bar represents 500 relative units.

illumination (Fig. 3). These observations indicate that illumination of PQ-depleted PSII membranes results in $^1O_2$ formation.

3.3. Effect of PQH_2-1 on $^1O_2$ formation in PQ-depleted PSII membranes

To test $^1O_2$ scavenging activity of exogenous plastoquinols in PQ-depleted PSII membranes, TEMPONE EPR spectra were measured in the presence of exogenous PQH_2-1. When 100 μM PQH_2-1 was added to PQ-depleted PSII membranes prior to illumination, the formation of TEMPONE EPR signal was prevented (Fig. 2B). Time profile of the light-induced TEMPONE EPR signal shows that TEMPONE EPR signal observed within the whole period of illumination corresponds to the TEMPONE EPR signal caused by the impurity of the spin trap compound (Fig. 3). Fig. 4 shows the effect of PQH_2-1 concentration on TEMPONE EPR signal in PQ-depleted PSII membranes. When PQ-depleted PSII membranes were illuminated for 90 min in the presence of increased concentration of PQH_2-1, TEMPONE EPR signal was gradually decreased. Based on these results, it is concluded that PQH_2-1 added to PQ-depleted PSII membranes serves as an effective scavenger of $^1O_2$.

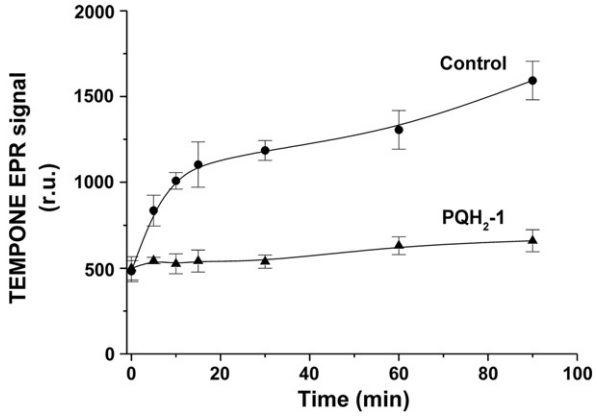

Fig. 3. Effect of exogenous plastoquinol (PQH_2-1) on the time dependence of TEMPONE EPR signal measured in spinach PQ-depleted PSII membranes. PQ-depleted PSII membranes were illuminated in the absence (control) and in the presence (PQH_2-1) of 100 μM exogenous plastoquinol added to the sample prior to illumination. The intensity of EPR signal was evaluated as the relative height of the central peak of the first derivative of the EPR absorption spectrum. Each data point represents the mean value of at least three experiments. Other experimental conditions were the same as described in Fig. 2.

3.4. Singlet oxygen scavenging activity of plastoquinols with a different side-chain length

In further, the effect of exogenous plastoquinols with a different side-chain length on $^1O_2$ scavenging activity was studied in PQ-depleted PSII membranes (Fig. 5). When PQ-depleted PSII membranes were illuminated for 90 min in the presence of plastoquinol molecules with a different side-chain length (PQH_2-n, n isoprenoid units in the side chain), light-induced TEMPONE EPR signal was significantly suppressed by all the homologues in the following order PQH_2-9 (by 73%) < PQH_2-4 (by 87%) < PQH_2-2 (by 91%) < PQH_2-1 (by 94%) (Fig. 5). The residual TEMPONE EPR signal observed for all the homologues corresponds to the TEMPONE EPR signal due to the impurity of the spin trap compound. The changes in the suppression of TEMPONE EPR signal by plastoquinol molecules with a different side-chain length are likely caused by different polarity/solubility of the plastoquinol homologues. These observations may suggest that the $^1O_2$ scavenging activity of exogenously added plastoquinol molecules to PQ-depleted PSII membranes is not dependent on the length of the side chain and that the side-chain does not contribute significantly to the overall quenching activity of the plastoquinol homologues. However, it cannot be excluded, for the reasons given above, that incorporation efficiency of different plastoquinol homologues into thylakoid membranes is different among the homologues.

4. Discussion

It is well known that $^1O_2$ is produced when the absorption of light energy by chlorophylls exceeds the capacity for its utilization. Under these circumstances, formation of the triplet excited state of chlorophyll occurs which reacts with the triplet state of molecular oxygen forming $^1O_2$ [10,11]. Singlet oxygen formation was demonstrated by phosphorescence at 1270 nm [14,38], chemical trapping [15], EPR spin trapping [16,39,40] and fluorescence quenching of DanePy [41–43]. In agreement with these reports, we have

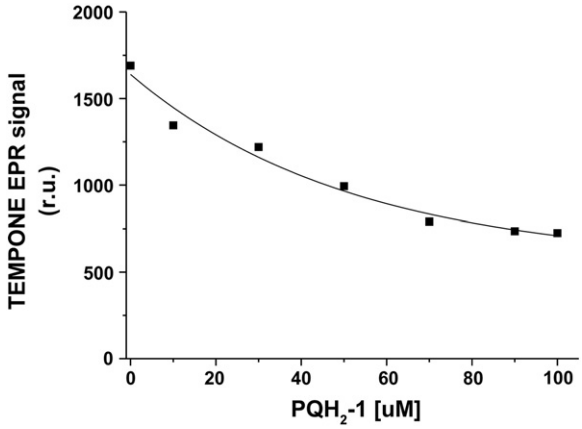

Fig. 4. Effect of various PQH_2-1 concentrations on TEMPONE EPR signal measured in spinach PQ-depleted PSII membranes. PQ-depleted PSII membranes were illuminated for 90 min in the presence of exogenous PQH_2-1 in the concentration as indicated in the figure. Other experimental conditions were the same as described in Fig. 2.

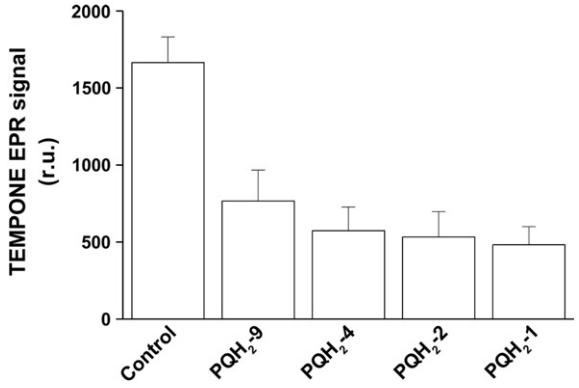

Fig. 5. Effect of various plastoquinols with a different side-chain length on TEMPONE EPR spectra measured in spinach PQ-depleted PSII membranes. PQ-depleted PSII membranes were illuminated for 90 min in the presence of 100 μM exogenous plastoquinol added to the sample prior to illumination. Other experimental conditions were the same as described in Fig. 2. The TEMPONE EPR signal observed in the all homologues included the TEMPONE EPR signal caused by impurity of the spin trap compound.

demonstrated that the exposure of PSII membranes deprived of intrinsic plastoquinone to excessive illumination results in the formation of $^1O_2$. Deleterious effect of $^1O_2$ on PSII is prevented via $^1O_2$ scavenging by carotenoids, α-tocopherol and plastoquinol [25,27,31,44–47]. It has been demonstrated that the addition of plastoquinone homologues to *Chlamydomonas reinhardtii* cultures grown in the presence of plastoquinone inhibitors prevented degradation of D1 protein [27,31].

In the present study, we provide evidence that plastoquinol serves as an efficient $^1O_2$ scavenger. Using EPR spin-trapping technique, we have demonstrated that the addition of exogenous plastoquinol significantly scavenged $^1O_2$ both in the chemical system as well as in the biological system. The presented data clearly show that exogenous plastoquinol efficiently scavenged the light-induced $^1O_2$ generated by rose bengal (Fig. 1). Similarly, $^1O_2$ generated by exposure of PQ-depleted PSII membranes to high-light intensity illumination was scavenged by exogenous plastoquinol (Figs. 2 and 3). Due to the fact that plastoquinol is a mobile molecule, which is able to diffuse in the thylakoid membranes for a long distance, plastoquinol may function as one of the most efficient scavenger of $^1O_2$ in the thylakoid membrane. In agreement with this, we have recently demonstrated that plastoquinol is the main prenyllipid which is synthesized under high light condition in *Arabidopsis* as a response to oxidative stress [48].

Due to the fact that oxidation of plastoquinol by $^1O_2$ may lead to its irreversible degradation, as it was shown in organic solvent where hydroxy-plastoquinones were formed [25], there is a need of making reservoir of plastoquinol in chloroplasts for its antioxidant action. Plastoglobuli, a globular plastid structure, have been previously shown to serve as the storage site for excess prenyllipids that cannot be stored in thylakoid membrane [49]. It has been demonstrated that under high-light conditions plastoquinol is predominantly accumulated in the plastoglobuli that are permanently attached to the thylakoid membrane [48,50]. It has been also shown that the number and size of plastoglobuli increased in chloroplast under oxidative stress [48,49]. It was suggested that the degraded plastoquinol can be replaced by newly synthesized plastoquinol by means of diffusion from the plastoglobuli to the thylakoid membrane and this maintains efficient $^1O_2$ scavenging under high-light illumination [48]. Addition of exogenous plastoquinol to PSII membranes used in this study substitutes the supply of plastoquinol from plastoglobuli to thylakoid membrane under oxidative stress. It should be noted that besides plastoquinol, plant defense systems contain another efficient $^1O_2$ scavengers, such as tocopherols, plastochromanol or hydroxy-plastochromanol [48,51], whose apparent antioxidant activity certainly depends on their relative content in chloroplasts and localization within thylakoid membranes.

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Small CAB-like proteins prevent formation of singlet oxygen in the damaged photosystem II complex of the cyanobacterium *Synechocystis sp.* PCC 6803

RAKESH KUMAR SINHA[1], JOSEF KOMENDA[2], JANA KNOPPOVÁ[2], MICHAELA SEDLÁŘOVÁ[3] & PAVEL POSPÍŠIL[1]

[1]*Department of Biophysics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Šlechtitelů 11, 783 71 Olomouc, Czech Republic,* [2]*Institute of Microbiology, Academy of Sciences, Opatovický mlýn, 37981 Třeboň, Czech Republic and Institute of Physical Biology, University of South Bohemia, 37333 Nové Hrady, Czech Republic and* [3]*Department of Botany, Faculty of Science, Palacký University, Šlechtitelů 11, 783 71 Olomouc, Czech Republic*

ABSTRACT

The cyanobacterial small CAB-like proteins (SCPs) are single-helix membrane proteins mostly associated with the photosystem II (PSII) complex that accumulate under stress conditions. Their function is still ambiguous although they are assumed to regulate chlorophyll (Chl) biosynthesis and/or to protect PSII against oxidative damage. In this study, the effect of SCPs on the PSII-specific light-induced damage and generation of singlet oxygen ($^1O_2$) was assessed in the strains of the cyanobacterium *Synechocystis sp.* PCC 6803 lacking PSI (PSI-less strain) or lacking PSI together with all SCPs (PSI-less/*scp*ABCDE$^-$ strain). The light-induced oxidative modifications of the PSII D1 protein reflected by a mobility shift of the D1 protein and by generation of a D1-cytochrome b-559 adduct were more pronounced in the PSI-less/*scp*ABCDE$^-$ strain. This increased protein oxidation correlated with a faster formation of $^1O_2$ as detected by the green fluorescence of Singlet Oxygen Sensor Green assessed by a laser confocal scanning microscopy and by electron paramagnetic resonance spin-trapping technique using 2, 2, 6, 6-tetramethyl-4-piperidone (TEMPD) as a spin trap. In contrast, the formation of hydroxyl radicals was similar in both strains. Our results show that SCPs prevent $^1O_2$ formation during PSII damage, most probably by the binding of free Chl released from the damaged PSII complexes.

Key-words: oxidative stress; photoinhibition; reactive oxygen species.

INTRODUCTION

In oxygenic photosynthetic organisms (cyanobacteria, algae and higher plants), the light energy absorbed by chlorophylls (Chls) and accessory pigments such as carotenoids and phycobilins is transferred to the reaction centre, where the excitation energy is converted to the chemical energy (Amerongen & Dekker 2003; Larkum 2003; Renger & Holzwart 2005; Renger 2008). To maintain the efficient and rapid energy transfer, Chls and accessory pigments are bound to the integral transmembrane proteins at the proper distance and orientation (Barter, Klug & van Grondelle 2005). In higher plants, the most abundant pigment-protein complex is light-harvesting complex (LHC) of photosystem II (PSII) (Kühlbrandt, Wang & Fujiyoshi 1994; Liu *et al.* 2004). This complex is mostly trimeric with each monomer polypeptide containing a unique Chl *a*/*b*-binding domain called CAB motif (Jansson 1999). Contrary to light-harvesting function of LHCII, other multiple helix CAB proteins such as PsbS (Bergantino *et al.* 2003; Johnson & Ruban 2010), early light-induced proteins (ELIPs) (Heddad & Adamska 2000; Adamska, Kruse & Kloppstech 2001) and stress-enhanced proteins (SEPs) (Engelken, Brinkmann & Adamska 2010) have a protective function. In cyanobacteria, major LHCs are either peripheral phycobilisomes attached to the cytoplasmic side of the membranes or transmembrane Chl a/*b*-binding proteins Pcb homologous to the CP43 antenna of PSII (Grossman *et al.* 1993; Scheer & Zhao 2008). Under stress conditions, cyanobacteria accumulate single-helix CAB-related proteins called small CAB-like proteins (SCPs) (Satoh *et al.* 2001) or high-light-induced-proteins (HLIPs) (Dolganov, Bhaya & Grossman 1995; Funk & Vermaas 1999). Although SCPs contain the conserved CAB motif, no conclusive experimental data have been provided so far on the coordination of Chls to this domain despite some reconstitution attempts supporting this view (Storm *et al.* 2008). The genome of the cyanobacterium *Synechocystis sp.* PCC 6803 contains five *scp* genes (*scp*A, *scp*B, *scp*C, *scp*D, *scp*E) (Funk & Vermaas 1999). The four genes, *scp*B-E code for small proteins with similarity to the first and third transmembrane helix of CAB protein, whereas the ScpA protein is a ferrochelatase containing CAB-like membrane-embedded C-terminal part (Funk & Vermaas 1999). Although the exact function of SCPs is still not clear, two different roles for SCPs have been proposed: (1) transient binding of Chl during biogenesis/turnover of Chl-proteins (Funk & Vermaas

Correspondence: P. Pospíšil. E-mail: pospip@prfnw.upol.cz

1999; Promnares *et al*. 2006; Vavilin, Yao & Vermaas 2007; Yao *et al*. 2007; Storm *et al*. 2008) and related protection against photo-oxidative damage and acclimation to high light (Dolganov *et al*. 1995; Havaux *et al*. 2003); and (2) regulation of Chl biosynthesis (Xu *et al*. 2002, 2004; Hernandez-Prieto *et al*. 2011).

When excitation energy absorbed by Chls in the antenna complexes exceeds its consumption by photochemical reactions, electron transport on the electron acceptor and the electron donor side of PSII is inhibited (Apel & Hirt 2004; Chow & Aro 2005; Vass & Aro 2008; Foyer & Shigeoka 2011). The PSII photoinactivation is accompanied by the production of reactive oxygen species (ROS) formed either by excitation energy transfer or by electron transport (Krieger-Liszkay, Fufezan & Trebst 2008; Pospíšil 2009, 2011). Excitation energy transfer from triplet Chl to molecular oxygen results in the formation of singlet oxygen ($^1O_2$). Triplet Chl is formed either by intersystem crossing from singlet Chl in the antenna complex or by charge recombination of primary radical pair [$P680^+Pheo^-$] in the PSII reaction centre (Krieger-Liszkay *et al*. 2008; Triantaphylides & Havaux 2009; Vass & Cser 2009; Pospíšil 2011). The electron leakage to molecular oxygen on the electron acceptor side of PSII brings about the production of superoxide anion radical ($O_2^{\bullet-}$), which initiates a cascade of reactions leading to the formation of hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^\bullet$) (Pospíšil 2009, 2011). The generated $^1O_2$ and $HO^\bullet$ may react with polyunsaturated fatty acid and amino-acid residues of nearby proteins leading to lipid peroxidation and oxidative damage of proteins, respectively (Stadtman 1993; Dean *et al*. 1997; Girotti 1998).

It is believed that extensive protein oxidation in the inactivated PSII complexes is prevented by the fast repair process comprising of the partial disassembly of the complex, the selective replacement of the inactive D1 protein by a newly synthesized copy and finally reassembly and activation of the complex (Aro *et al*. 2005; Yamamoto *et al*. 2008; Nixon *et al*. 2010). During degradation of the damaged D1 protein, Chls released from the protein enhance the risk of formation of triplet Chl and subsequently $^1O_2$. It has been proposed that SCPs temporarily bind at least some of released Chls and thus eliminate their photodynamic potential via interaction with carotenoids that are also hypothetically bound to SCPs (Xu *et al*. 2004). Nevertheless, no direct evidence has been provided on the protective role of SCPs against formation of deleterious $^1O_2$.

The current study provides direct evidence on the protective role of SCPs against formation of $^1O_2$ and related protein oxidation in the photoinactivated PSII both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Strain and culture conditions

PSI-less (Shen, Boussiba & Vermaas 1993) and PSI-less/*scp*ABCDE$^-$ (Vavilin *et al*. 2007) strains of *Synechocystis sp.* PCC 6803 were grown in a continuous white light (10 μmol m^{-2} s^{-1}) at 25 °C in BG-11 medium supplemented with 5 mM glucose and 5 mM sodium bicarbonate as the main carbon source (Rippka *et al*. 1979). The cell culture was placed on a multi-position magnetic stirrer RT 5 power (IKA Werke GmbH**,** Staufen, Germany) and permanently stirred to obtain constant CO_2 concentration in the growth medium. The cells were studied during the end of log phase at a density of approximately 10^8–10^9 cells mL^{-1}. Cell density was assessed by a manual microscopic cell count. For the determination of Chl concentration, the cells were pelleted at 10 000 *g* for 3 min and extracted with 80% methanol. The Chl concentration was determined by the absorbance at 646 and 663 nm according to Lichtenthaler (1987).

Preparation of thylakoid membranes

Thylakoid membranes for the two-dimensional (2D) gel electrophoresis were isolated from *Synechocystis sp.* PCC 6803 cells using the method described by Komenda & Barber (1995) with the following modifications. The cell suspension (25 μg of Chl) was washed, broken and finally resuspended in 10 mM $CaCl_2$, 10 mM $MgCl_2$, 25 mM Mes (pH 6.5) and 25% glycerol. For large-scale isolation of the membranes for electron paramagnetic resonance (EPR) spin-trapping spectroscopy, the cell culture was spun down for 5 min at 8000 *g* and the pellet was resuspended in KPN buffer (50 mM K-phosphate, pH = 8, 100 mM NaCl). After several washing steps, the cells were resuspended in KPN buffer containing a mini ethylenediaminetetraacetic acid (EDTA)-free ROCHE protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany). Prior to cell breaking, the cell culture with the same volume of glass beads was put in 15 mL tubes and kept in ice for 30 min. Cells were broken using a bead beater (BioSpec Products Inc., Bartlesville, OK, USA) with glass bead sizes of 150–200 μm in diameter over six cycles, each cycles comprising of 1 min of beating and 3 min of cooling. The unbroken cells, cell debris and thylakoid membranes were decanted off by repeated washing of the glass beads with KPN buffer. The decanted material was centrifuged at 3000 *g* for 3 min to remove unbroken cells and cell debris. The supernatant was centrifuged at 40 000 *g* for 20 min. The pellet was resuspended in 4 mL of KPN buffer along with an addition of 1 mL of glycerol and stored in –80 °C until use. The extraction procedure was carried out on ice in a dark room.

Photoinhibitory treatment

For assessment of the oxygen evolution and $^1O_2$ generation, the cell suspension or thylakoid membranes were placed in a spectroscopic cuvette (2 mL) and exposed to continuous white light (1000 μmol photons m^{-2} s^{-1}). For assessment of Chl fluorescence spectra and protein damage, the cell suspension (50 mL, 1 μg Chl mL^{-1}) was transferred to plan-parallel cuvettes in which the cultures were bubbled with air and exposed to high light intensity illumination

(2500 μmol photons m^{-2} s^{-1}) for 20 min. For assessment of the oxygen evolution, Chl fluorescence spectra and protein damage, the high light intensity illumination was performed in the presence of 100 μg mL^{-1} tetracycline, an inhibitor of protein synthesis. The illumination was performed using a halogen lamp with a light guide (Schott KL 1500, Schott AG, Mainz, Germany) under slow continuous stirring using a tiny bar magnet. The light intensity was measured by quantum radiometer LI-189 (Li-Cor Inc., Lincoln, NE, USA).

PSII activity measurements

Steady-state rate of oxygen evolution was measured with a Clark oxygen electrode (oxygen electrode chamber DW1, Hansatech, King's Lynn, Norfolk, UK) connected to an Oxyrecorder 3015 and supplied with Oxywin software 3.0 (Photon System Instruments, Brno, Czech Republic). After photoinhibitory treatment, the suspension of *Synechocystis* cells (10 μg Chl mL^{-1}) in the presence of artificial electron acceptors 0.4 mM 2,5-dimethyl-*p*-benzoquinone (DMBQ) and 2 mM potassium ferricyanide was placed in an oxygen electrode chamber and the rate of oxygen evolution was measured under saturating light conditions (2500 μmol photons m^{-2} s^{-1}). Prior to the measurements, the Clark oxygen electrode was calibrated with air-saturated water at 25 °C and with the addition of a few crystals of sodium dithionite. For the statistical analysis, Origin 5.0 software (Microcal Software, Inc., Northampton, MA, USA) has been used for calculation of the mean value and standard deviation with at least three different replicates.

Low temperature Chl fluorescence emission

Chl fluorescence emission spectra at 77 K were measured using an Aminco Bowman Series 2 luminescence spectrometer (Spectronic Unicam, Rochester, NY, USA). The same number of cells for each strain was excited at 435 nm (bandwidth 4 nm) and Chl fluorescence emission spectra were recorded in the range 550–800 nm. Subsequently, the spectra were corrected for the sensitivity of the photomultiplier and normalized to the 570 nm fluorescence maximum of rhodamine, which was used as an internal standard.

Gel electrophoresis and immunobloting

The protein complexes from the isolated thylakoid membranes were solubilized in 1% β-n-dodecyl maltoside (DM) and analysed by a clear variant of blue-native electrophoresis at 4 °C in a 5–14% gradient polyacrylamide gel according to Schägger & von Jagow (1991). In this variant, Coomassie Blue was omitted from all solutions and the upper electrophoretic buffer contained 0.05% sodium deoxycholate and 0.02% DM. Samples with the same Chl content (1 μg) were loaded onto the gel. The protein composition of the complexes was analysed by electrophoresis in a denaturing 12–20% linear gradient polyacrylamide gel containing 7 M urea (Komenda, Lupínková & Kopecký 2002). Complete lanes from the native gel were excised, incubated for 30 min in 25 mM Tris (pH 7.5) containing 1% sodium dodecyl sulphate (SDS; w/v) and placed on top of the denaturing gel; two lanes were analysed in a single denaturing gel. Proteins separated in the gel were stained by Sypro Orange and then subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with the specific primary antibody raised in rabbit against residues 58–86 of the spinach D1 polypeptide, then with secondary antibody-horseradish peroxidase conjugate and finally with chemiluminescent substrate West Pico. The Sypro Orange fluorescence of proteins in the gel as well as chemiluminescence of the blot was recorded by LAS-4000 imaging system (Fuji, Tokyo, Japan) and quantification of bands was performed by ImageQuant TL software (GE Healthcare, Uppsala, Sweden).

Singlet oxygen imaging by confocal laser scanning microscopy and image analysis

Singlet Oxygen Sensor Green (SOSG) reagent® (Molecular Probes Inc., Eugene, OR, USA) was chosen to visualize $^1O_2$ production in the intact PSI-less and PSI-less/*scp*ABCDE$^-$ *Synechocystis* cells (Flors *et al.* 2006; Driever *et al.* 2009). The cells of both strains were harvested in the middle of the log-phase to facilitate SOSG uptake along with nutrients. The suspensions were diluted to a cell density of approximately 10^8–10^{10} cells mL^{-1}, stained with 250 μM SOSG and kept at 37 °C for 15 min to enhance the efficiency of fluorochrome uptake through mucoid sheath surrounding the cyanobacterial cell wall. Subsequently, the samples were incubated for 3 h at room temperature, either in dark or with exposure to the photoinhibitory treatment. Cell suspensions in 50% deuterium oxide (D_2O) or 10 mM histidine served as a positive or a negative control, respectively.

Singlet oxygen imaging was performed by a confocal laser scanning microscope, Olympus Fluorview 1000 confocal unit, combined with inverted microscope IX 80 (Olympus Czech Group, Prague, Czech Republic). The transmission images were obtained by transmitted light detection module with 405 nm excitation using a near-ultraviolet (UV) laser diode (LD) laser and Nomarski DIC filters (Olympus Czech Group, Prague, Czech Republic). Simultaneously, visualized fluorescence channels result from excitation by a 488 nm line of argon laser, representing the signals for SOSG signal detected by 505–525 nm emission filter and Chl fluorescence with 650–750 nm emission filter set. The proper intensity of lasers was set according to unstained samples at the beginning of each experiment (Sedlářová *et al.* 2011).

The numbers of total cells per image and cells with SOSG signal superior to background were recorded from green channel, the percentage counted and mean value and standard deviation were calculated from three representative microphotographs per variant. For each strain the samples kept in dark and illuminated for 3 h in water or D_2O were compared. The intensity of signal (ranging from 0 to 4095 levels of brightness) was evaluated using software FV10-ASW 3.0 Viewer (Olympus). Data are presented as

calculation of means and standard deviations. Statistical analysis included testing of differences between strains and treatments using two-way analysis of variance (Tukey's multiple comparison test) in NCSS 2007 software (NCSS, Kaysville, UT, USA). Values from at least three independent replicates in each variant have been used for analysis.

EPR spin-trapping spectroscopy

For detection of $^1O_2$, the spin-trapping was accomplished by 2, 2, 6, 6-tetramethyl-4-piperidone (TEMPD) (Sigma, St Louis, MO, USA). Thylakoid membranes (150 μg Chl mL^{-1}) were illuminated on the ice with continuous stirring in the presence of 50 mM TEMPD and 40 mM Mes (pH 6.5). In some measurements, 10 mM histidine (Sigma, St. Louis, MO, USA) or 50% D_2O (Sigma) was added to the sample prior to the illumination as indicated in the figure legend. After illumination, the sample was centrifuged at 5000 *g* for 4 min to separate 2, 2, 6, 6-tetramethyl-4-piperidone-1-oxyl (TEMPONE) from the thylakoid membranes. The separation of the two phases was carried out to prevent the reduction of TEMPONE by non-specific reducing component in the thylakoid membranes. After centrifugation, the upper phase was immediately transferred into the glass capillary tube (Blaubrand® intraMARK, Brand, Hamburg, Germany) and frozen in the liquid nitrogen until its use. Prior to data collection, the capillary tube was taken away from the liquid nitrogen and EPR spin-trapping data were collected at room temperature. For detection of HO$^\bullet$, the spin-trapping was accomplished by a 4-pyridyl-1-oxide-*N*-*tert*-butylnitrone (POBN)/ethanol spin trapping system (Pou *et al*. 1994). Thylakoid membranes (150 μg Chl mL^{-1}) were illuminated on the ice with continuous stirring in the presence of 50 mM POBN, 170 mM ethanol and 40 mM Mes (pH 6.5). After illumination, the sample was immediately transferred into the glass capillary tube and EPR spectra were measured. EPR spectra were recorded using an EPR spectrometer MiniScope MS200 (Magnettech GmbH, Berlin, Germany). Signal intensity was evaluated as a height of the central peak of EPR spectrum. EPR conditions were as follows: microwave power, 10 mW; modulation amplitude, 1 G; modulation frequency, 100 kHz; sweep width, 100 G; scan rate, 1.62 G s^{-1}.

RESULTS

PSII photoinactivation in PSI-less and PSI-less/*scp*ABCDE$^-$ *Synechocystis* cells

Light-induced inhibition of the PSII in the PSI-less and PSI-less/*scp*ABCDE$^-$ cells was assessed by monitoring steady-state rate of oxygen evolution measured in the presence of artificial electron acceptors (Fig. 1). Exposure of the intact cells of both strains to high irradiance (1000 μmol photons m^{-2} s^{-1}) in the presence of tetracycline to stop the PSII repair cycle resulted in the gradual and parallel decrease in the rate of oxygen evolution, which was suppressed almost completely in both strains after 3 h of illumination (Fig. 1). In compliance with preceding study by Vavilin *et al*. (2007), our results indicate that the rates of PSII photoinactivation reflected by the loss of oxygen evolution are similar in PSI-less and PSI-less/*scp*ABCDE$^-$ cells upon exposure to high irradiance.

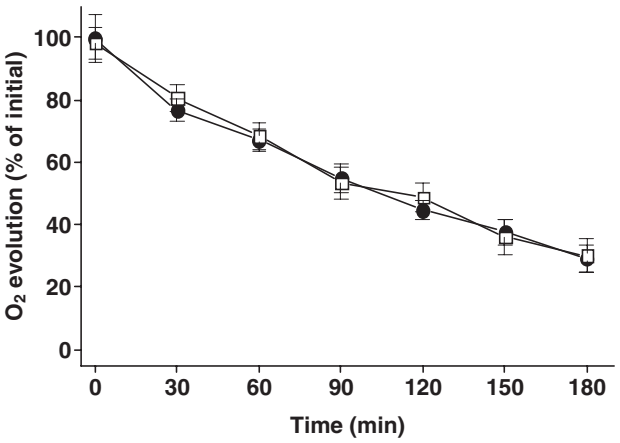

Figure 1. Photoinactivation in the photosystem I (PSI)-less and PSI-less/*scp*ABCDE$^-$ *Synechocystis* cells measured by assessment of the rate of oxygen evolution. The cell suspensions of *Synechocystis* PSI-less (square) and PSI-less/*scp*ABCDE$^-$ (circle) strains [10 μg chlorophyll (Chl) mL^{-1}] after addition of protein synthesis inhibitor tetracycline (100 μM) were exposed to high irradiance (1000 μmol photons m^{-2} s^{-1}). After indicated period, the aliquot of the suspension was withdrawn for measurement of the light-saturated steady state rate of oxygen evolution using a Clark oxygen electrode in the presence of artificial electron acceptors 2, 5-dimethyl-p-benzoquinon (DMBQ; 0.4 mM) and potassium ferricyanide (2 mM). Each data point represents the mean value of at least three measurements.

Low temperature Chl fluorescence emission and PSII protein oxidation in PSI-less and PSI-less/*scp*ABCDE$^-$ *Synechocystis* cells

A nearly complete inhibition of oxygen evolution was also reached in the cells of both strains when they were illuminated at 2500 μmol photons m^{-2} s^{-1} for 20 min in the presence of tetracycline (not shown). This treatment was used for assessment of low temperature fluorescence spectra and PSII protein oxidation to maximally limit the time for action of cellular proteases that largely eliminate impaired and oxidized proteins during longer incubations. In both strains, the 77 K fluorescence emission spectra measured in non-illuminated cells exhibited two major peaks at 685 and 696 nm (Fig. 2a,b, full line). However, the ratio of fluorescence emission at 696 and 685 nm (F696/F685) was different in each strain reaching 1.7 in the PSI-less strain (Fig. 2a) and 1.1 in the PSI-less/*scp*ABCDE$^-$ strain (Fig. 2b). Interestingly, in the fluorescence emission spectrum of the PSI-less/*scp*ABCDE$^-$ strain there was also contribution of fluorescence emission at around 670 nm that was absent in the PSI-less strain (Fig. 2b, full line). This observation indicates the presence of Chls in the specific environment

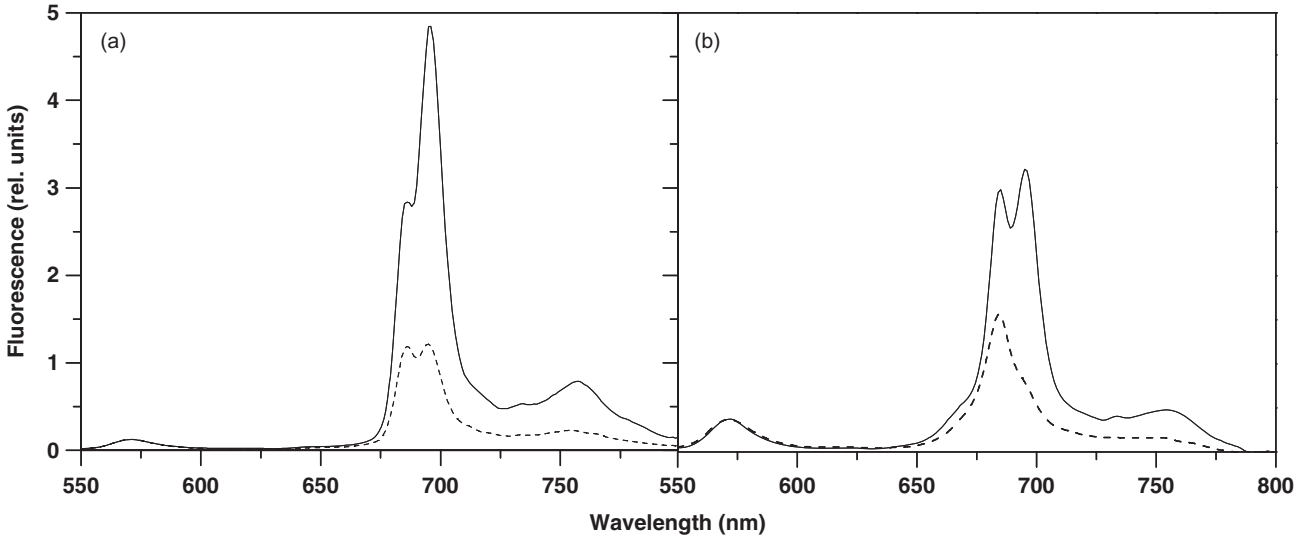

Figure 2. Low temperature chlorophyll fluorescence emission spectra measured in the cells of the photosystem I (PSI)-less (a) and PSI-less/*scp*ABCDE$^-$ (b) *Synechocystis* strains. 77 K chlorophyll (Chl) fluorescence spectra (excitation at 435 nm) were measured in cells of both strains before (full line) and after (dashed line) high light treatment (2500 μmol photons m^{-2} s^{-1} of white light for 20 min in the presence of 100 μM tetracycline. The same amount of cells of each strain was excited at 435 nm. Spectra were corrected for the sensitivity of the photomultiplier and normalized to 570 nm (the fluorescence peak of rhodamine used as an internal standard).

because of the absence of SCPs. After high light treatment, the fluorescence emission at 685 and 696 nm was pronouncedly decreased (Fig. 2a,b, dashed line). The fluorescence emission at 696 nm belongs to Chl bound to native CP47 either in unassembled CP47 or in the photochemically active PSII (Komenda *et al.* 2010). This peak decreased in both strains by 75% reflecting the extensive functional inactivation of PSII, which corresponded to nearly complete inhibition of oxygen evolution in the cells (data not shown).

In order to correlate the above-described functional impairment of PSII with oxidative damage of PSII proteins, the analysis of protein complexes and their subunits was performed before and after 20 min illumination at 2500 μmol photons m^{-2} s^{-1} using a combination of native polyacrylamide gel electrophoresis (PAGE) in one direction and denaturing SDS–PAGE in the second direction (2D CN/SDS–PAGE). In the control non-illuminated cells of both strains, the large PSII subunits D1, D2, CP43 and CP47 were detected almost exclusively in two large complexes representing the dimeric and monomeric PSII core complexes [RCC(2) and RCC(1) (Fig. 3, control stained gel]. The amount of RCC(2) was higher in both strains, the ratio of RCC(2)/RCC(1) reached the values about 2 in the PSI-less strain and 1.4 in the PSI-less/*scp*ABCDE$^-$ strain (Table 1). Only negligible amount of the monomeric PSII core complex lacking CP43 (RC47) was detected in the non-illuminated cells of both strains (Fig. 3, control anti-D1). As the main target of the photo-oxidative damage in PSII is the D1 protein, we monitored the light-induced changes in the PSII complexes and possible oxidative protein damage by Western blotting using antibodies specific for the D1 protein (Fig. 3, anti-D1). As shown by densitometric scanning of the D1 signal in the particular complexes, exposure of cells of both strains to high irradiance did not lead to the change in the overall level of the D1 protein indicating no significant degradation of the D1 protein (Table 1, sum of D1). However, the light treatment caused a large change in the content of the individual complexes (Fig. 3, Table 1). At least 50% of the RCC(2) disappeared with concomitant increase in the level of RCC(1) and, more importantly, with the appearance of the RC47 complex (Fig. 3, HL anti-D1), which was previously considered as an indicator of light-induced damage of PSII in cyanobacteria under conditions with an ineffective PSII repair cycle (Komenda & Masojídek 1995). Interestingly, the light treatment was also accompanied by an appearance of the D1-cytochrome b-559 adduct (Fig. 3, D1-cyt) in the gel region corresponding to RCC(1) and RC47 complexes. This protein adduct was previously detected as a result of reaction between the oxidized His258 residue of the D1 protein and the N-terminal amino group of the α-subunit of cytochrome b-559 (Lupínková *et al.* 2002). As the formation of this adduct is related to the action of $^1O_2$, its appearance in the PSII complexes after light treatment could be used as an indicator of the $^1O_2$-mediated oxidative damage. While the intensity of the adduct was similar in RCC(1) and RC47 in the cells of the PSI-less strain, its level in the RC47 complex of the PSI-less/*scp*ABCDE$^-$ strain was apparently higher than in other complexes (Fig. 3, arrowhead). Moreover, the migration of the typical D1 band in this RC47 complex was slower in comparison with the D1 band in RCC(2) and RCC(1) in this strain and in all the PSII complexes of PSI-less strain (Fig. 3, arrowhead). The light-induced shift in the D1 mobility was previously related to the oxidative damage of the protein and mostly $^1O_2$ was

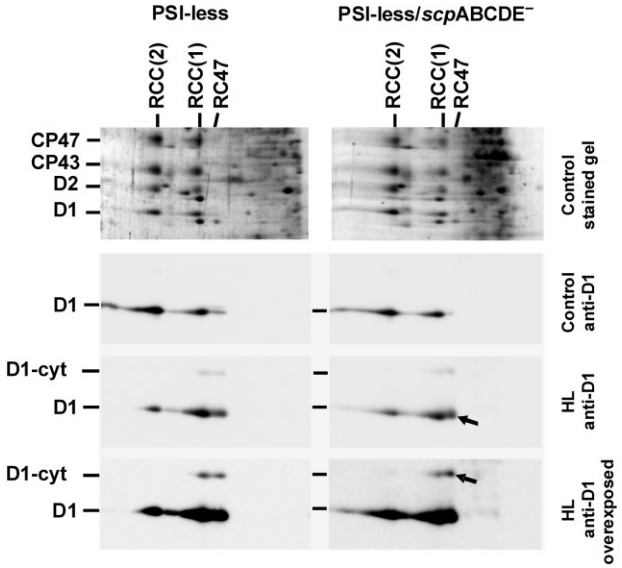

Figure 3. Two-dimensional (2D) analysis of the D1 protein in the cells of the photosystem I (PSI)-less and PSI-less/*scp*ABCDE⁻ *Synechocystis* strains. Cells of both strains were illuminated at 2500 μmol photons m^{-2} s^{-1} of white light for 20 min in the presence of 100 μM tetracycline. Membranes isolated from non-illuminated (control) and illuminated (HL) cells were analysed by 2D clean native/sodium dodecyl sulphate–polyacrylamide gel electrophoresis (CN/SDS–PAGE). The proteins in the gel were stained with Sypro Orange (stained gel), subsequently blotted onto PVDF membrane and immunodecorated using antibodies specific for the D1 protein (anti-D1). RCC(2) and RCC(1) designate the dimeric and monomeric PSII core complex, respectively; RC47 indicate the monomeric PSII core complex lacking CP43. Arrowheads indicate D1 adduct and D1 mobility shift in the RC47 complex of the PSI-less/*scp*ABCDE⁻ strain.

found as ROS responsible for this modification (Komenda *et al*. 2002; Lupínková & Komenda 2004). Thus, the monitoring of the changes in PSII proteins and their complexes in illuminated cells of the studied strains supported the view that the oxidative $^1O_2$-mediated protein damage reflected by the mobility shift of the D1 protein, and its adduct generation is largely prevented in the strain containing SCPs.

Singlet oxygen imaging in PSI-less and PSI-less/*scp*ABCDE⁻ *Synechocystis* cells

To monitor $^1O_2$ formation in the intact cells of the PSI-less and PSI-less/*scp*ABCDE⁻ strains, $^1O_2$ imaging was performed by the green fluorescence of SOSG using laser confocal scanning microscopy (Fig. 4, middle columns). SOSG is a unique fluorescence probe for $^1O_2$ detection that reacts selectively with $^1O_2$ without any undesirable interaction with $O_2^{\bullet-}$ and $HO^{\bullet}$ (Molecular Probes 2004; Flors *et al*. 2006, Ragas *et al*. 2009). In the absence of $^1O_2$, SOSG exhibits a blue fluorescence with excitation at 372 and 393 nm and emission at 395 and 416 nm. In the presence of $^1O_2$, SOSG fluorescence is green shifted with excitation and emission at 504 and 525 nm, respectively. In addition to SOSG fluorescence, the red channel corresponding to Chl fluorescence (Fig. 4, right columns) and Nomarsky DIC channel (Fig. 4, left columns) were visualized.

In the dark, the intact cells of PSI-less and PSI-less/*scp*ABCDE⁻ strains exhibited weak green fluorescence emission in a limited number of cells (Fig. 4, middle column). Exposure of cells of PSI-less and PSI-less/*scp*ABCDE⁻ strains to high irradiance for 3 h enhanced the number of cells emitting the green fluorescence. The green fluorescence was even more pronounced when water in the media was replaced by D_2O known to prolong the lifetime of $^1O_2$ by several folds compared with H_2O (Fig. 4). To confirm that SOSG fluorescence is linked solely to $^1O_2$ formation, the effect of $^1O_2$ scavenger on SOSG fluorescence was tested. The addition of hydrophilic $^1O_2$ scavenger histidine in the intact cells of PSI-less and PSI-less/*scp*ABCDE⁻ strains completely suppressed SOSG fluorescence emission (Fig. 4).

To evaluate the differences in $^1O_2$ production between both strains, the intensities of SOSG fluorescence signals within confocal images were analysed in details by image analysis (Fig. 5). In the PSI-less strain, the mean signal intensity increased by approximately 15% and the

Table 1. Quantification of the D1 signals in photosystem II (PSII) complexes RCC(2), RCC(1) and RC47 resolved by CN/SDS–PAGE from cells of the PSI-less and PSI-less/*scp*ABCDE strain before (Contr) and after (HL) high light treatment (see Fig. 3)

	PSI-less Contr		PSI-less HL		PSI-less SCP-less Contr		PSI-less SCP-less HL	
Complexes/cells	%[a]	% init[b]	%[a]	% init[b]	%[a]	% init[b]	%[a]	% init[b]
RCC(2)	63 ± 6	100	19 ± 4	32 ± 7	58 ± 9	100	28 ± 5	50 ± 8
RCC(1)	32 ± 3	100	60 ± 10	196 ± 32	41 ± 3	100	52 ± 11	130 ± 30
RC47	5 ± 1	100	21 ± 3	436 ± 83	1 ± 0.5	100	20 ± 3	570 ± 110
Sum of D1	100	100	100	104 ± 8	100	100	100	102 ± 5

[a]Percentage of the D1 protein in the particular complex related to the overall amount of D1 in all complexes of the particular sample; means of three measurements ± standard deviation (SD).
[b]Percentage of the D1 protein in the particular complex related to the D1 level found in the same complex in the control sample; means of three measurements ± SD. SCP, small CAB-like protein; CN/SDS–PAGE, clean native/sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

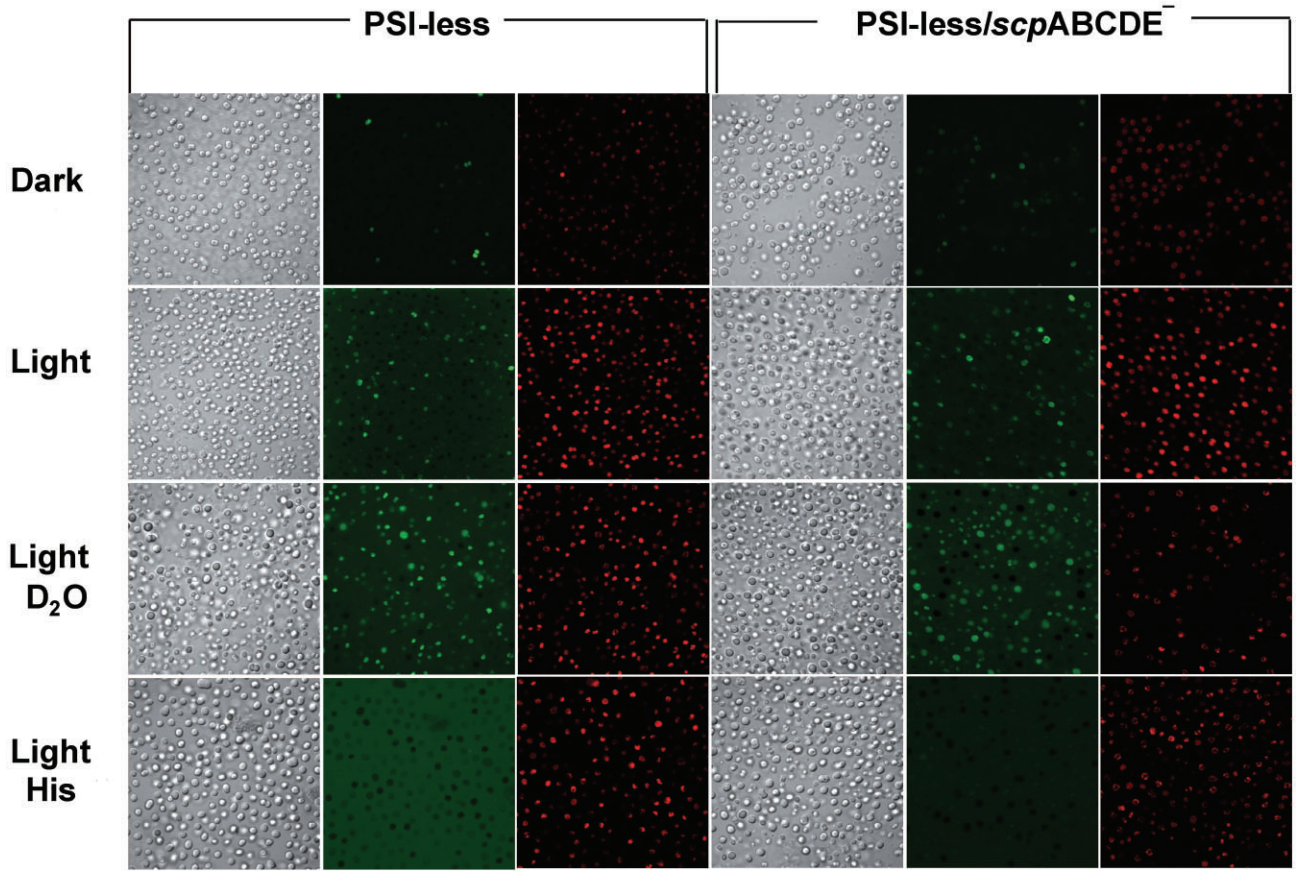

Figure 4. Singlet oxygen imaging in the photosystem I (PSI)-less and PSI-less/*scp*ABCDE$^-$ *Synechocystis* cells by confocal laser scanning microscopy. The suspension of *Synechocystis* cells with the density of 10^8–10^{10} cells mL^{-1} was stained with 250 mM Singlet Oxygen Sensor Green (SOSG) for 15 min. Singlet oxygen imaging was performed in the cell suspension placed on the microscopic slide in the dark or exposed to high irradiance (1000 μmol photons m^{-2} s^{-1}) for 3 h. In some measurements, 10 mM histidine and 50% deuterium oxide were added to the sample prior to the illumination. For each sample three channels are presented: Nomarsky DIC (left row), SOSG fluorescence (λ_{em} = 505–525 nm) (middle row) and chlorophyll (Chl) fluorescence (λ_{em} = 650–750 nm) (right row).

percentage of cells with SOSG fluorescence raised from 8 to 21% after illumination for 3 h (Fig. 5a–c). On the other hand, in the PSI-less/*scp*ABCDE$^-$ strain the mean SOSG fluorescence increased by 30% and the percentage of cells with the fluorescence signal grew from 15 to 47% after illumination for 3 h (Fig. 5a,b,d). Whereas the enhancement in intensity of SOSG fluorescence after 3 h of high irradiance was rather negligible (25%), the increase in percentage of cells with SOSG fluorescence was pronounced (120%) in PSI-less/*scp*ABCDE$^-$ strain in comparison with PSI-less strain. In both strains, the changes in $^1O_2$ production visualized by SOSG fluorescence were amplified after replacing the water in growing media by D_2O. These observations reveal that the exposure of the intact cell to high irradiance results in $^1O_2$ formation.

Singlet oxygen formation in the thylakoid membranes isolated from the PSI-less and PSI-less/*scp*ABCDE$^-$ *Synechocystis* cells

In order to quantify $^1O_2$ production in both strains, $^1O_2$ production was measured in the thylakoid membranes isolated from the PSI-less and PSI-less/*scp*ABCDE$^-$ strains by EPR spin-trapping spectroscopy (Fig. 6). The spin trapping was accomplished by hydrophilic nitroxide spin trap TEMPD, which is known to react with $^1O_2$, forming the nitroxyl radical TEMPONE (Lion, Delmelle & van de Vorst 1976; Lion, Gandin & van de Vorst 1980). The addition of TEMPD to the thylakoid membranes prior to illumination does not cause any formation of TEMPONE EPR signal. The TEMPONE EPR signal observed in non-illuminated thylakoid membranes was caused by impurity of spin trap compound. Exposure of thylakoid membranes isolated from the PSI-less strain to high-light intensity illumination resulted in the generation of TEMPONE EPR spectra (Fig. 6a). Similarly, illumination of thylakoid membranes isolated from PSI-less/*scp*ABCDE$^-$ strain caused the generation of TEMPONE EPR spectra (Fig. 6b). When the intensity of TEMPONE EPR signal was plotted against the illumination period, a gradual increase in TEMPONE EPR signal was observed (Fig. 6c). In the initial period of illumination, no significant difference in TEMPONE EPR signal was observed. However, in the later period of illumination, more pronounced TEMPONE EPR signal was observed in

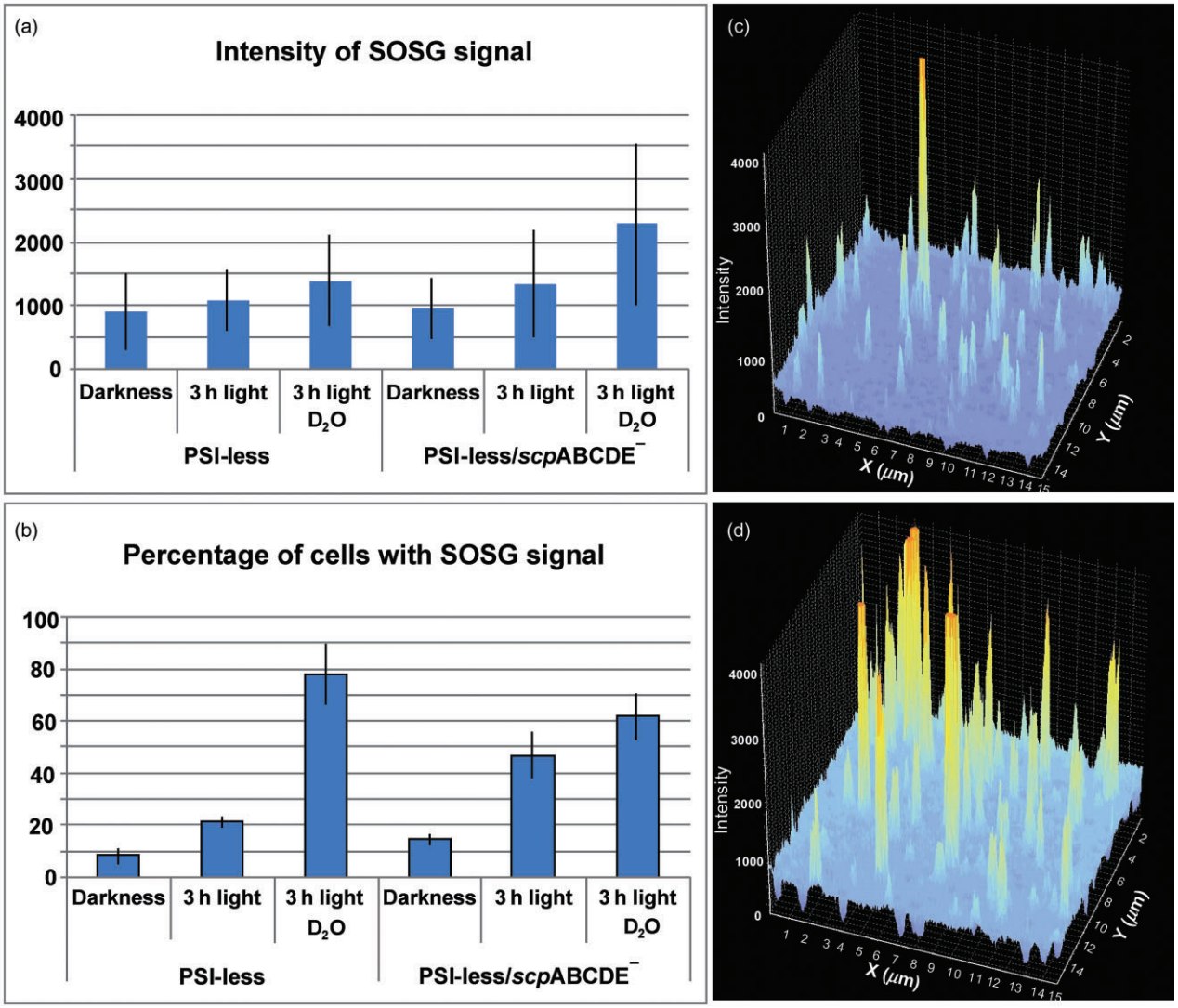

Figure 5. Signal intensities and percentage of cells with Singlet Oxygen Sensor Green (SOSG) fluorescence within confocal laser scanning microscopy images of photosystem I (PSI)-less and PSI-less/*scp*ABCDE$^-$ *Synechocystis* cells. The relative intensity of SOSG signal (a) and the ratio of cells with SOSG signal and the total number of cells (b) were determined in three representative microphotographs per variant and expressed as mean value and standard deviation. Demonstration of the SOSG signal intensity distribution within an image of PSI-less (c) and PSI-less/*scp*ABCDE$^-$ *Synechocystis* cells (d). In both images (c, d), the axes X and Y represent μm, and the Z axis means the levels of brightness, ranging between 0 and 4095. Other experimental conditions as in Fig. 4.

thylakoid membranes isolated from PSI-less/*scp*ABCDE$^-$ strain. These results reveal that the absence of SCP results in the increase in $^1O_2$ formation during the later period of illumination, whereas $^1O_2$ formation was unaffected during the initial period of illumination.

Light-induced TEMPONE EPR spectra were measured in the thylakoid membranes illuminated in the presence of $^1O_2$ scavenger (histidine) and $^1O_2$ enhancer (D_2O) (Fig. 7). When histidine was added in the thylakoid membranes prior to illumination, a complete suppression of TEMPONE EPR spectra was observed in both the PSI-less and PSI-less/*scp*ABCDE$^-$ strains (Fig. 7). The residual TEMPONE EPR signal observed in the presence of histidine corresponds to the TEMPONE EPR signal caused by the impurity of the spin trap compound. When water in the buffer solution was replaced by D_2O, TEMPONE EPR signal increased in both the PSI-less and PSI-less/*scp*ABCDE$^-$ strain (Fig. 7). The obtained results confirmed that the observed TEMPONE EPR signals were specific for $^1O_2$ and implied that $^1O_2$ formed in the interior of the membrane diffused out of the membrane.

Hydroxyl radical formation in the thylakoid membranes isolated from the PSI-less and PSI-less/*scp*ABCDE$^-$ *Synechocystis* cells

To evaluate the possible effect of SCPs on the generation of other ROS, the production of HO$^\bullet$, another dangerous ROS produced by PSII under the high light intensity illumination (Pospíšil *et al.* 2004), was measured in the thylakoid

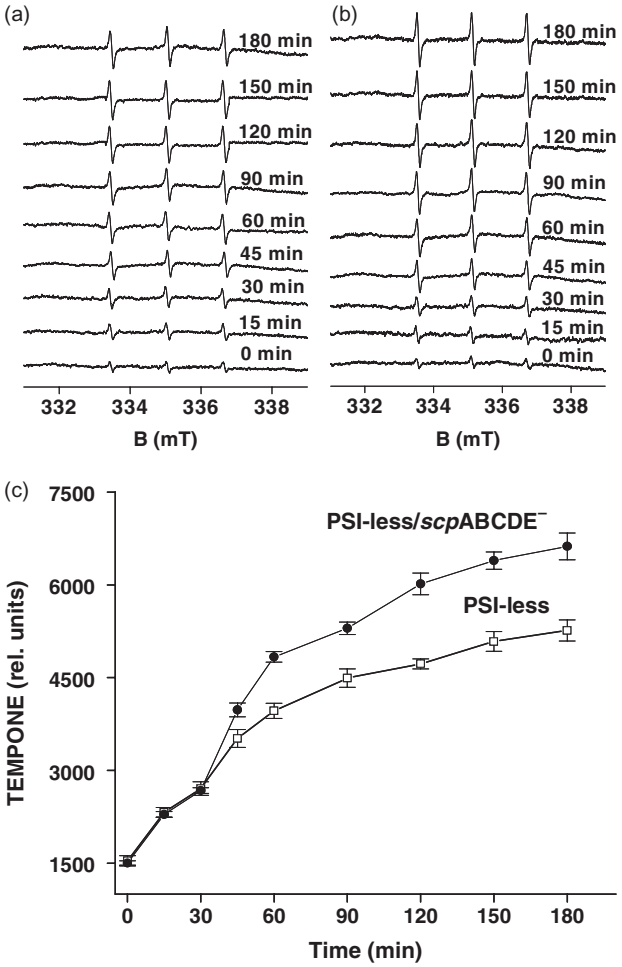

Figure 6. Singlet oxygen formation in the thylakoid membranes isolated from the photosystem I (PSI)-less and PSI-less/*scp*ABCDE⁻ *Synechocystis* cells detected by electron paramagnetic resonance (EPR) spin-trapping spectroscopy. Light-induced 2, 2, 6, 6-tetramethyl-4-piperidone-1-oxyl (TEMPONE) EPR spectra were measured in the thylakoid membranes isolated from the PSI-less (a) and PSI-less/*scp*ABCDE⁻ (b) *Synechocystis* cell. (c) Time profile of light-induced TEMPONE EPR spectra measured in the thylakoid membranes isolated from the PSI-less (square) and PSI-less/*scp*ABCDE⁻ (circle) *Synechocystis* cell. Thylakoid membranes (150 μg of Chl mL^{-1}) were exposed to high light intensity illumination (1000 μmol photons m^{-2} s^{-1}) in the presence of 50 mM 2, 2, 6, 6-tetramethyl-4-piperidone (TEMPD) and 40 mM Mes (pH 6.5) for the time periods indicated in the figure. The intensity of EPR signal was evaluated the as the relative height of the central peak of the first derivative of the EPR absorption spectrum. Each data point represents the mean value of at least three measurements.

membranes isolated from the PSI-less and PSI-less/*scp*ABCDE⁻ strains by EPR spin-trapping spectroscopy (Fig. 8). Light-induced production of HO$^\bullet$ was assessed using a POBN/ethanol spin-trapping system (Pou *et al.* 1994). In this system, the interaction of HO$^\bullet$ with ethanol yields α-hydroxyethyl radical [CH(CH$_3$)HO$^\bullet$] known to react with POBN, while a stable α-hydroxyethyl radical adduct of POBN [POBN-CH(CH$_3$)OH adduct] is formed. When thylakoid membranes isolated from the PSI-less and PSI-less/*scp*ABCDE⁻ strains were illuminated in the presence of POBN, POBN-CH(CH$_3$)OH adduct EPR spectra were detected (Fig. 8a,b). We have previously demonstrated that in the thylakoid membranes, HO$^\bullet$ is produced mainly by PSI (Šnyrychová, Pospíšil & Nauš 2006; Khatoon *et al.* 2009). Because of the absence of PSI in the PSI-less and PSI-less/*scp*ABCDE⁻ strains, HO$^\bullet$ is expected to be produced predominantly by PSII. The observation that DCMU has no effect on HO$^\bullet$ production confirmed that HO$^\bullet$ is produced by PSII (data not shown). Figure 8c shows that no significant difference in HO$^\bullet$ production was observed between the PSI-less and PSI-less/*scp*ABCDE⁻ strains. Based on these observations we concluded that the absence of SCP has no effect on HO$^\bullet$ production during the whole period of illumination.

DISCUSSION

$^1O_2$ formation in the PSI-less strains and suppressive effect of SCPs

Singlet oxygen imaging by the green fluorescence of SOSG shows that $^1O_2$ is formed in the intact cells of both PSI-less and PSI-less/*scp*ABCDE⁻ strains exposed to high irradiance (Figs 4 & 5). Moreover, during the initial period of this exposure no significant difference in $^1O_2$ formation in thylakoid isolated from both strains was observed (Fig. 6c). These observations reveal that in the initial period of illumination $^1O_2$ formation in both strains proceeds via the

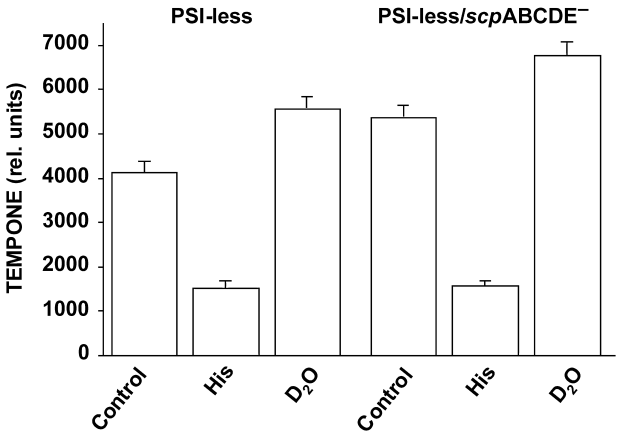

Figure 7. Effect of histidine and deuterium oxide on $^1O_2$ formation in the thylakoid membranes isolated from the photosystem I (PSI)-less and PSI-less/*scp*ABCDE⁻ *Synechocystis* cells. Light-induced 2, 2, 6, 6-tetramethyl-4-piperidone-1-oxyl (TEMPONE) electron paramagnetic resonance (EPR) spectra were measured in the thylakoid membranes isolated from the PSI-less and PSI-less/*scp*ABCDE⁻ *Synechocystis* cell in the absence and presence of 10 mM histidine and 50% deuterium oxide. Other experimental conditions were the same as in Fig. 4. The TEMPONE EPR signals observed in all samples also included the TEMPONE EPR signal caused by impurity of the spin trap compound.

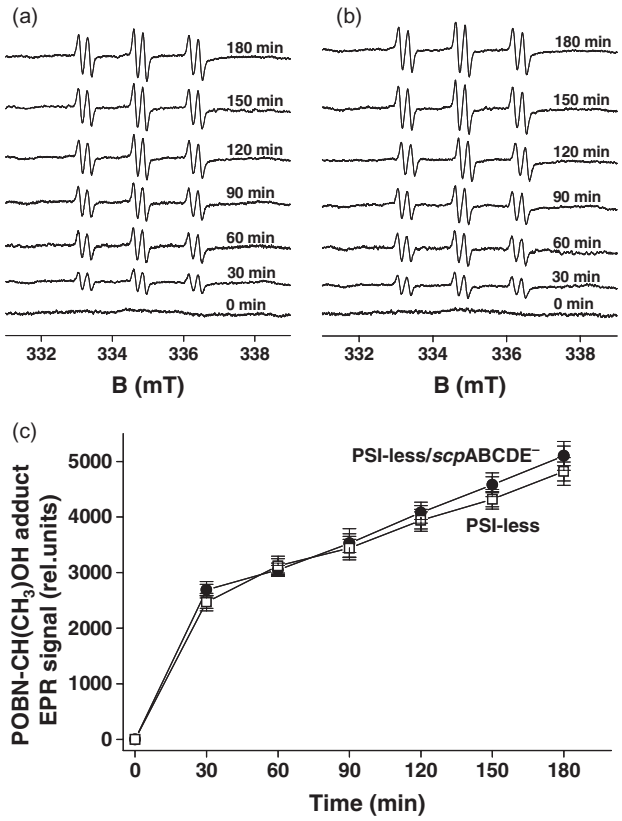

Figure 8. Hydroxyl radical formation in the thylakoid membranes isolated from the photosystem I (PSI)-less and PSI-less/*scp*ABCDE⁻ *Synechocystis* cells detected by electron paramagnetic resonance (EPR) spin-trapping spectroscopy. Light-induced 4-pyridyl-1-oxide-*N*-*tert*-butylnitrone (POBN) [POBN-CH(CH_3)OH adduct] spectra were measured in the thylakoid membranes isolated from the PSI-less (a) and PSI-less/*scp*ABCDE⁻ (b) *Synechocystis* cell. (c) Time profile of light-induced POBN-CH(CH_3)OH adduct EPR spectra measured in the thylakoid membranes isolated from the PSI-less (square) and PSI-less/*scp*ABCDE⁻ circle) *Synechocystis* cell. Thylakoid membranes [150 μg of chlorophyll (Chl) mL^{-1}] were exposed to high light intensity illumination (1000 μmol photons m^{-2} s^{-1}) in the presence of 50 mM POBN, 170 mM ethanol and 40 mM Mes (pH 6.5). Because of the instability of POBN-CH(CH_3)OH adduct (the adduct is stable up to 30 min), for period of illumination longer than 30 min thylakoid membranes were first pre-illuminated in the absence of spin trap system and then illuminated for additional 30 min in the presence of 50 mM POBN and 170 mM ethanol to complete the illumination period. The intensity of EPR signal was evaluated as the relative height of the central peak of the first derivative of the EPR absorption spectrum. Each data point represents the mean value of at least three measurements.

same reaction mechanisms comprising of either intersystem crossing from the weakly-coupled Chl in the antenna complexes or by charge recombination of the primary radical pair [$P680^+Pheo^-$] in the PSII reaction centre. In the antenna complexes, the strong coupling between Chl and carotenoid molecules requires that both molecules are within van der Waals distance i.e. the distance between π–systems is less than 4 Å (Edge & Truscott 1999; Polívka & Sundström 2004; Pogson, Rissler & Frank 2005). The three-dimensional crystal structure of PSII from thermophilic cyanobacteria *Thermosynechococcus elongatus* showed that only three Chls in the CP47 antenna (Chl 17, Chl 22, Chl 27) and two Chls in CP43 (Chl 17, Chl 22) are strongly coupled with β-carotene (Loll *et al*. 2005; Müh, Renger & Zouni 2008). It shows that majority of Chls in PSII might be a potential source of $^1O_2$, which we detected in cells of both strains. The data obtained by confocal microscopy showed only low percentage of *Synechocystis* cells with the fluorescence signal of SOSG detected in darkness in both strains. Following illumination the number of cells with SOSG fluorescence increased, especially in PSI-less/*scp*ABCDE⁻ strain (Fig. 5). Despite this, there was just a small fraction of cells exhibiting high fluorescence intensity in cells of both strains. We assume that this was caused by the limited amount of fluorochrome that could penetrate into the cells and react with $^1O_2$. The amount of $^1O_2$ above this limit could not be detected and was probably quenched by various cellular components. Conversely, in the presence of D_2O, the enhancement in the cells with SOSG fluorescence is most likely caused by the increase in permeability of plasmatic membrane leading to enhanced uptake of the fluorochrome. Similar effect was reported previously by Brink *et al.* who suggested that channel structure by deuterium/hydrogen exchange increases junctional membrane permeability for fluorescein (Brink 1983; Brink, Verselis & Barr 1984). The data obtained by EPR spin-trapping spectroscopy demonstrated that after prolonged illumination, $^1O_2$ formation in thylakoid membranes isolated from the PSI-less/*scp*ABCDE⁻ strain was enhanced (Fig. 6c). In these membranes, the extensive damage of the PSII complexes is expected to result in the release of Chls from their binding sites. These 'free' Chls cannot transfer their excitation energy and thus serve as highly potent photosensitizers generating triplet Chl by intersystem crossing. Thus, we propose that enhancement in $^1O_2$ formation observed in thylakoid membranes isolated from PSI-less/*scp*ABCDE⁻ strain after prolonged high light treatment is caused by the action of Chls released from damaged protein. These Chls emit low temperature fluorescence with maximum at shorted wavelengths than Chls bound to specific protein-binding sites. Indeed, in the PSI-less/*scp*ABCDE⁻ strain we observed a small fraction of such Chls and the relative contribution of this fraction to the overall Chl fluorescence significantly increased after strong illumination (Fig. 2b). In contrast, we believe that Chls released from damaged proteins in the PSI-less strain might be temporarily bound to SCP and therefore, no apparent contribution of shorter wavelengths to fluorescence emission is observed in cells of this strain even after illumination (Fig. 2a). The hypothesis is further supported by the observation that unlike $^1O_2$, there is no significant difference between the PSI-less and PSI-less/*scp*ABCDE⁻ strains in the production of $HO^\bullet$, another ROS generated by PSII (Fig. 8). To fulfil such a role effectively, SCPs have to be able to bind both Chls and carotenoids. Based on the sequence similarity between SCP and the first and third CAB

transmembrane helices of plant LHCII, SCPs have been proposed to exhibit a capability of binding both these pigments (Xu *et al*. 2004; Storm *et al*. 2008). Moreover, an *in vitro* study with ScpBE and ScpCD pairs showed that Chls might be coordinated to SCPs and fluorescence resonance energy transfer and circular dichroism indicated that Chls interact with carotenoids in the reconstituted SCP pairs (Storm *et al*. 2008). This interaction is essential for the effective quenching of triplet Chls by carotenoids and it seems likely that carotenoids are permanently bound, whereas Chls are just transiently coordinated to SCPs in the proper distance and orientation with respect to carotenoids. Finally, in line with putative binding of carotenoids to SCPs, Xu and coworkers (2004) showed that depletion of SCPs caused a decrease in the cellular carotenoid content of the SCP-less mutants.

SCPs protect against PSII protein damage

Singlet oxygen is able to cause extensive damage to proteins (Stadtman 1993). In the photoinactivated PSII, $^1O_2$ has been shown to primarily photo-oxidize the D1 protein as demonstrated by the shift of electrophoretic mobility of the D1 band (Komenda *et al*. 2002; Lupínková & Komenda 2004) and by the formation of the adduct with the α-subunit of cytochrome b-559 both *in vitro* and *in vivo* (Lupínková *et al*. 2002). In this study, the same shift in the mobility and increased accumulation of the D1-cytochrome b_{559} adduct was observed in high light-treated cell of the PSI-less/*scp*ABCDE$^-$ strain but not in the cells of the PSI-less strain. The PSI-less strain has been previously shown to accumulate SCPs even without exposure to light stress, most probably because of the sufficiently stressing effect of the missing PSI (Funk & Vermaas 1999; Promnares *et al*. 2006). Therefore, the observed difference in the high light response between both strains can be easily correlated with the presence of SCPs even in the presence of protein synthesis inhibitor. It is interesting that the higher level of $^1O_2$ observed in the PSI-less/*scp*ABCDE$^-$ strain did not lead to the faster inactivation of the PSII, even in the later stage of illumination. The 'free' Chls are assumed to be released from PSII only after its inactivation and therefore, their sensitization product, $^1O_2$, may affect only other active PSII complexes in the vicinity. However, it has been observed that externally produced $^1O_2$ does not cause functional inactivation of PSII (Nishiyama *et al*. 2004) and in agreement with this, the strain lacking SCPs does not exhibit a faster photoinactivation of PSII.

The high light treatment caused the large decrease in the level of the dimeric PSII complex RCC(2) in both strains. This decrease follows inactivation of electron transfer processes and reflects the early light-induced structural change, which is similar in both strains (Fig. 3, Table 1). The absence of mobility shift as well as the absence of the D1-cytochrome b_{559} adduct in RCC(2) is in agreement with general assumption that the dimeric PSII is the native functional form of PSII (Loll *et al*. 2005). In contrast, the major difference between both strains was observed in the RC47 complex. This complex is assumed to represent both the assembly intermediate during *de novo* assembly of PSII as well as the product of partial disassembly during the PSII repair cycle (Nixon *et al*. 2010). The complex accumulates in the later stages of high light treatment when the D1 replacement cannot occur because of inhibition of protein synthesis, absence of the protease or when the D1 protein is truncated at N-terminus or extensively oxidatively modified (for review see Nixon *et al*. 2010). The higher abundance of the D1-cytochrome b_{559} adduct and mobility shift of the D1 band in the RC47 complex of the PSI-less/*scp*ABCDE$^-$ strain is in a good agreement with increased generation of $^1O_2$ observed in thylakoids from this strain in the later stage of the high light treatment. SCPs are bound to the CP47 antenna of the PSII complex in the vicinity of the PsbH protein (Promnares *et al*. 2006) and this might indicate that Chls released from this antenna during the high light treatment will be the primary targets of SCPs. This fact may explain why the CP47 antenna is among the large Chl-binding PSII subunits with the lowest rate of light-induced damage and turnover (Komenda & Masojídek 1995). On the other hand, because of the primary damage localized at the D1 protein, we assume that SCPs should also be able to eliminate deleterious effects of Chls released from D1.

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