PhD Thesis

Molecular Mechanism of UV-B Damage and Repair of Photosynthetic Apparatus in Cyanobacterium Synechocystis sp. PCC 6803

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Abbreviations:

ATP	Adenosine triphosphate
BA	Benzyl alcohol
CP47, CP43	Protein subunits from the photosystem II complex
D1	Protein subunit of photosystem II
D2	Protein subunit of photosystem II
DCMU	[3-(3,4-dichlorphenyl)-1,1'-dimethylurea]
FADH	Reduced form of flavine adenine dinucleotide
GTP	Guanosine triphosphate
NADPH	Nycotinamide-adenine-dinucleotide phosphate reduced form
P_{680}	Primary electron donor in PSII
PBS	Phycobilisome
Pheo	Pheophytin
PQ	Plastoquinone
PSI,	Photosystem I
PSII	Photosystem II
Q_A, Q_B	Primary and secondary quinone electron acceptor of Photosystem II, respectively
RUBISCO	Ribulose-bisphosphate-carboxylase/oxygenase
UV-A	Ultraviolet light (320- 400 nm)
UV-B	Ultraviolet light (280- 320 nm)
UV-C	Ultraviolet light (200- 280 nm)

A cyanobacterial story

Once upon the time, the Earth was very different, the dark land was washed by the waters of an ocean very unlike the one we know. All life was practically in water, a water full of reduced components, chemical soup, toxic for us, but a sweet home for the few unicellular organisms especially microbes which were inhabiting it. It was a peaceful life, and the monotony of the life cycles was scarcely disturbed by electrical storms which were touching the surface of the ocean. And life was going on…until something had happened. On the thin layer of the surface where the light of the sun battled the eternal dark of the deep, a new living been emerged… and it was a very different one, one which was about to change all life on Earth as it was known at the time. Otherwise small and not significant, the little cells could have been easily pass unnoticed, but they were doing something no one had done before, and that was photosynthesis. They were living a new kind of life, taking power from the mighty sun and using the surrounding water they were able to go on with their lives and produce something which was changing the life on Earth, and that was oxygen. Slowly the waters around them were oxidized, poisoning and killing all the living beings. In the end oxygen emerged at the surface changing the composition of the atmosphere and opening the path for the new, aerobic, organisms to conquer the solid earth. And they were coming out, slowly and timid at the beginning since the life-giving sun was also a very dangerous one. Its spectrum was containing ultraviolet light and other short wavelength radiation, which was a killer combination for all life. But slowly that problem was solved also. As the cyanobacterial oxygen was accumulating and reaching the high atmospheric layers it was transformed into a new molecule, ozone, which was absorbing the dangerous UV light. This allowed life on Earth to develop, and evolve, using photosynthesis to stock the energy of the sun, and building a new world. And the life evolved until it reached us, grand-, grand-, grandchildren of the small unicellular cyanobacteria of which we completely forgot. We conquered the Earth and used it for our interest and for developing our society, little thinking on the consequences. In the end we realized that our actions are destroying our home and life on it. We realized that the celestial barrier that keeps us safe from the dangerous UV radiations, the ozone layer, is getting thin, and realized that together with it we are perishing too. Then, we, so big, so clever and so powerful have turned again to the smallest of all photosynthetic organisms, cyanobacteria, to learn how the miracle (of photosynthesis) is happening and how to protect ourselves. And there we are now, and that's what this PhD. thesis is about.

1 Introduction

1.1 General Considerations

Solar radiation is not only the vital source of energy for the biosphere on our planet but also acts as an adverse environmental factor for various forms of life. The efficiency of light induced damage increases with decreasing the wavelength.

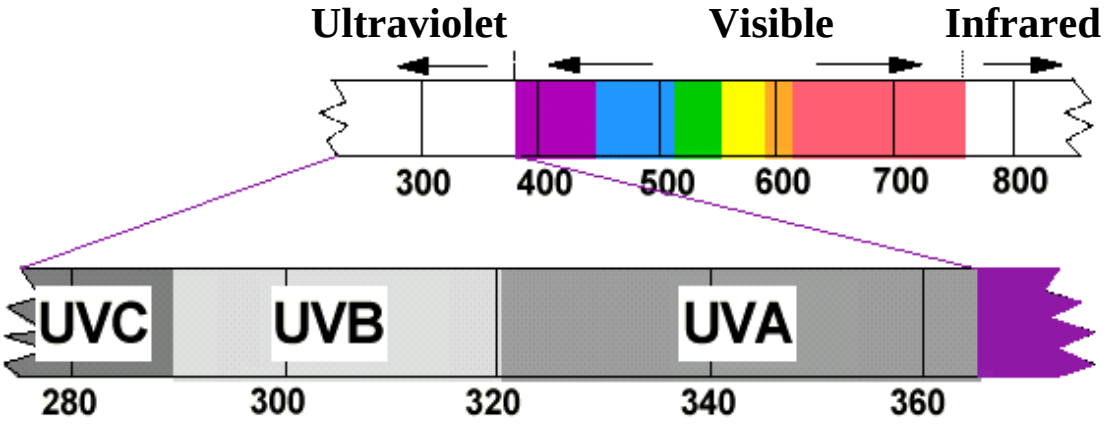

Figure 1. Segment of the electromagnetic spectrum that is relevant from photosynthetic point of view.

Photosynthetically relevant solar radiation that reaches the Earth (Fig. 1) is divided into three main spectral regions: UV-B (280-320 nm), UV-A (320-400nm) and photosynthetically active radiation, PAR (400-700 nm). Among those, the UV-B region is selectively attenuated by the stratospheric ozone layer (Green et al. 1974; Madronich 1993). On the contrary, UV-A and PAR radiation have no selective absorber and are affected mainly by light scattering. The biologically most damaging wavelengths below 280 nm, such as those of the UV-C (200-280 nm) region, are absorbed almost completely by the atmosphere and, therefore, are insignificant for biological processes under natural conditions (Fig. 2).

UV radiation, terrestrial life, and ozone depletion have a notable relationship to oxygenic photosynthesis. The present-day ozone shield that protects terrestrial life from UV-B radiation is formed in the stratosphere from oxygen by short wavelength (< 242 nm) UV radiation. In the prebiotic phase of the Earth's evolution, the atmosphere contained insignificant amounts of oxygen and ozone, thus damaging short wavelength radiation could reach the surface without significant attenuation. It seems probable that primary life forms developed in oceans protected by the water layer against the UV radiation. After developing of the oxygen evolving capacity by photosynthesizing bacteria, biotically produced oxygen

slowly accumulated in the atmosphere and was partly converted into ozone in the upper layers by the UV radiation. The gradually formed ozone layer served as a protective shield against the biologically damaging UV-B radiation and made it possible for marine life forms to conquer terrestrial habitats. Paradoxically, it seems that the site of oxygen production in plants, the water-oxidation complex, is one of the main targets of UV radiation. Recent reduction of the stratospheric ozone layer, due to industrial activities (Molina and Rowland 1974; Stolarski et al. 1992; Kerr and McElroy 1993), leads to an increased level of UV-B radiation reaching the Earth's surface and ecologically significant depths of the oceans, with negative effects in all life forms.

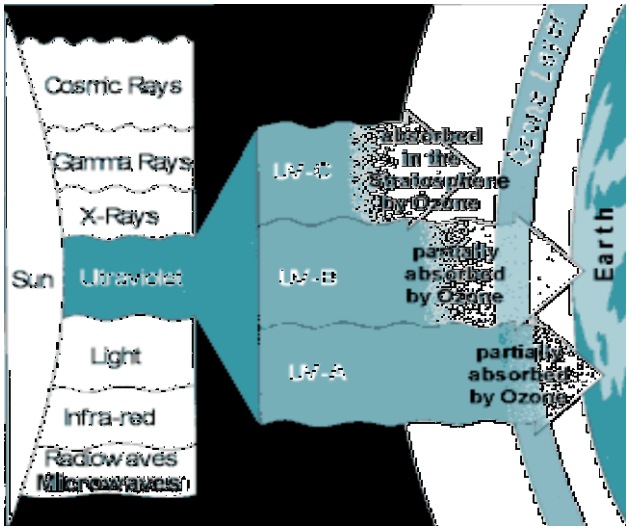

Figure 2. Differential absorption of UV radiation by Earths atmosphere and ozone layer.

Cyanobacteria are photosynthetic prokaryotic organisms that evolve O_2 (Bryant, 1994). Fossil evidence indicates that cyanobacteria existed over 3 billion years ago and it is thought that they were the first oxygen evolving organisms on earth (Wilmotte, 1994). Cyanobacteria are presumed to have evolved in water in an atmosphere that lacked O_2. Initially, the O_2 released by cyanobacteria reacted with ferrous iron in the oceans and was not released into the atmosphere. Geological evidence indicates that the ferrous Fe was depleted around 2 billion years ago, and earth's atmosphere became aerobic. The release of O_2 into the atmosphere by cyanobacteria has had profound effects on the evolution of life. One of the most important of these effects is the formation of the stratospheric ozone layer.

The photosynthetic apparatus of cyanobacteria is similar to that of chloroplasts. The main difference is in the antenna system. Cyanobacteria depend on chlorophyll a and specialized protein complexes (phycobilisomes) to gather light energy (Sidler, 1994). They do

not contain chlorophyll b. As in chloroplasts, the chlorophyll a is located in membrane bound proteins. The phycobilisomes are bound to the outer side of the photosynthetic membrane and act to funnel exciton energy to the photosystem II reaction center. They are composed of phycobiliproteins, protein subunits that contain covalently attached open ring structures known as bilins that are the light absorbing pigments. Primary photochemistry, electron transport, phosphorylation and carbon reduction occur very similarly as in chloroplasts. Cyanobacteria have a simpler genetic system than plants and algae that enable them to be easily modified genetically. Because of this cyanobacteria have been used as a model to understand photosynthesis in plants. By genetically altering photosynthetic proteins, researchers can investigate the relationship between molecular structure and mechanism (Barry et al., 1994).

In plants, the thylakoid membrane is asymmetrically arranged (Albertson 1995). It is differentiated into grana and stroma lamellae, the stacked and unstacked regions, respectively. These regions have different biochemical compositions and functions. In contrast, cyanlobacteria lack this heterogeneity of the tylakoid membrane. The major light-harvesting complex in cyanobacteria and red algae is the extrinsic phycobilisome, which is usually connected to PSII (MacColl 1998). It is composed of phycobiliproteins and linker polypeptides. The composition vary among the organisms and also within individual organism in response to environmental factors. Cyanobacteria contain sub-cellular structures, inclusion bodies, for storage of carbon, nitrogen, phosphates and lipids. RUBISCO is located in carboxysomes, one of the inclusion bodies.

1.2 Basics of Photosynthesis

Photosynthesis is the physico-chemical process by which plants, algae and photosynthetic bacteria use light energy to drive the synthesis of organic compounds. In plants, algae and certain types of bacteria, the photosynthetic process results in the release of molecular oxygen and the removal of carbon dioxide from the atmosphere that is used to synthesize carbohydrates (oxygenic photosynthesis). Other types of bacteria use light energy to create organic compounds but do not produce oxygen (anoxygenic photosynthesis). Photosynthesis provides the energy and reduced carbon required for the survival of virtually all life on our planet, as well as the molecular oxygen necessary for the oxygen consuming organisms. In addition, the fossil fuels currently being burned to provide energy for human activity were produced by ancient photosynthetic organisms. Although photosynthesis occurs

in cells or organelles that are typically only a few microns across, the process has a profound impact on the earth's atmosphere and climate. Each year more than 10% of the total atmospheric carbon dioxide is reduced to carbohydrate by photosynthetic organisms. Most, if not all, of the reduced carbon is returned to the atmosphere as carbon dioxide by microbial, plant and animal metabolism, and by biomass combustion. In turn, the performance of photosynthetic organisms depends on the earth's atmosphere and climate. Over the next century, the large increase in the amount of atmospheric carbon dioxide created by human activity is certain to have a profound impact on the performance and competition of photosynthetic organisms. Knowledge of the physico-chemical process of photosynthesis is essential for understanding the relationship between living organisms and the atmosphere and the balance of life on earth

The overall equation for photosynthesis is deceptively simple. In fact, a complex set of physical and chemical reactions must occur in a coordinated manner for the synthesis of carbohydrates. To produce a sugar molecule such as sucrose, plants require nearly 30 distinct proteins that work within a complicated membrane structure. Research into the mechanism of photosynthesis centers on understanding the structure of the photosynthetic components and the molecular processes that use radiant energy to drive carbohydrate synthesis. It involves several disciplines, including physics, biophysics, chemistry, structural biology, biochemistry, molecular biology and physiology, and serves as an outstanding example of the success of multidisciplinary research.

1.3 Principles of Photosynthetic Energy Transformation

The energy that drives photosynthesis originates in the center of the sun. A small fraction of the visible light incident on the earth is absorbed by plants. Through a series of energy transducing reactions, photosynthetic organisms are able to transform light energy into chemical free energy in a stable form that can last for hundreds of millions of years (e.g. fossil fuels).

The photosynthetic process in plants and algae occurs in small organelles known as chloroplasts that are located inside the cells. The more primitive photosynthetic organisms, for example oxygenic cyanobacteria, prochlorophytes and anoxygenic photosynthetic bacteria, lack organelles. The photosynthetic reactions are traditionally divided into two stages - the "light reactions," which consist of electron and proton transfer events and the "dark reactions," which consist of the biosynthesis of carbohydrates from CO_2. The light

reactions occur in a complex membrane system (the photosynthetic membrane) that is made up of protein complexes, electron carriers, and lipid molecules. The photosynthetic membrane is surrounded by water and can be thought of as a two-dimensional surface that defines a closed space, with an inner and outer water phase. The protein complexes embedded in the photosynthetic membrane have a unique orientation with respect to the inner and outer phase (Fig. 3). The asymmetrical arrangement of the protein complexes allows some of the energy released during electron transport to create an electrochemical gradient of protons across the photosynthetic membrane.

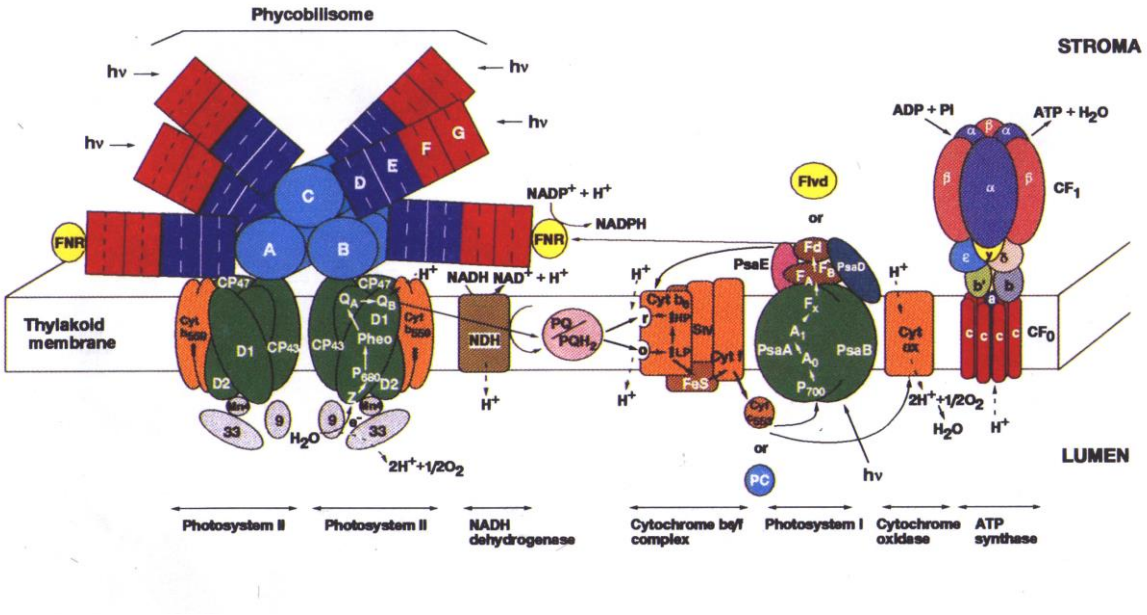

Figure 3. Diagram showing the major respiratory and photosynthetic electron transport components of cyanobacteria.

The light reactions convert energy into several steps (Fig.4). The first step is the conversion of a photon to an excited electronic state of an antenna pigment molecule located in the antenna system. The antenna system consists of hundreds of pigment molecules (mainly chlorophyll or bacteriochlorophyll and carotenoids) that are anchored to proteins within the photosynthetic membrane which form a specialized complex known as a reaction center. The electronic excited state is transferred over the antenna molecules as an exciton. Some excitons are converted back into photons and emitted as fluorescence, some are converted to heat, and some are trapped by a reaction center. Excitons trapped by a reaction center provide the energy for the primary photochemical reaction of photosynthesis - the transfer of an electron from a donor molecule to an acceptor molecule. Both the donor and acceptor molecules are

attached to the reaction center protein complex (Fig. 3). Once primary charge separation occurs, the subsequent electron transfer reactions are energetically downhill.

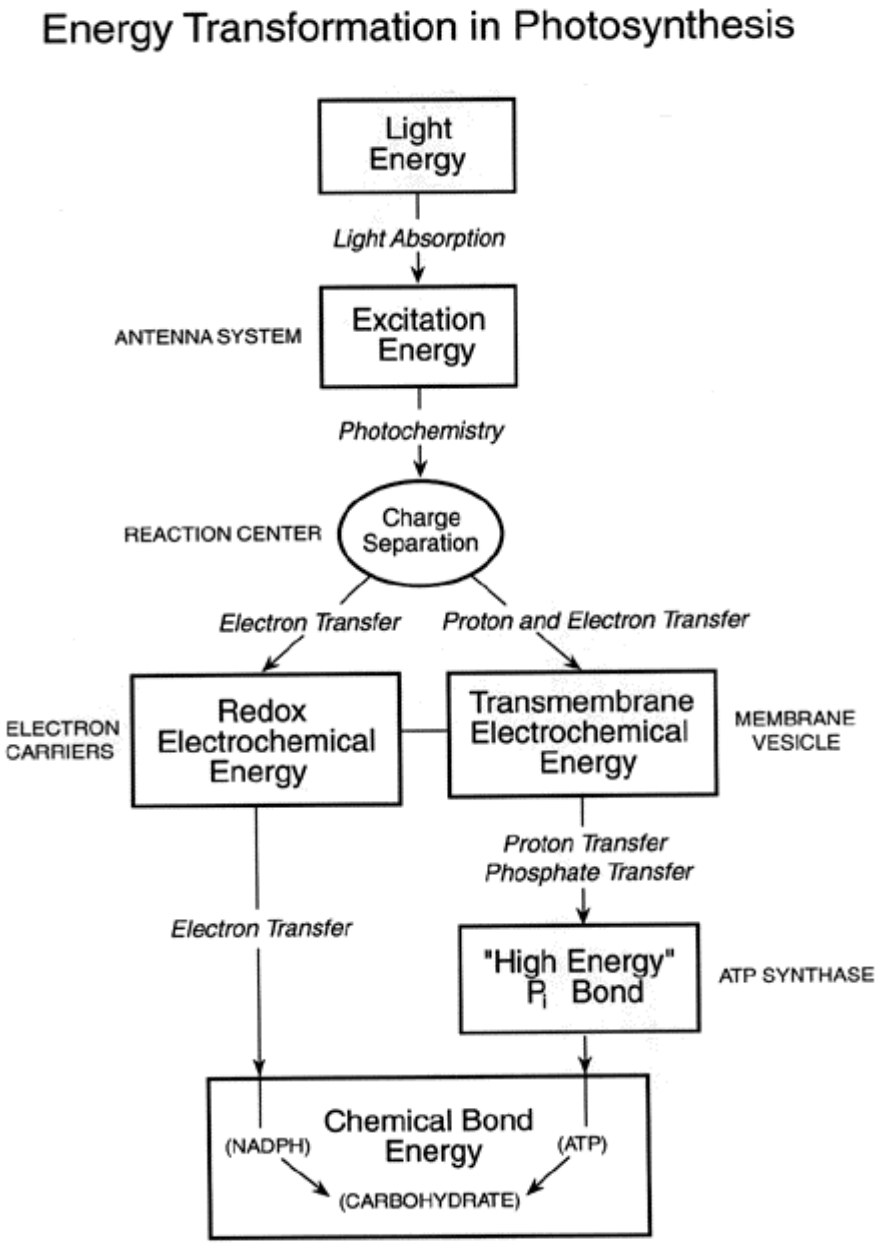

Figure 4. The scheme of the energy transformation in photosynthesis, from the absorption of light to the formation of chemical bonds in carbohydrates.

In oxygenic photosynthetic organisms, two different reaction centers, known as photosystem II and photosystem I, work concurrently but in series. In the light photosystem II feeds electrons to photosystem I, via intermediate carriers. The net reaction is the transfer of electrons from a water molecule to $NADP^+$, producing the reduced form, NADPH (Fig. 3). In the photosynthetic process, much of the energy initially provided by light energy is stored as redox free energy in NADPH, to be used later in the reduction of carbon. In addition, the

electron transfer reactions concentrate protons inside the membrane vesicle and create an electric field across the photosynthetic membrane. In this process the electron transfer reactions convert redox free energy into an electrochemical potential of protons. The energy stored in the proton electrochemical potential is used by a membrane bound protein complex (ATP-Synthase) to covalently attach a phosphate group to adenosine diphosphate (ADP), forming adenosine triphosphate (ATP). Protons pass through the ATP-Synthase protein complex that transforms electrochemical free energy into a type of chemical free energy known as phosphate group-transfer potential (or a high-energy phosphate bond) (Klotz, 1967) (Fig. 3). The energy stored in ATP can be transferred to another molecule by transferring the phosphate group. The net effect of the light reactions is to convert radiant energy into redox free energy in the form of NADPH and phosphate group-transfer energy in the form of ATP (Fig. 4).

1.4 Light Absorption - The Antenna System

Plant photosynthesis is driven primarily by visible light (wavelengths from 400 to 700 nm) that is absorbed by pigment molecules (mainly chlorophyll a and b and carotenoids) (Fig. 5). In chlorophyll b, the CH_3 in ring II is replaced by CHO group. Plants appear green because of chlorophyll, which is so plentiful that whole regions of the earth appear green from space. Light is collected by 200-300 pigment molecules that are bound to light- harvesting protein complexes located in the photosynthetic membrane. The light-harvesting complexes surround the reaction centers that serve as an antenna. The three-dimensional structure of the light-harvesting complex (Kühlbrandt et al., 1994) shows that the protein determines the position and orientation of the antenna pigments.

The light-harvesting complexes of photosystem II of cyanobacteria (and red algae) are the phycobilisomes (PBS), large multi-protein complexes associated with the cytoplasmic stroma side of the thylakoid membrane. PBS are primarily composed of phycobiliproteins, a brilliantly colored family of water soluble proteins bearing covalently attached, open-chain tetrapyrolles known as phycobilins. In addition, phycobilisomes also contain smaller amounts of “linker polypeptides”, most of which do not bear chromophores. These components are absolutely required for proper assembly and functional organization of the structure. Phycobilisomes are constructed from two main structural elements: a core substructure and peripheral rods that are arranged in a hemidiscoidal fashion around that core.

The core of most hemidiscoidal phycobilisomes is composed of three cylindrical subassemblies. The peripheral rods radiate from the lateral surfaces of the core substructure, which are not in contact with the tylakoid membrane. Absorbed light energy is transferred by

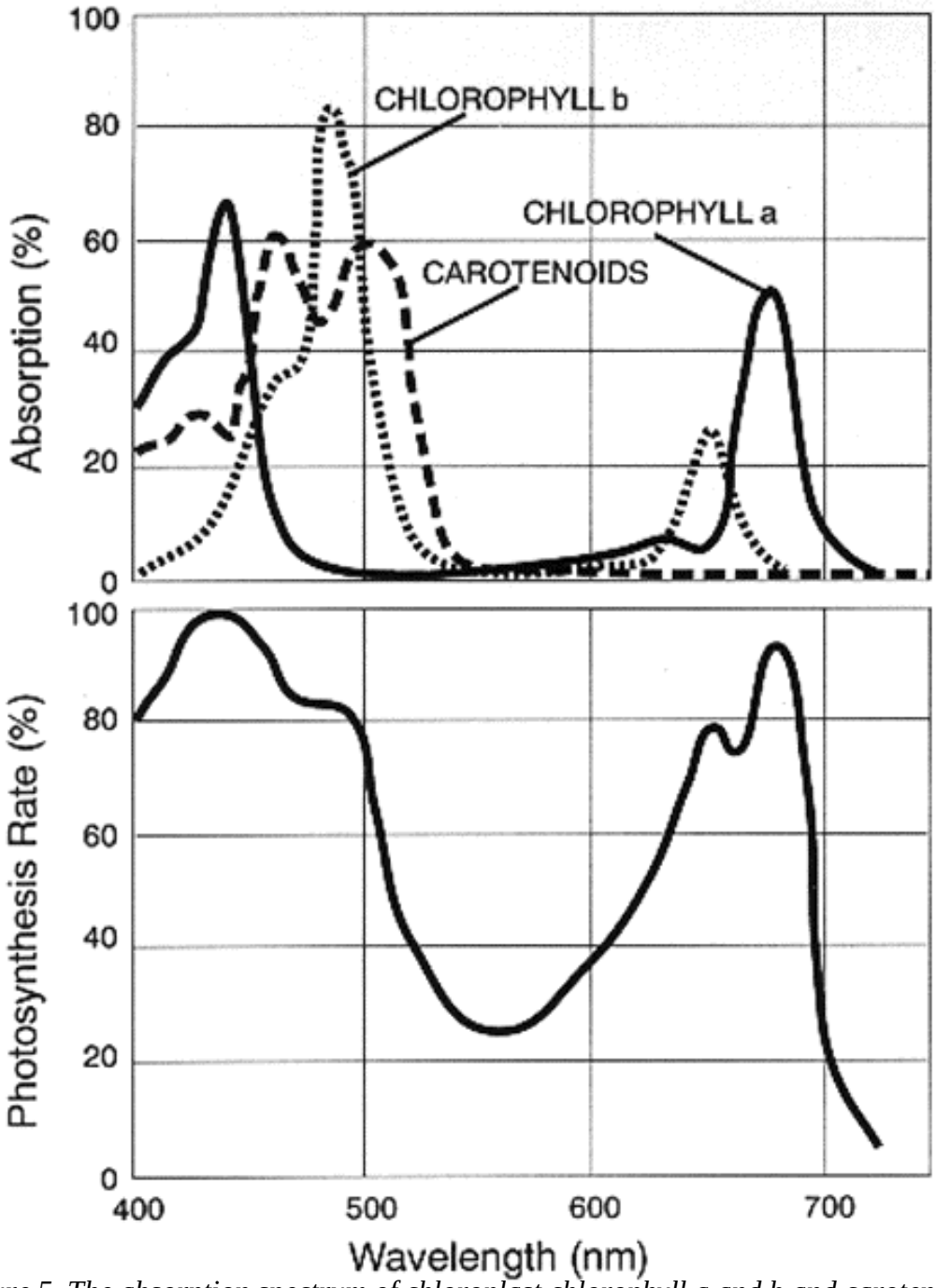

Figure 5. The absorption spectrum of chloroplast chlorophyll a and b and carotenoids (upper panel) along with the action spectrum of photosynthesis of a chloroplast (lower panel).

very rapid radiation-less downhill energy transfer from phycoerythrin or phycoerythrocyanin (if present) to C-phycocyanin and then to allophycocyanin species that

act as the final energy transmitters from the phycobilisome to the PSII or (partially) PSI reaction centers.

1.5 Primary Photochemistry - Photosystem II Reaction Center

Photosystem II uses light energy to drive two chemical reactions - the oxidation of water and the reduction of plastoquinone. The photosystem II complex is composed of more than twenty polypeptides and at least nine different redox components (chlorophyll, pheophytin, plastoquinone, tyrosine, Mn, Fe, cytochrome b559, carotenoid and histidine) have

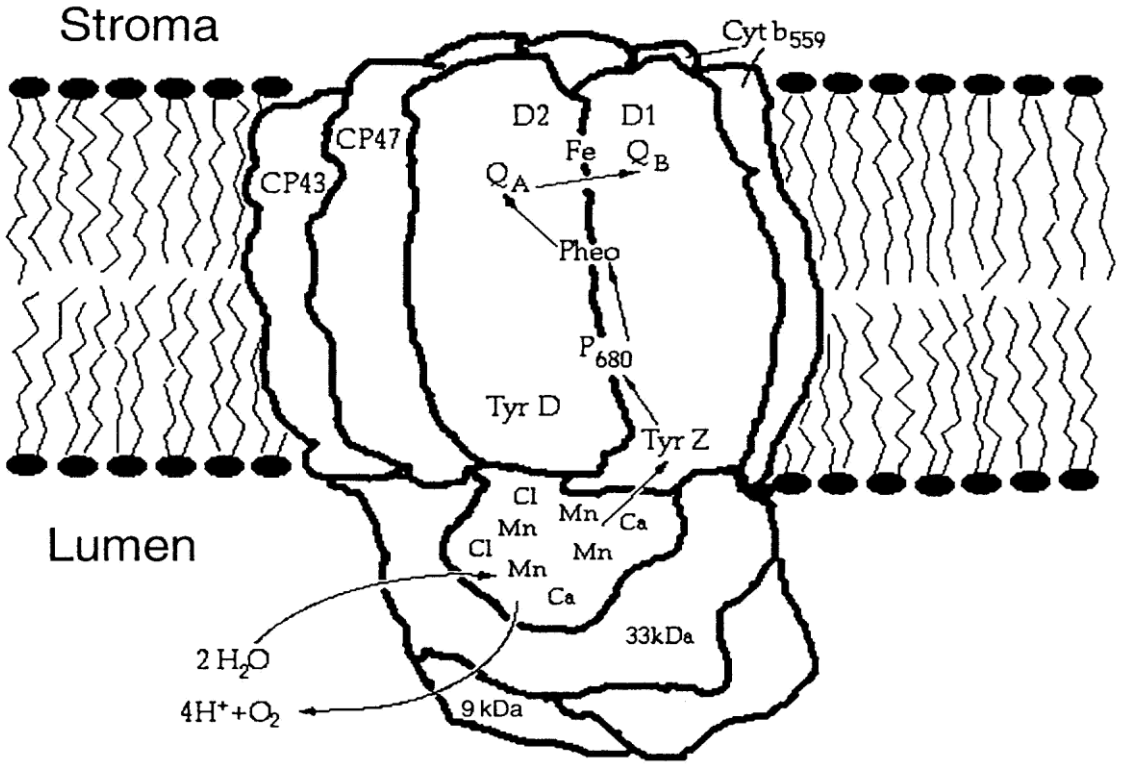

Figure 6. Schematic illustration of PSII protein complex. The direction of electron transport among the cofactors is indicated by arrows.

been shown to participate in light-induced electron transfer (Debus, 1992) (Fig. 6).
These essential redox components are bound to or contained by two key polypeptides that form the heterodimeric reaction center core of photosystem II (D1 and D2). There is also indication that the D1 polypeptide provide ligands for the $(Mn)_4$ cluster (Debus 2001; Zouni et al. 2001).

Photochemistry in photosystem II is initiated by charge separation between P680 and pheophytin, creating $P680^+/Pheo^-$. Primary charge separation takes about a few picoseconds (Fig. 7). Subsequent electron transfer steps have been designed through evolution to prevent

the primary charge separation from recombining. This is accomplished by transferring the electron within 200 picoseconds from pheophytin to a plastoquinone molecule (Q_A) that is permanently bound to photosystem II.

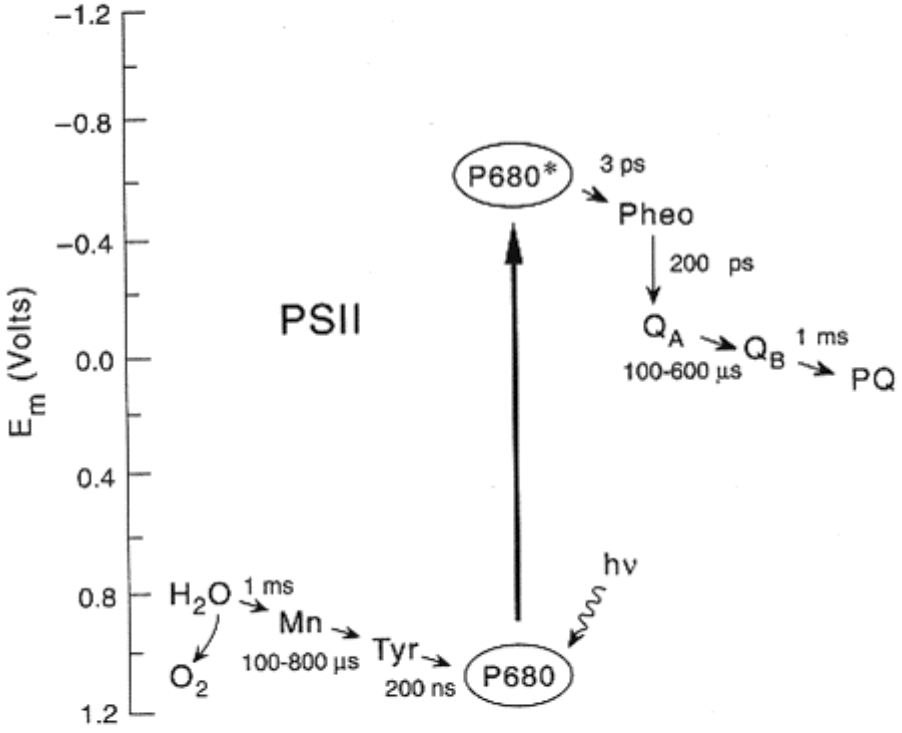

Figure 7. Light driven photochemical reactions of photosystem II. The different redox components taking part in the reactions are positioned according to their redox potentials. The time range of the reactions is shown at the respective arrows.

Although plastoquinone normally acts as a two-electron acceptor, it works as a one-electron acceptor at the Q_A-site. The electron on Q_A^- is then transferred to another plastoquinone molecule that is loosely bound at the Q_B-site. Plastoquinone at the Q_B-site differs from Q_A in that it works as a two-electron acceptor, becoming fully reduced and protonated after two photochemical turnovers of the reaction center. The full reduction of plastoquinone requires the addition of two electrons and two protons. The reduced plastoquinone then debinds from the reaction center and diffuses into the hydrophobic core of the membrane. After which, an oxidized plastoquinone molecule finds its way to the Q_B-binding site and the process is repeated. Because the Q_B-site is near the outer aqueous phase, the protons added to plastoquinone during its reduction are taken from the outside of the membrane.

Photosystem II is the only known protein complex that can oxidize water, resulting in the release of O_2 into the atmosphere. Despite years of research, little is known about the molecular events that lead to water oxidation. Energetically, water is a poor electron donor. The oxidation-reduction midpoint potential of water is +0.82 V (pH 7). In photosystem II this reaction is driven by the oxidized reaction center, $P680^+$ (the midpoint potential of $P680/P680^+$ is estimated to be +1.2 V at pH 7). $P680^+$ oxidizes tyrosine Tyr-Z on the D1 protein which extracts an electron from the tetramanganese cluster which forms the catalytic site of the water oxidizing complex (Zouni et al. 2001). X-ray absorption spectroscopy shows that Mn undergoes light-induced oxidation. Water oxidation involves two molecules of water and requires four sequential turnovers of the reaction center. This was shown by an experiment demonstrating that oxygen release by photosystem II occurs with a periodicity of four (Joliot et al., 1969; Joliot and Kok, 1975). Each photochemical reaction creates an oxidant that removes one electron. The net reaction results in the release of one O_2 molecule, the deposition of four protons into the inner water phase, and the transfer of four electrons to the Q_B-site (producing two reduced plastoquinone molecules) (Renger, 1993; Klein et al., 1993; and Lavergne and Junge , 1993).

1.6 The D1 polypeptide.

The D1 polypeptide is one of the most important subunits of the PSII protein complex, which together with D2 polypeptide form the core complex of PSII (Fig. 6). Also, D1 has a central role in the functionality of PSII by totally or partially harboring components like Q_B, TyrZ, P_{680}, pheophytin and the Mn cluster (Fig. 8). D1 also plays an important role in regulation and protection of PSII. In plants, the 32kDa D1 polypeptide is synthesized on tylakoid membrane bound polysomes, and insertion into the membrane occurs in a co-translational manner (Klein et al. 1988; Kim et al. 1991). In addition, it appears that D1 is co-translationally assembled with other PSII polypeptides like D2 or CP47 (Zhang et al. 1999). In most oxygenic photoautotrophs the D1 polypeptide is synthesized with a 8 to 10 amino acid C-terminal extension that is removed later in order to form the mature D1 (Marder at al. 1984; Mattoo and Edelman 1987;Takahashi et al. 1988; Nixon et al. 1992; Jansson and Maenpaa 1997). For cyanobacteria and red algae the extension is 16 amino acids long. The D1 precursor has to be processed to assemble into a functional PSII (Diner et al. 1988; Nixon et al. 1992; Anbudurai et al. 1994; Fujita et al. 1995; Olemuller et al. 1996; Trost et al. 1997).

The three dimensional structure at 1.8 Å has been resolved for the protease from the green algae *Scenedesmus obliquus* (Liao et al. 2000). Other post-translational modifications of the D1 protein in plants are NH_2-processing, N-acetylation of the threonine residue in position 2 (Michel et al. 1988; Sharma et al. 1997), phosphorylation (Rintamaki and Aro 2001) and transient palmitolyation (Mattoo and Edelman 1987). These characteristics of the D1 protein indicate that it has a central functional and regulatory role in PSII.

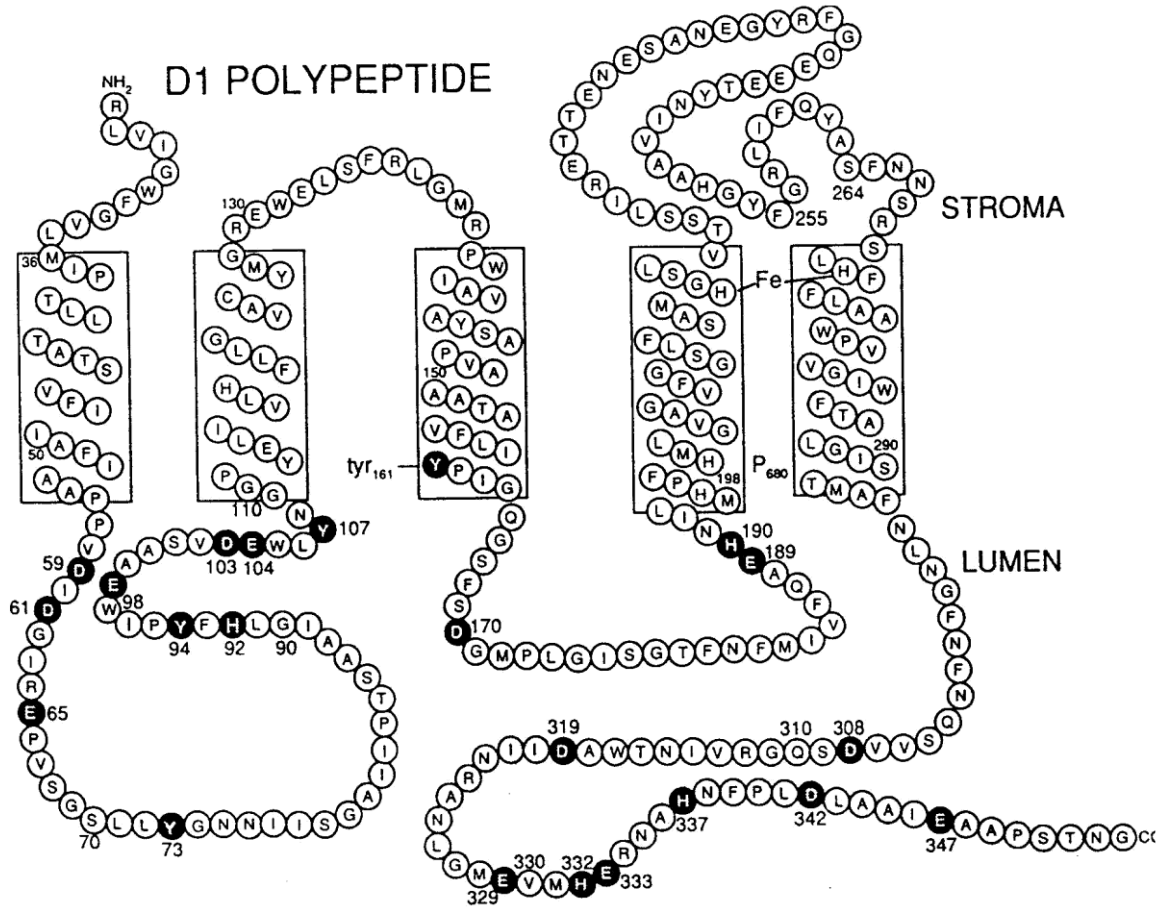

Figure 8. Aminoacid sequence and folding pattern for the D1 plypeptide. The potential Mn-cluster binding sites are highlighted.

The D1 polypeptide is encoded by the *psbA* gene. In plants and algae *psbA* is located in the plastome, in most cases as a single copy gene. In contrast, the cyanobacterial *psbA* gene belongs to small multigene families (Mohamed et al. 1993; Golden 1994).

In the cyanobacterium *Synechocystis* 6803 the psbA gene family contains three members, psbA1, psbA2 and psbA3 (Jansson et al. 1987; Jansson et al. 1998). The psbA1 gene is silent and its presence is enigmatic (Mohamed et al. 1993; Jansson et al 1998). The psbA2 and psbA3 genes encode identical proteins, and their coding regions are 99% similar

(Ravnikar et al. 1989; Metz et al 1990). They are, however, differentially expressed in a light dependent manner (Mohamed and Jansson 1989, 1991; Mohamed et al. 1993).

There is a significant difference between cyanobacteria and higher plants concerning the regulation of the function of the psbA genes. The main regulation of psbA gene expression in cyanobacteria is at the transcriptional level (Golden 1994; Jansson et al. 1998). In contrast, chloroplast regulation is mainly post-transcriptional at the level of translational initiation (Somanchi and Mayfield 1999). In addition, it seems that D1 translation elongation and membrane insertion are performed in a regulated manner for both cyanobacteria (Tyystjarvi et al. 2001) and chloroplasts (Choquet and Vallon 2000; Andersson and Aro 2001). The target for the ribosome-nascent D1 complexes in chloroplasts seems to be mediated by the binding to the signal recognition particle cpSRP54 (Nilsson et al. 1999). Pigment availability seems important in the assembly process for the stability of the D1 polypeptide, and it appears that the assembly of the pigments precedes the C-terminal processing and assembly in a functional PSII reaction center (Kim et al. 1994; He and Vermaas 1998; Muller and Eichacker 1999).

The damages at the PSII level during photoinhibition are targeted mainly to the reaction centre polypeptide D1 (Sharma et all 1997). The damaged polypeptide has to be removed and replaced by newly synthesized form. The removal of the D1 polypeptide is most probable triggered by a conformational change within PSII that is related to the occupancy of the Q_B- binding site in the case of acceptor side induced photoinactivation (Anderson and Aro 2001). This is followed by proteolytic degradation. In addition, in *Synechocystis* 6803 there seems to be a correlation between D1 degradation and the availability of newly synthesized D1 polypeptide and with *psbA* gene transcription (Komenda et al. 2000; Tyystjarvi et al. 2001)

The Photosystem II D1 protein in cyanobacteria is encoded by the psbA multigene family (Golden 1995). The cyanobacterium Synechocystis PCC 6803 contains two functional genes psbA2 and psbA3 encoding proteins with the same amino acid sequence (Jansson et al. 1987) while in Synechococcus PCC 7942 there are three genes psbA1, psbA2 and psbA3 encoding two different forms of the D1 protein (Golden et al. 1986). As a response to increased irradiance, the „low light“ D1:1 form, which is encoded by psbA1 is depleted from the cells and it is replaced by the „high light“ D1:2 form encoded by psbA2 and psbA3 (Schaefer and Golden 1989, Clarke et al. 1993). On the other hand, when the high light-treated cells are transferred back to low irradiance, D1:2 is very quickly replaced by D1:1

(Bustos et al. 1990). Similar exchange of the two D1 forms occurs after exposure to UV-B radiation.

1.7 UV-B and Visible Damage of Photosystem II

1.7.1 Mechanism of Photoinhibition and Recovery of PSII

Loss of the photosynthetic activity induced by light energisation in excess of the potential of the photosynthetic electron transport chain is usually referred to as photoinhibition. The process occurs both *in vivo* as well as in isolated, tylakoids and submembrane particles. Photoinhibition is a mutistep process including photoinctivation of reaction centre II and break-down of its D1 and to some extent the D2 polypeptides. Photoinactivation includes reversible and irreversible stages. Recovery of photosynthetic activity depends on the extent of damage attained prior to return of the experimental material to recovery conditions, as well as on the nature of the experimental system. Complete recovery can be obtain only *in vivo* and is related to *de novo* synthesis and replacement of PSII polypeptides degraded during the process.

PSII was demonstrated to be the major target in light-induced damage of the photosynthetic apparatus (Krause et al. 1990). However experimental results indicate that PSI can be affected as well, apparently by inactivation of Fe-S center (Sonoike et al. 1994; 1995; 1998). Photoinhibition is a complex phenomenon, comprising various phases, some of which overlap and are thus difficult to resolve. According to general views, visible light can inactivate PSII by two different mechanisms, which are called acceptor side and donor side-induced photoinhibition (Aro et al. 1993; Barber and Andersson 1992; Prasil et al. 1992).

1.7.1.1 Acceptor Side-Induced Photoinhibition of PSII.

When photosynthetic systems are subjected to light intensities that exceed the saturation level of the photosynthetic electron transport, the quantum yield of oxygen evolution decreases (Powles 1984). The majority of the available results suggests that this photoinduced inhibition is related to the sequential modification of the PSII acceptor side at the level of the O_A and/or Q_B acceptors. The main steps in this modification are:

1. Inhibition of electron transfer from Q_A to Q_B (Ohad et al. 1990; Vass et al. 1992), most likely due to lack of reducible plastoquinone molecules under the strong

illumination that keeps the plastoquinone pool and Q_B fully reduced (Vass et al. 1992)

2. Formation of highly stable, singly reduced and doubly reduced forms of Q_A, which are most likely neutralized by protonation (Vass et al. 1992; Setlik et al. 1990; Styring et al. 1990).
3. Release of doubly reduced and protonated Q_AH_2 from its binding site (Vass et al. 1992; Styring et al. 1990; Van Mieghem et al. 1989; Koivuniemi et al. 1993).
4. Modification or release of the nonheme iron located between the Q_A and Q_B acceptors at the interface of the D1 and D2 proteins (Gleiter et al. 1992; Vass et al. 1995).

An important consequence of the acceptor side modification of the PSII is the light induced production of the triplet state of the reaction center chlorophyll, $^3P_{680}$, which is observed in the presence of stable reduced, neutralized Q_A forms and after the release of Q_AH_2 (Vass et al. 1992; Vass and Styring 1992; Vass and Styring et al. 1993; Kirilovsky et al. 1994; Vass et al. 1993). $^3P_{680}$ strongly interacts with oxygen (Vass and Styring 1992; Durrant et al. 1990), and this interaction has been suggested to lead to the formation of highly reactive singlet oxygen, which can damage the protein structure in the vicinity of its formation site (Vass et al. 1992; Vass and Styring 1992; Durrant et al. 1990). Light induced $^1O_2$ oxygen production was indeed detected by the method of EPR spin trapping in photoinhibited thylakoid membranes (Hideg et al. 1994a; Hideg et al. 1994b) as well as in isolated reaction center complexes of PSII using chemical trapping (Telfer et al. 1994a) or measuring near-infrared luminescence (Macpherson et al. 1993; Telfer et al. 1994b).

Photoinhibition of PSII electron transport is followed by degradation of the D1 reaction center protein. Under the condition of acceptor side induced photoinhibition, D1 degradation is blocked by anaerobic conditions (Vass et al. 1992; Hundall et al. 1990) and by low temperatures (0-4 C) (Richter et al. 1990; Kettunen et al. 1991) and retarded by serine-type protease inhibitors (Virgin et al. 1991; Misra et al. 1991; Virgin et al. 1992). These observations indicate that D1 degradation is a two-step process. In the first step, the protein is damaged by photochemical events occurring during illumination of acceptor side-impaired PSII (Richter et al. 1991; Aro et al. 1991), whereas in the second step, the damaged protein is degraded by serine-type proteolytic activity (Virgin et al. 1991; Misra et al. 1991; Virgin et al. 1992). Since D1 degradation is related to singlet oxygen formation (Vass et al. 1992; Mishra et al. 1994; Mishra and Ghanotakis 1994), it seems likely that the protein damage is initiated

by singlet oxygen attack on crucial residues, like histidines (Hideg et al. 1994a; Mishra et al. 1994) or β-carotenes (Telfer et al. 1994b).

Characteristic degradation products of the D1 protein under acceptor side-induced photoinhibition are 23 kDa N-terminal (De La Rivas et al. 1992; Greenberg et al. 1987) and 10 kDa C-terminal fragments (De La Rivas et al. 1992). These fragments locate the site of primary cleavage in the stroma-exposed loop between the fourth and fifth helices (De La Rivas et al. 1992; Greenberg et al. 1987). A further degradation product is a 16 kDa C-terminal fragment that indicates a cleavage at the lumen-exposed loop between the third and fourth helices (Virgin et al. 1990).

Even though it has been possible to identify fragments produced after primary cleavage it has been difficult to identify the proteases involved in the degradation of the D1 polypeptide and the others PSII proteins. The proteolytic activity is associated with PSII (Anderson and Aro 2001) and was early suggested to be autoproteolytic (Shipton and Barber 1991). CP43 has been one candidate for proteolysis of D1 (Salter et al. 1992). Other possible candidates, like a Clp-type protease, have also been proposed or correlated to photoinhibition (Mattoo et al. 1999; Choquet and Vallon 2000). The protease seems to be serine type in both cyanobacteria and plants (Misra et al. 1991; Virgin et al. 1991; Komenda et al. 1999). Homologous proteins of four families of well characterized proteases in *Escherichia coli* have been found in *Arabidopsis thaliana* chloroplasts (Adam et al. 2001). Three of these belong to the serine type, the Clp, Deg and Lon proteases. The fourth one is the ATP-dependent metallo-protease FtsH of which one, the thylakoid FtsH1, has been shown to be responsible for the secondary cleavage of D1 (Lindahl et al. 2000). Recently DegP2, a DegP homolog in *Arabidopsis* , located at the outer stroma-exposed thylakoid surface, was overexpressed in *E. coli* and the isolated protease could cleave D1 protein producing a 23 kDa N-terminal fragment (Haussuhl et al. 2001). DegP2 has a requirement for the nucleotide GTP.

In *Synechocystis 6803*, there are three Deg protease homologous, DegP, DegQ and DegS (Funk et al. 2001). It has been shown that expression of these homologs is light dependent and that deletion mutant strains show no obvious phenotype (Funk et al. 2001). It is possible that the cyanobacterial proteolytic systems differ from those of higher plants or that the different isomers in cyanobacteria were able to compensate for the missing ones. These results may also indicate that the DegP2 is not he true primary endopeptidase even in plants. This could explain the contradiction between the results of Haussuhl showing that the DegP2 protease is over-expressed at low temperature and the observation that D1 degradation slows down by decreasing the temperature (Aro et al. 1990). As mentioned above, FtsH1 seems to

be responsible for a secondary cleavage of D1 (Lingdahl et al. 2000). In *Synechocystis* 6803 there are four possible FtsH homologs. Two of them, slr0228 and sll1463 have been inactivated. The strain having inactivated slr0228 contained reduced amounts of functional PSI (Mann et al. 2000). The slr0228 gene shares significant sequence similarities with FtsH2. Recent data from studies on the slr0228-strain shows that the D1 repair cycle was slower in this mutant compared to the control strain (Silva et al. 2001). It was also shown that His-tagged PSII preparations contained FtsH and that FtsH could serve as a potential protease and/ or chaperone (Lindahl et al. 2000).

1.7.1.2 Donor Side-Induced Photoinhibition of PSII

Light-induced inactivation of PSII electron transport and protein structure can also take place when the rate of electron donation from the water-oxidizing complex is unable to keep up with the rate at which electrons are transferred from P_{680} towards the acceptor side components. In this situation, long-lived and highly oxidizing radicals (Tyr-Z^+ and/or P_{680}^+) can accumulate, which have the potential to induce rapid inactivation of PSII electron transport and protein damage (Blubaugh and Cheniae 1990; Blubaugh et al. 1991; Eckert et al. 1991). In contrast to the acceptor side-induced mechanism of photoinhibition, which occurs under high-intensity illumination, the donor side-induced mechanism can occur under both low and high light intensities (Blubaugh and Cheniae 1990; Blubaugh et al. 1991; Eckert et al. 1991).

Donor side-induced photoinactivation is observed mainly in isolated PSII preparations in which the function of the water-oxidation complex is deliberately inactivated before the onset of photoinhibitory illumination (Blubaugh and Cheniae 1990; Blubaugh et al. 1991; Jegerschold et al. 1990). So far, there is no unambiguous indication for the occurrence of this mechanism in photosynthetic systems that had the intact donor side at the beginning of the illumination period. However, the increased light sensitivity observed in a number of mutants in which the water-oxidation function is directly or indirectly disturbed is likely to be related to donor side-induced photoinhibition.

Degradation of the D1 protein also occurs under the conditions of donor side-induced photoinactivation. However, the main feature of D1 protein degradation differ significantly from those observed under the conditions of the acceptor side-induced mechanism:

1. In donor side-inhibited PSII, the D1 protein is degraded in the absence of oxygen (De La Rivas et al. 1992; Jegerschold and Styring 1991).

2. The main cleavage of the D1 protein occurs at the lumenal exposed loop, between the first and second transmembrane helices as indicated by the formation of 24 kDa C-terminal and 9 kDa N-terminal fragments (Barbato et al. 1991).
3. There is no indication for the involvement of a proteolytic step in the protein degradation.
4. Singlet oxygen is not produced, instead, hydroxyl radicals are the main active oxygen species that accompany D1 degradation (Hideg et al. 1994a).

A 16 kDa C-terminal fragment is also detected under the donor-side photoinhibitory conditions (Barbato et al. 1992). This indicates a cleavage site at the lumenal-exposed loop between the third and fourth helices, which seems to be close to the secondary cleavage site observed under acceptor side-induced photoinhibition (Virgin et al. 1990).

In contrast to the clear distinction and separation of the acceptor side-induced and donor side-induced mechanisms of photoinhibition in *in vitro* studies, it is not yet clear what is the dominating mechanism *in vivo*. It is realistic to suppose that under high-light stress conditions, overreduction of the acceptor side components and consequently acceptor side-induced photoinhibition can develop. This view is supported by the *in vivo* detection of D1 fragments (De La Rivas et al. 1992), which are typical for the acceptor side-induced mechanism (Greenberg et al. 1987). Under low light conditions, which can also induce D1 degradation, overreduction of the PSII acceptor side is not very likely. Thus, the donor side-induced mechanism could be expected to occur. However, compelling evidence for light-induced damage of the PSII donor side in intact *in vivo* systems as the prerequisite for subsequent donor side-induced photoinhibition has not yet been provided. It has also been suggested by the group of Ohad that low-light-induced D1 protein degradation involves $^3P_{680}$ formation due to charge recombination between stable charge separation states (Keren et al. 1995) and consequently leads to $^1O_2$ production. It is clear, however, that once the capacity of the water-oxidizing complex to donate electrons to P_{680} is impaired by other stress factors, the PSII centers are expected to suffer rapid inactivation by the donor side-induced mechanism of photodamage.

1.7.2 The Mechanism of UV-B Induced Damage of PSII

The mechanisms of damage induced by UV-B light to the electron transport and protein structure of PSII has been addressed by a number of studies both in isolated and intact systems. According to an experimentally well supported model the primary UV-damage

occurs at the donor side of PSII, at the Mn cluster of water oxidation (Renger et al. 1989; Vass et al. 1996; Post et al. 1996). However, UV-B induced modification or loss in the function of the Q_A and Q_B quinone acceptors (Vass et al. 1996; Jansen et al. 1998), the Tyr-D and Tyr-Z donors (Vass et al. 1996; Yerkes et al 1990) have also been observed. One manifestation of the UV-B effect on the acceptor side components is an apparent resistance against electron transport inhibitors, which act by replacing the mobile plastoqiunone electron acceptor at the Q_B binding site. This effect, which is most likely related to the UV-B induced modification of the Q_B binding site, has been observed not only in oxygen evolving organisms (Renger et al. 1989 Hideg 1993; Vass et al. 1999), but also in isolated reaction centers of the purple bacterium *Rhodobacter sphaeroides* (Tandori et al. 1996), that have analogous reaction center units and quinone acceptors as PSII. Another important site of UV-B damage was proved to be the water oxidation process which conclusion was derived from the S state dependence of the UV damage. The most sensitive states are the S_2 and S_3 oxidation states. During the S-state transitions the catalytic Mn cluster of water oxidation is sequentially oxidized. Mn ions bound to organic ligands (such as amino acids) have pronounced absorption in the UV-B and UV-A regions in the Mn(III) and Mn(IV) oxidation states, which dominate the higher S states, but not in the Mn(II) oxidation state, which occur in the lower S-states (Bodini et al. 1976). Thus the high UV sensitivity of PSII in the S_2 and S_3 states indicates that UV absorption by the Mn(III) and Mn(IV) ions could be the primary sensitizer of UV-induced damage of the water-oxidizing machinery. Beside the electron transport components, UV-B light also damages the D1 and D2 reaction center subunits of PSII, leading eventually to their degradation (Greenberg et al. 1989; Trebst and Depka 1990; Friso et al. 1993; Barbato et al. 1995), which can be repaired via *de novo* protein synthesis in intact cells (Sass et al. 1997; Mate et al. 1998).

The mechanisms by which the PSII redox components are impaired by UV-B are not completely clear. In the case of quinone acceptors and Tyrosine donors, a direct destruction of the molecules could occur. However, the possibility that the redox function of these components is impaired due to damage of their protein environment cannot be excluded.

Damage or alteration of the protein binding site of the catalytic Mn cluster is also the most likely scenario for the inactivation of the water-oxidizing function, since the Mn ions themselves are not expected to be modified by UV-B. The S_1 to S_2 and S_2 to S_3 redox transitions of the Mn cluster are accompanied by absorption changes in the UV-B region (Dekker et al. 1984; Lavergne 1991), closely resembling the action spectrum of PSII inactivation in the UV-B region. Thus, absorption of UV-B quanta within the Mn cluster may

directly damage the protein environment. However, the site of absorption and damage by UV-B are not necessarily identical, there may be energy transfer from a sensitizing species to a sensitive site. Since the Mn cluster of water oxidation appears to be the most fragile component of the electron transport chain, UV-B absorption by another redox component or by the protein matrix may lead to a conformational change of the protein surrounding the catalytic Mn site and thus render it inactive.

The protein environment in the vicinity of the Mn cluster can also be damaged by free radical species. It appears that free radicals, predominantly hydroxyl radicals, are formed in the UV-B irradiated PSII preparations. One possibility for the formation of the free radicals is that they arise from the decomposition of hydrogen peroxide by UV-B as observed in model reactions (Czapski 1984). Hydrogen peroxide has been suggested to appear as an intermediate during water oxidation (Renger 1978; Wydrzynsky et al. 1989). Thus, UV-B-induced decomposition of H_2O_2 could produce highly reactive hydroxyl radicals within the water-oxidizing complex and promote extensive damage to the protein environment.

1.7.3 Photodamage by Simultaneous UV-B and Visible Illumination

Comparison of the main characteristics of photodamage induced by visible and UV-B spectral ranges reveals several similarities, but also significant differences. Both UV-B and visible light damage primarily the PSII complex within the photosynthetic apparatus, but unlike visible light UV-B has a wide array of targets in the plant cells. Within the PSII complex, the two types of radiation seem to have common action sites, such as the quinone electron acceptors and the donor side components. However, the UV-B action is concentrated on the water-oxidizing complex both in vitro and in vivo, in contrast to photoinhibition by visible light, whose main effect in functional PSII centers appears to be at the acceptor side. Both UV-B and visible light damages the protein structure of PSII reaction center by attacking primarily the D1 protein. However the damage sites induced by UV-B and the donor and acceptor side mechanisms of photoinhibition by visible light are clearly different.

Thus, in spite of the predominant damage of PSII and the often similar symptoms of its inactivation, UV-B and visible light exert their damaging effect via different mechanisms.

Since both UV-B and visible light can influence the function of PSII electron transport, the interaction of the two light regimes can lead to a wide range of effects. Experimental observations indicate that low-intensity UV-B and relatively weak visible light can lead to a synergistic enhancement of photodamage to the function and protein structure of

PSII (Teramura et al. 1980; Bornman and Sundby-Emanuelson 1995; Jensen et al. 1993). On the other hand, high-intensity visible light seems to be able to ameliorate the effects of UV-B under certain conditions (Warner and Caldwell 1983).

1.7.3.1 The Potential of UV-B Radiation to Aggravate Visible Light-Induced Photodamage.

UV-B impairment of electron donation from the water-oxidizing complex to P_{680} can obviously create a situation that facilitates the accumulation of P_{680}^{+} and Tyr-Z^{+} upon illumination by visible light. Thus, the presence of UV-B radiation together with visible illumination may enhance the inactivation of PSII by promoting donor side-induced photoinhibition. Although this hypothesis seems well established and Tyr-Z^{+} accumulation had been observed in UV-B irradiated PSII membranes (Vass et al. 1996), no serious experimental effort has been devoted to investigate it.

The D1 protein is the most rapidly turned over protein component in plants (Greenberg et al. 1987). Even under normal light conditions, D1 is continuously degraded and resynthesized in as short a time as 30 min (Greenberg et al. 1987). This rapid turnover is generally viewed as a repair process that is operated to remove and replace the light-damaged D1 protein copies in PSII (Aro et al. 1993). Others, however, consider it part of a protection mechanism by which the dissipation of the excess light energy can be regulated buring the periods of high visible illumination (Critchley and Russel 1994). Whatever the precise role of D1 turnover is, this cycle requires continuous de novo protein synthesis. The limited ability of PSII activity to recover after intense UV-B radiation (Renger et al.1986; Larkum and Wood 1993; Chow et al. 1993) may indicate the impairement of the D1 protein turnover cycle. Such an effect may be a consequence of UV-B damage to nucleic acids that are not fully repaired.

An important defence strategy of photosynthetic organisms to cope with photodamage by visible light is the dissipation of excess excitation energy in the form of heat in the light-harvesting antenna. The main mechanism of heat dissipation is the xanthophyll cycle (Demmig-Adams 1990) that can be impaired by UV-B radiation and increase the damaging effect of this radiation on plants.

1.7.3.2 The Potential of Visible Light to Aggravate or Ameliorate UV-B-Induced Photodamage

The synergistic enhancement of PSII damage by simultaneous UV-B and visible light can also be viewed from the aspect of visible illumination promoting the UV-B damage. This view can be based on the differential UV-B sensitivity of PSII electron transport components depending on their redox state. The UV-B damage of the water-oxidizing complex could be induced by absorption due to redox transitions of the water-oxidizing complex, by oxidized tyrosine radicals, or perhaps by semi-reduced quinone electron acceptors. These UV-B-sensitive redox states are all formed and accumulated by light-driven electron transport through PSII, although Q_B^- and Tyr-D^+ are quite stable in the dark. Consequently, the damaging effects of UV-B exerted on these redox components are expected to be enhanced in the presence of background illumination by visible light.

Simultaneous illumination with UV-B and visible light does not always enhance damage. There are reports which show that the presence of relatively high, but probably not photoinhibitory levels of visible of visible light can ameliorate the UV-B effect (Warner and Caldwell 1983)

1.8 DNA as Target for UV-B Radiation

DNA is one of the most notable targets of ultraviolet radiation. Irradiation in both the UV-C and UV-B regions results in a multitude of DNA photoproducts (Sancar and Sancar 1988), which may be a source of mutations. The most common DNA photoproducts are cyclobutane-type pyrimidine dimers and the pyrimidine(6,4)pyrimidone dimer (Hutchinson 1987). The most common pyrimidine base to form dimers is thymine (Fig. 9). Once formed the photoproducts may be the cause of mutation if they are not eliminated in time by repairing processes. One of the most common repair processes is photoreactivation (Fig. 9).

Most DNA repair processes remove the damaged nucleotides and several adjacent residues, then replace the excised region using information encoded in the complementary (undamaged) strand. Two processes, however, directly change the damaged bases, rather than removing them. These two processes are photoreactivation, and the process catalyzed by O6-alkylguanine alkyltransferase. The enzyme responsible for photoreactivation is called photoreactivating enzyme or DNA photolyase. It repairs cyclobutane pyrimidine dimers in the

presence of visible light. A wavelength of 370 nm is most effective. The photoreactivating enzyme binds to DNA, in a light-independent process, at the site of pyrimidine dimers

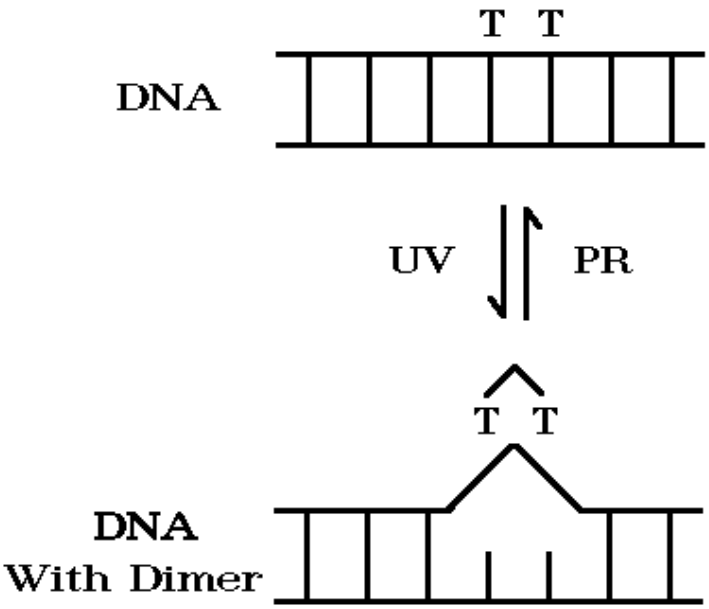

Figure 9. Schematic representation of thymine dimer formation as an effect of UV light damage and photoreparatory process.

. In the presence of visible light, the bonds linking the pyrimidine rings are broken, after which the enzyme can dissociate in the dark. The photoreactivating enzyme contains two chromophores. One chromophore is flavin adenine dinucleotide in the reduced state, $FADH^-$. The second chromophore in some photolyases is 5,10-methenyltetrahydrofolate and in others is 8-hydroxy-5-deazaflavin. Mechanistic studies suggest a process akin to photosynthesis, with the second chromophore functioning as a light-harvesting factor, and $FADH^-$ functioning like the photochemical reaction center, translating light energy to facilitate the transfer of an electron to the dimer and breaking the pyrimidine-pyrimidine bonds by a free radical mechanism. Photolyase has been detected in numerous eukaryotic and prokaryotic systems.

2. Aim of Study

- Investigation of the UV-B damaging effect on PSII and the recovery of the activity under visible light conditions.
- Elucidation of the relationship between visible light and UV-B light in damaging the PSII activity.
- Comparison of the two different cyanobacterial response strategies to UV-B damage from Synechocystis sp PCC 6803 and Synechococcus sp PCC 7942.
- Comparison of the UV-B induced damage at the phycobilisome and photosystem II level.
- Determination of the importance of UV-B induced DNA-damage, in relation to the damage at the photosynthetic electron transport level.

3. Materials and Methods

3.1 Experimental Models

The unicellular cyanobacterium *Synechocystis sp.* PCC 6803 is widely used as model system for studies of functional, structural and regulatory aspects of the photosynthetic machinery, especially using a mutagenesis approach (Bryant 1994; Jansson et al. 1998). The main advantages of this system are the following:

- The similarities in the composition and functional organization of the complexes in the tylakoids that are involved in photosynthesis to those of higher plants.
- *Synechocystis sp.* PCC 6803 is, like many other cyanobacteria, naturally competent, which means that it is easily transformed, and the added DNA can incorporate into the chromosome by homologous recombination.
- *Synechocystis sp.* PCC 6803 has a short generation time and grows photoautotrophically as well as photoheterotrophically. In studies of photoautotrophically deficient mutans like those where important proteins from the composition of PSII has been depleted, glucose can support growth.
- The entire genomic DNA sequence (3.57 Mb, approximate 3200 protein coding genes) of *Synechocystis sp.* PCC 6803 has been sequenced and it available on the Internet at: www.kazusa.or.jp/cyano/ .
- *Synechocystis sp.* PCC 6803 expresses bacterial selectable marker genes. Antibiotic resistance marker genes are usually used for mutagenesis studies.

During the experiments we used different strains, wild-types as well as mutants of the two cyanobacterial species: *Synechocystis sp.* PCC 6803 and *Synechococcus sp.* PCC 7942. Both cyanobacteria species were grown in BG-11 media with the composition as described in Table I.

3.2 Culture Conditions

The cells were cultured in 500 ml flasks containing not more then 200 ml of medium. We grow the cells at 30C, under 40 $\mu E\ m^{-2}\ s^{-1}$ illumination in the presence of 5% CO_2 in a Gallenkamp termostated rotary incubator

As starter cultures we used smaller volume (50 ml) culture from which we used 1 ml to initiate the cultures we used for experiments. One such culture we used for about 3 weeks

and then pass it to another flask. Normally not more then 2 or 3 passages were done. The starter cultures were inoculated from samples of cells preserved in deep freezer at –80 C. These samples are aliquots of 1 ml of cells at around 10-15 μg Chl/ml to which 8% DMSO was added to increase the resistance to freezing.

Stock Solution I (100 ml): Citric acid……..0.3g $FeNH_4$-citrat……..0.3g EDTA……0.05g Stored at 4 C.	Stock Solution II (1000 ml): $NaNO_3$..…….30g K_2HPO_4……..0.78g $MgSO_4x7H_2O$……..1.5g Stored at –20 C.
Stock Solution III (100 ml): $CaCl_2x2H_2O$……..1.9g Stored at 4 C.	Stock Solution IV (100 ml): $Na_2CO_3x10H_2O$…….5.03g or Na_2CO_3…….….2g Stored at 4 C.
Stock Solution V (1000 ml): H_3BO_4…….…….5.03g $Na_2MoO_4x2\ H_2O$…….0.39g $MnCl_2x4H_2O$…….…….2g $CuSO_4x5\ H_2O$……0.079g $ZnSO_4x7\ H_2O$……0.222g $Co(NO_3)_2x6\ H_2O$……..…0.04g Stored at –20 C.	
For 1000 ml BG-11 use: Stock I……..2 ml Stock II……...50 ml Stock III……..….2 ml Stock IV……..…….1 ml Stock V…….……1ml HEPES…….……..…..4.768g Sterilize by autoclave at 120 C for 20 min.	

Table I Composition and preparation of BG-11 media used for the cultivation of cyanobacteria.

3.3 Tylakoids Isolation.

Thylakoid membranes were isolated from spinach as described earlier (Turcsanyi and Vass 2000) and were stored at –80 C until use in 0.4M sucrose, 5 mM $MgCl_2$, 10 mM NaCl and 40mM Hepes (pH 7.5) at concentrations of 2-3 mg/ml.

3.4 Light Treatment

UV-B light was provided by a Vilbert-Loumart VL-215M lamp, in combination with a 0.1 mm cellulose acetate filter yielding 6 μE m^{-2} s^{-1} intensity at the surface of the samples.

Visible light was produced by an array of Halogen spot lamps in the 0-1300 μE m^{-2} s^{-1} intensity range. Samples were illuminated at 10 as well as 6.5 μg Chl/ml.

At the beginning of the treatment the cells that were in the logarithmic growth phase were harvested by centrifugation at 7000 rpm for 10 min. The cells were resuspended in fresh BG-11 medium and the chlorophyll concentration was set to the desired value. They were allowed to stay in the incubator for 1 hour to recover after centrifugation. Before starting the treatment controls were measured and the mean of these measurements is plotted on graphs as time zero. During the treatment the temperature was maintained constant at 30 C. Also the influence of the environmental light during the treatment was minimized. During the course of the treatment measuring points were recorded at periods of usually 30 min (exceptions were the longer experiments when the periods at which the points were measured were longer) and in this way we were careful that the volume of the cells that we extracted from the sample was not significant and it did no affect the kinetics of the treatment.

When we needed to block the protein synthesis, 100 μg/ml lincomycin, was added about 10 to 15 minutes before the start of the treatment.

3.5 Fluorescence Relaxation Measurements.

In fluorescence experiments, light serves to drive photosynthetic reactions and, at the same time, to induce fluorescence emission. To separate these roles, two different light sources are frequently used. The actinic light delivers energy that is sufficient to induce substantial change in the state of photosynthetic apparatus. On the contrary, the mean energy of the measuring light is so low that the impact of the induced photosynthetic reactions can be neglected. In most of the presently used fluorometers, either the actinic or the measuring light

is modulated, so that the detection system can separate the fluorescence excited by the measuring light.

The PSI fluorometer (Fig. 10) is based on a non-periodic, user-definable modulation of both, the actinic and measuring flashes, that is why it is called double-modulation fluorimeter.

We measured the decay of flash induced fluorescence in intact cyanobacterial cells as well as in tylakoids. 1 ml samples of cells were dark adapted for 10 minutes prior to the measurement. We measured the decay in absence as well as in presence of DCMU [3-(3,4-dichlorphenyl)-1,1'-dimethylurea]. The curves were recorded using the Fluorwin software that was provided together with the apparatus and were plotted on a logarithmic time scale.

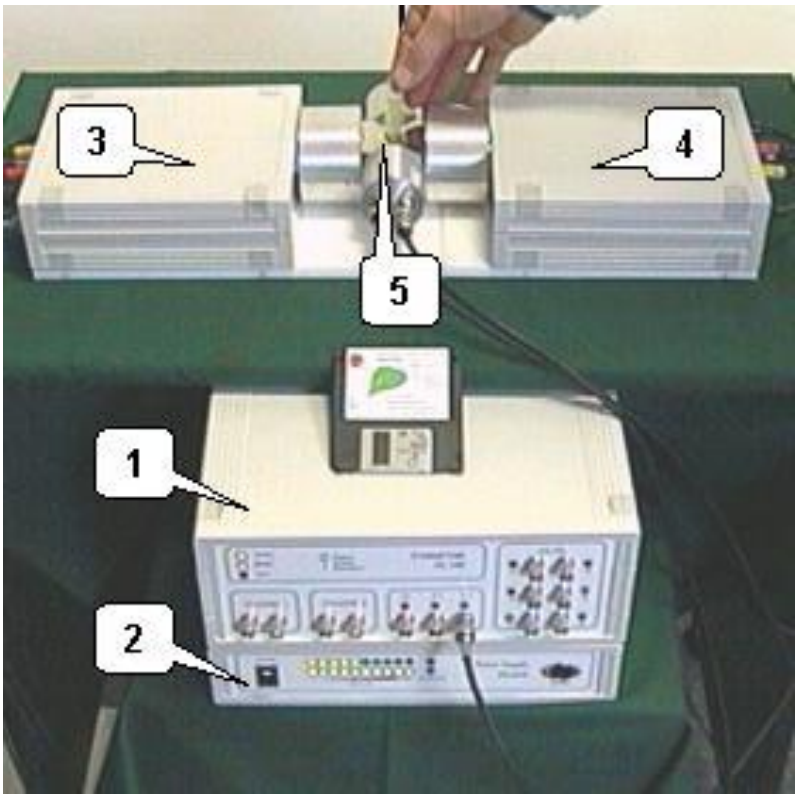

Figure 10 . The typical conformation of a double modulated fluorometer from PSI Czech Republic.1- control box; 2- power supply; 3 and 4-LED units responsible for generating the actinic and measuring flashes; 5- sample holder.

DCMU (Fig. 11.A) is a herbicide that is frequently used in photosynthetic experiments because of its capacity to block the electron transfer between Q_A and Q_B (Fig. 11.B).

DCMU inhibits electron transport because it competes with plastoquinone (PQ) for the Q_B binding site and it blocks it irreversibly. In this way Q_A^- can not be reduced by Q_B and it recombines back with redox components from the donor side of the electron transport chain

especially with the S states of the water oxidation complex. In this way, by adding DCMU to a sample we can get information about the integrity of the donor side of the electron transport chain.

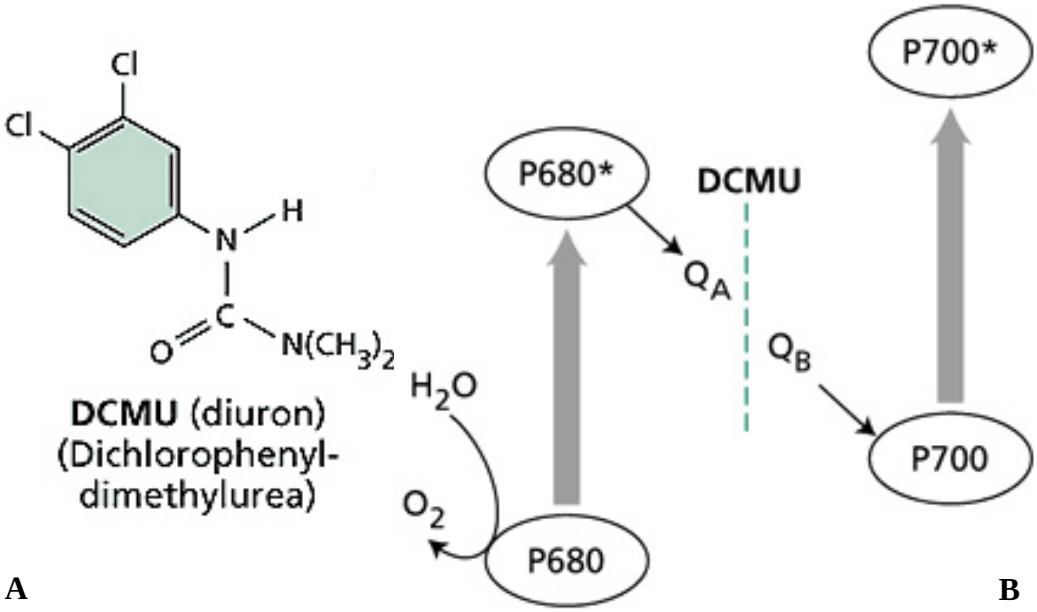

Figure 11. The herbicide DCMU (A) and its acting site in the electron transport chain (B).

3.6 Continuous Oxygen Evolution Measurements.

An oxygen electrode is a specialized form of electrochemical cell which consists of two electrodes immersed in an electrolyte solution. Application of a polarizing voltage (i.e. a voltage which generates new positive and negative poles) across the electrodes results in ionisation of the electrolyte and the flow of current through the electrode. The magnitude of this current flow is proportional to the concentration of oxygen dissolved in the electrolyte which in turn is proportional to the oxygen concentration of the surrounding media. Such an electrode system was first used by Clark (1956) to measure oxygen in blood samples and consequently has become known as the Clark Electrode. The Hansatech Oxygen Electrode Disc is a high-precision Clark Electrode based on a design by Delieu and Walker (Delieu and Walker 1981). The sensor consists of a platinum cathode and silver anode which are set in an epoxy resin disc (A, Fig. 12.) with the cathode (B, Fig.12) at the center of a dome which is

surrounded by the anode (C, Fig. 12) set into a well which also serves as the electrolyte reservoir.

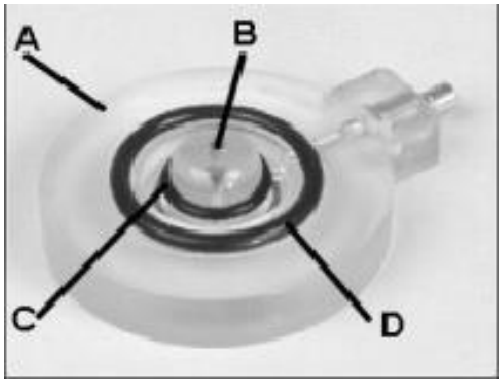

*Fig. 12. The Hansatech Oxygen Sensor. The electrodes are set into an epoxy resin disc (**A**) with the platinum cathode (**B**) at the center of a dome surrounded by a well which contains the silver anode (**C**). When set up a uniform electrolyte layer is held over the dome area of the electrode by a paper spacer and P.T.F.E. (polytetrafluoroethylene) membrane which is secured by an O-ring around the dome. The well which houses the anode also serves as a reservoir with electrolyte drawn up to the cathode by the paper spacer acting as a wick. An outer O-ring groove (**D**) surrounds the whole apparatus. The outer O-ring seals the electrodes into the base of the Hansatech DW electrode chamber.*

The membrane is sufficiently permeable to permit diffusion of oxygen from the contents of the reaction chamber whilst remaining impermeable to most poisons of the oxygen reduction reaction which occurs at the platinum electrode. Since oxygen is continuously consumed at the cathode the solution in the reaction chamber must be stirred to maintain a stable oxygen gradient across the membrane. This is achieved by placing a magnetic follower in the reaction chamber and mounting the electrode unit on top of the integral magnetic stirrer. The permeability of the membrane ensures that the oxygen concentration of solutions in the reaction chamber and electrolyte solutions are equal and thus the electrical signal generated through electrochemistry in the sensor unit is directly proportional to the oxygen concentration of the solution in the reaction chamber. The electrode disc is sensitive enough to detect diffusion of oxygen from air in the reaction chamber into the reaction solution.

For measuring oxygen evolution we used 2 ml samples of cyanobacterial suspension. We added 2,5 (dimethyl) p-benzoquinone as electron acceptor, which takes the electrons from PSII and keeps the photosystem oxidized. In this way the oxygen evolution rate we measured was proportional with the PSII activity.

3.7 Flash Induced Oxygen Evolution

If a sample of dark-adapted photosynthetic membranes or cyanobacterial cells is exposed to a sequence of very brief, intense flashes, a characteristic pattern of oxygen production is observed. Little or no oxygen is produced on the first two flashes, and maximal oxygen is released on the third flash and every fourth flash thereafter, until eventually the yield per flash damps to a constant value.

For the measurements of this type of oxygen evolution we used the so called Joliot type oxygen electrode (Fig. 13) that is different in organisation than the Clarke type electrode used in measurements of continuous oxygen evolution but functions according to the same principle. For the measurements we used a minimal amount of cells deposited to the electrode surface by centrifugation and that will ideally form a layer one cell thick.

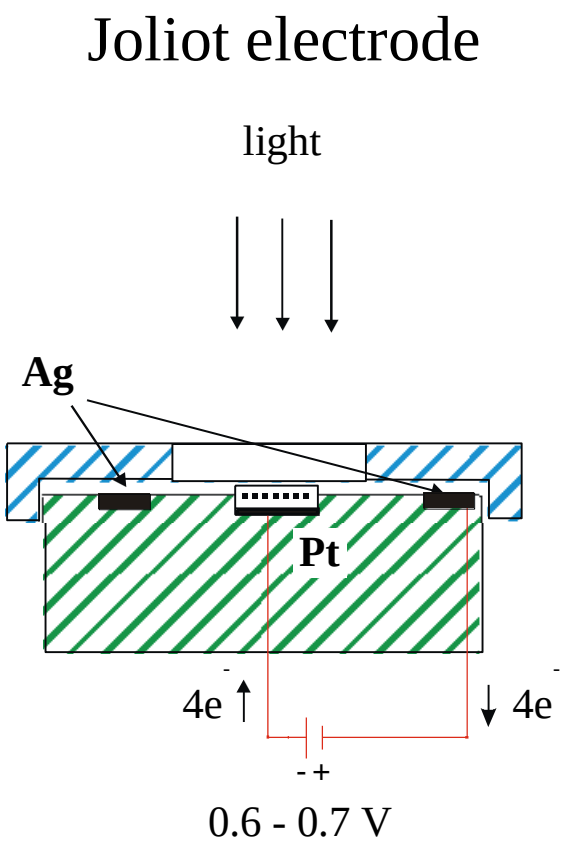

Figure 13. Schematic illustration of a Joliot type oxygen electrode similar to the one we used on the measurements of flash induced oxygen evolution.

We exposed the sample to a series of twenty flashes of saturating light and measure the pattern of released oxygen. The data from the electrode was processed and displayed by a PC using a specially created software.

3.8 Thermoluminescence Measurements

One of the fundamental discoveries on the photosynthesis field in the 1950s was the detection of thermally stimulated light emission from photosynthetic material that was previously preilluminated. (Arnold and Sherwood 1957). This phenomenon was named thermoluminescence (TL) and is characteristic not only to photosynthetic material but to a wide range of materials (minerals, semiconductors, inorganic and organic crystals as well as complex biological systems). All these materials share the capacity of storing radiant energy in thermally stabilized trap states.

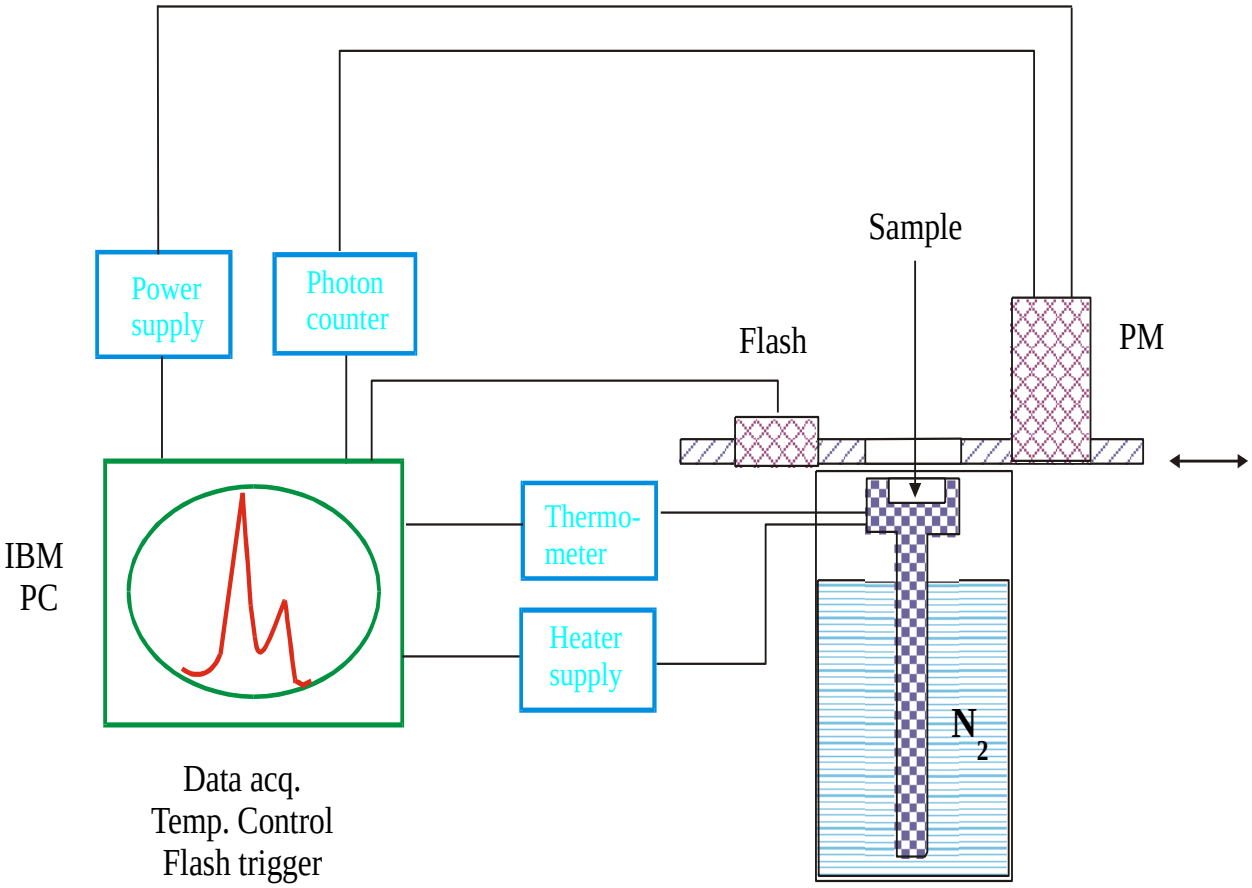

Figure 14. Representation of the home made TL–measuring apparatus that was used in our experiments.

The phenomenon of thermoluminescence (TL) can be described as emission of light at characteristic temperatures from samples that had been exposed to electromagnetic or particle radiation prior to their warming up in the dark (Chen and Kirsh 1981).

By its nature TL can be, and it is a useful tool in the study of the photosynthetic events. The main redox components that participate in the TL emission are the reduced forms of the Q_A and Q_B quinone electron acceptors, the S_2 and S_3 redox states of the water oxidation

complex, Tyr-D• and Tyr-Z•. Consequently, TL is used as a powerful tool to monitor the function and activity of the above redox components through PSII enriched membranes and thykaloids to intact leaves and whole cells of cyanobacteria or algae. For our experiment we used a home-made TL device (Fig. 14).

For the measurements we filtered 50 μgChl of cells on a disk of filter paper and we added to it 400 μl of BG-11 medium in the sample holder. The measuring protocol contains a preillumination phase 30 seconds long followed by 3 min of dark adaptation. When we wanted to record the Q band we added DCMU at the beginning of this dark adaptation phase. After the dark adaptation the sample was excited according to the experimental protocol with flashes of light (one or more) or with continuous illumination for 30 seconds. At the end of the excitation period we cooled the sample to the temperature from where we started the measurement (-40 C or 0 C) and then we recorded the luminescence generated by the sample while we shift it up to 70 C. The recorded data points were plotted and we obtained the TL curves.

3.9 Data Processing and Interpretation.

Most of the software used for recording the data were created in our laboratory for this specific purpose except the FluorWin program used in flash induced fluorescence decay measurements that was made by P.S.I Czech Republic.
For plotting and processing our data we used the Microcal Origin Profesionnal 5.0 software.

4 Results and Discussions

4.1 UV-B as Damage Factor of the Photosynthetic Electron Transport

In intact *Synechocystis* cells UV-B radiation induces a decrease in oxygen evolution as a consequence of decrease in the photosynthetic activity. Under our experimental conditions we obtain a decrease of about 50 % of the initial oxygen evolution rate in about 120 minutes, with a high initial rate of the decrease to about 60 % in the first 30 minutes (Fig. 15). The *Synechocystis* cells have the capacity of recovery after UV-B damage if put in visible light. The rate of the recovery is directly dependent on light intensity if this does not exceed the photoinhibition values (data not shown). The rate of recovery is also dependent on the extent of the damage. Under normal light conditions (45 $\mu Em^{-2}s^{-1}$) from a damage value of 50 % of initial oxygen evolution activity, the recovery is complete in about two hours (Fig. 15).

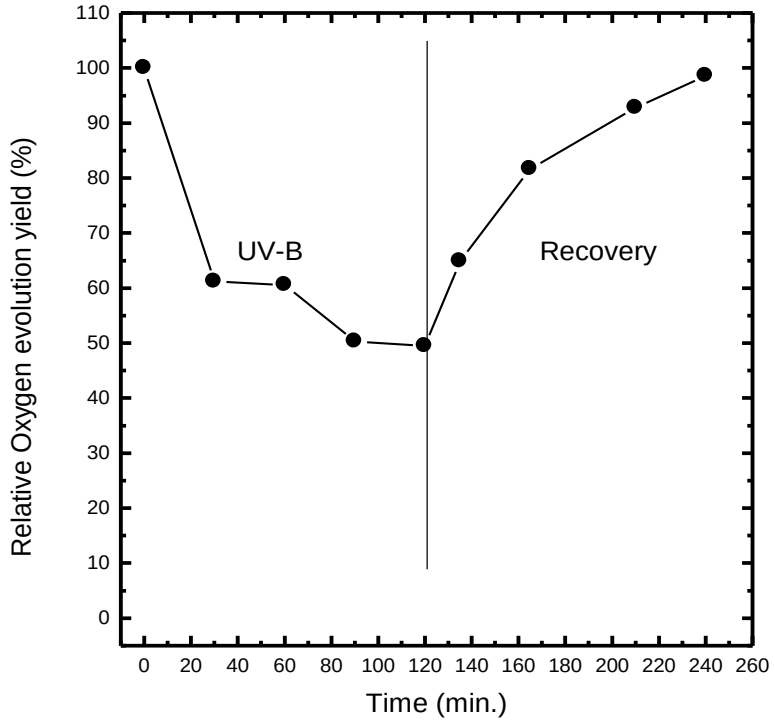

Figure 15. UV-B induced damage of photosynthetic activity of Synechocystis PCC 6803, and recovery under visible light. The oxygen evolution values are expressed

From our study it is evident that temperature is also one factor which influences the extent of the damage caused by UV-B (data not shown) and the rate of recovery (Fig. 16).

After an initial damage of 33 % of oxygen evolution activity the recovery at 20 C goes very slow reaching a value of about 45% in 150 min. If we shift the temperature of the sample to 30 C, maintaining all the other parameters, the cells start to recover much faster reaching the 70% value in 30 min (Fig. 16).

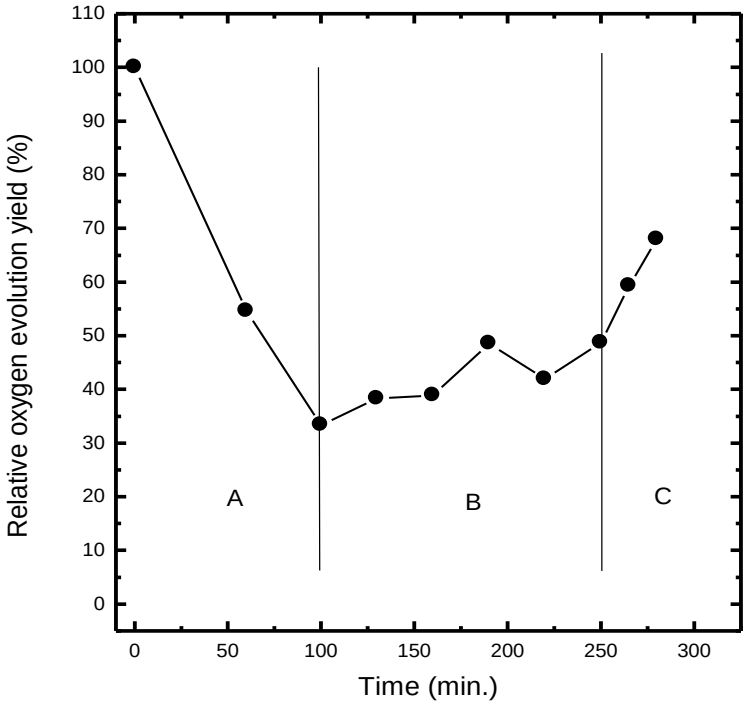

Figure 16. Effect of temperature on the rate of recovery following UV-B damage in Synechocystis PCC 6803. A: UV-B damage at 30 C; B: Recovery in visible light ($45 \mu Em^{-2}s^{-1}$) at 20 C; C: Recovery under the same light intensity at 30 C.

The modifications in the recovery rate as a consequence of the temperature shift can be the result of the modifications in the physical status of the membrane. It was shown that modifications of the saturation degree of the fatty acids in the tylakoid membrane is inducing modifications of the efficiency of PS II protein subunits assembly into the membrane (Kanervo et al. 1995). In order to study how does membrane fluidity affect the recovery process after UV-B damage we used a chemical agent, benzyl alcohol (BA), known to have a fluidizing effect on biological membranes. First we checked the effect of BA on the oxygen evolution of the *Synechocystis* cells under control conditions and we got no significant changes. We used two different concentrations of BA, 10 mM and 20 mM which are in the range of the usually applied concentrations. During a period of 120 minutes, no significant changes in the oxygen evolution were observed (Fig. 17).

For checking a longer time effect of BA we cultured the cyanobacterial cells in the presence of different concentrations of BA (5, 10, 20 and 30 mM) and monitored the growth of the cells daily by measuring the absorbance at 580 nm during a 6-day period (Fig. 18).

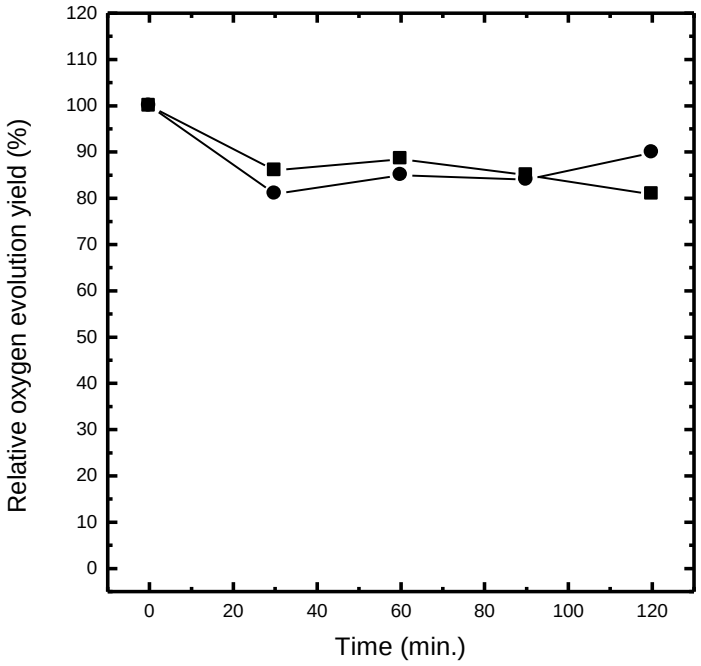

Figure 17. Effect of the benzyl alcohol on the oxygen evolution rate of Synechocystis PCC 6803 (circles -20 mM BA; squares10 mM BA).

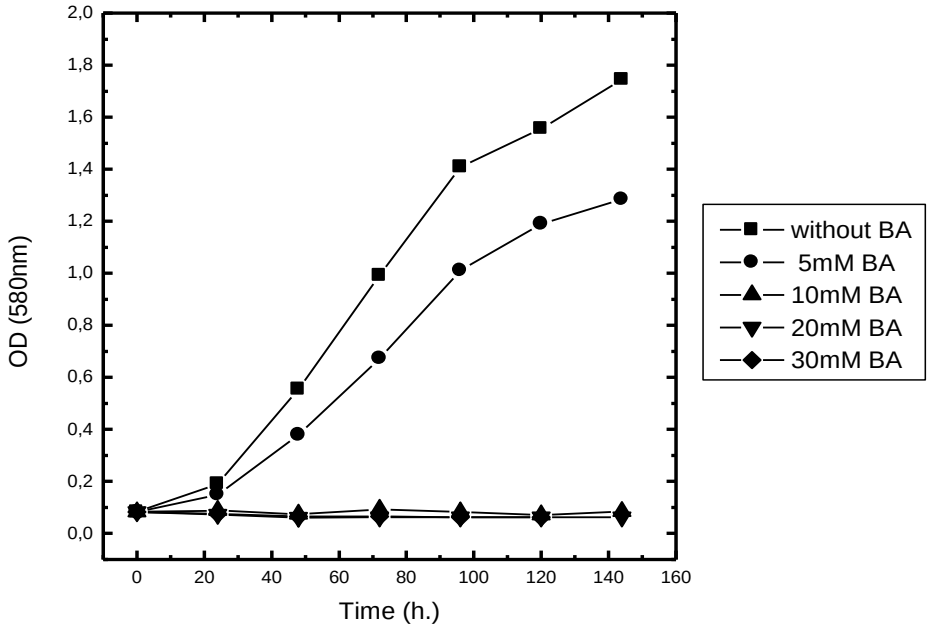

Figure 18. Influence of different concentrations of benzyl alcohol on the growth of Synecocystis PCC 6803

This is the time for the cells under our culturing conditions to reach the stationary phase of the growth. The experiment showed that concentrations higher than 5 mM drastically inhibit the growth of the cells. At 5 mM BA, the growth is inhibited to an extent of 70.5% of the control, without BA (Fig. 18).

Concerning the UV-B damage and recovery, the BA has an inhibitory effect on recovery at 10 mM (Fig. 19) or higher concentrations (data not shown). Starting from a value of 30 % of the initial oxygen evolution activity, the sample without BA recovered fully in a two-hour period. The sample which contained 10 mM BA was recovering to 45 % starting from a 20 % value in the same period of time (Fig. 19). In the case of using a 5 mM BA concentration, after an initial drop the BA-treated sample is more resistant to UV-B damage and is recovering faster (Fig. 20). Using a mutant strain of *Synechocystis* which contains the luciferase gene under the *psb*A2 promoter, we checked the expression of the gene in the presence of BA. Exposing the cells to 45 $\mu Em^{-2}s^{-1}$ visible light, at the temperature of 25^0C we observed, in about two hours, a 23-fold increase of the bioluminescence emission (Fig. 21).

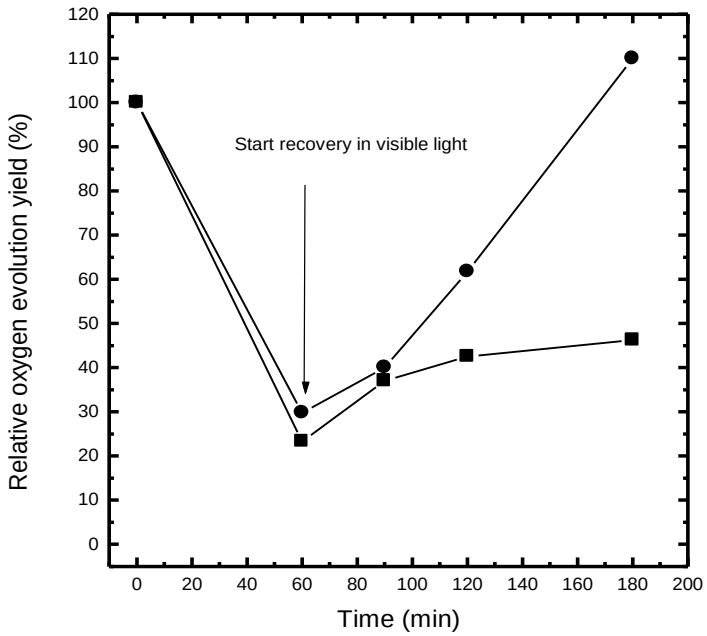

Figure 19. The effect of 10 mM benzyl alcohol on UV-B induced damage and recovery of PS II in Synechocystis PCC 6803. Symbols: treated cells (squares) were compared to non-treated cells (circles). Oxygen evolution rate is shown as percentage of the initial rate. Measurements were done at 30^0C. Intensity of visible light was 45$\mu Em^{-2}s^{-1}$.

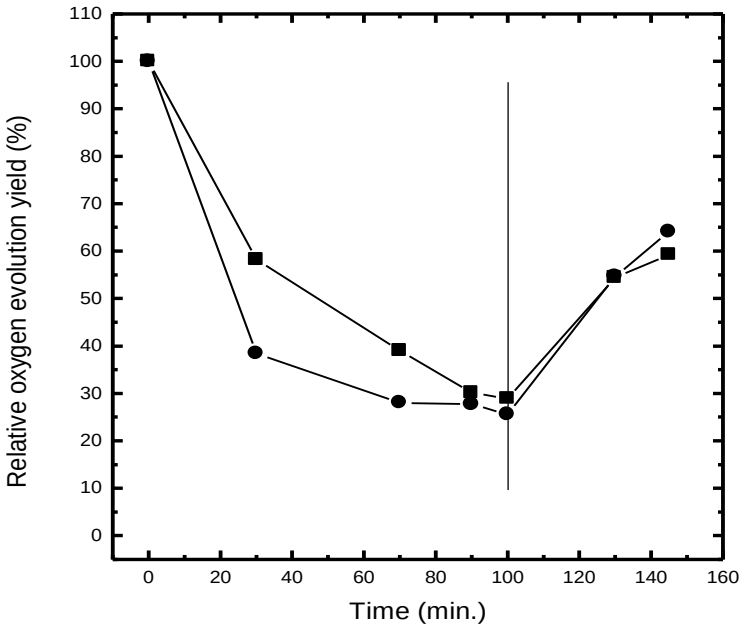

Figure 20. The effect of 5mM benzyl alcohol on UV-B induced damage and recovery of PS II in Synechocystis PCC 6803. Symbols: treated cells (squares) were compared to non-treated cells (circles). Oxygen evolution rate is shown as percentage of the initial rate. Measurements were done at 30^0C. Intensity of visible light was $45 \mu Em^{-2}s^{-1}$.

If we add 10 mM BA to the cells the extent of the increase was only 3.5 -fold. This shows a clear inhibitory effect of the BA at the level of luciferase production (Fig. 21).

From our results we can conclude that at the intensity that we used UV-B acts as a damaging factor for the electron transport chain. Under visible light conditions the photosynthetic cyanobacterium *Synechocystis* 6803 is capable of full recovery from severe (about 50%) damages caused by UV-B irradiation. The change in the temperature during the recovery phase induced the modification of the rate of the recovery. This effect was probably caused by the temperature induced modification of the membrane, which influences the turnover of membrane protein subunits of the PSII, especially D1 and D1. Considering this the addition of a fluidizing agent such as benzyl alcohol is expected to induce an increased resistance to UV-B damage and a faster recovery. However, BA led to increased damage and decreased recovery. BA also acted as an efficient inhibitor of the growth of the cyanobacterial cells over longer periods of time but it showed no inhibitory effect on the oxygen evolution rate during the duration of one experiment. One possibility is that BA acts

as a protein synthesis inhibitor somewhere at the transcriptional level. Its membrane-fluidising effect manifest itself to some extent at low concentrations when its toxic effect is less obvious.

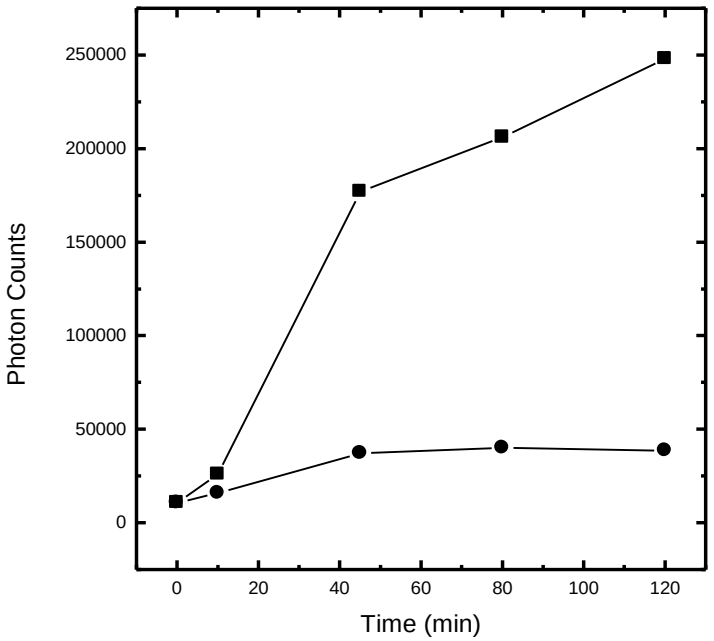

Figure 21. The effect of 10mM benzyl alcohol on luminescence emission during visible light illumination at 25^0C, of Synechocytis PCC 6803 strain containing the luciferase construct under $psbA_2$ promoter. Symbols: treated cells (ciecles) non-treated cells (squares). Intensity of visible light was 45 $\mu Em^{-2}s^{-1}$. Light emission was measured at 30^0C in 1 ml samples of liquid cell suspension (chlorophyll concentration: 5μg/ml)

4.2 The Interaction of UV-B and Visible Light in Damaging the Photosynthetic Apparatus.

4.2.1 Inactivation of Oxygen Evolution in Thylakoids

To clarify the role of visible light in UV-induced damage of PSII, we used isolated spinach thylakoids as a simple experimental system, which were exposed to UV-B and visible light in separate and simultaneous protocols. Illumination with 325 μE $m^{-2}s^{-1}$ visible light induced a 35-40% loss of oxygen evolving activity in 2h; whereas exposure to 6μE $m^{-2}s^{-1}$ UV-B light resulted in 55-60% inhibition (Fig. 22). Illumination of the thylakoid membranes with the mixture of UV-B and visible light enhanced the extent of the damage reaching 70%

inhibition at the end of 2h treatment. The relative activity values, normalized to one in the non-irradiated control samples, were plotted on a semilogarithmic time scale (Fig. 22). In this representation the relative activity gives the fraction of centers that survives photodamage, i.e. the probability that oxygen evolution is not inactivated by photon exposure for the given period of time.

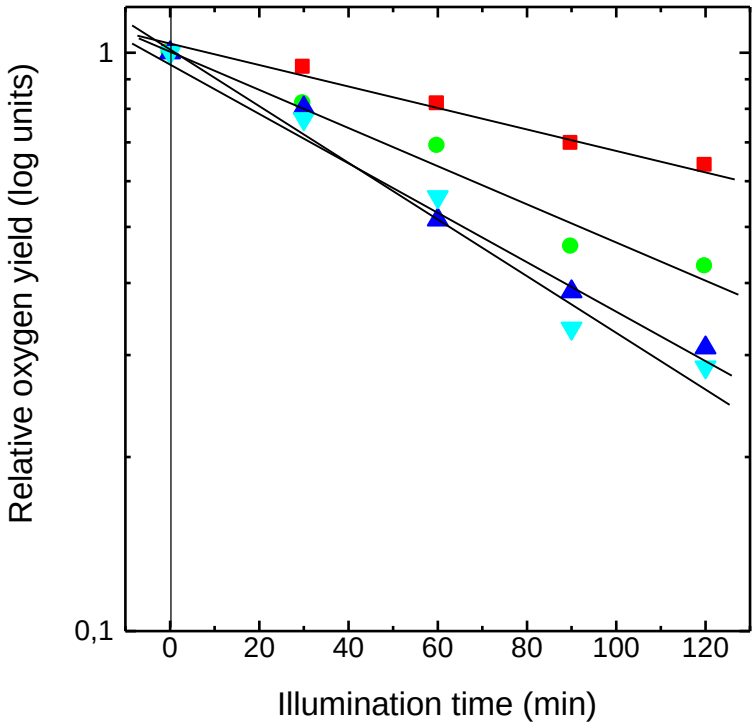

Figure 22. Semilogarithmic inactivation curves of oxygen evolution measured in isolated thylakoids. Samples were illuminated by 325 μE m^{-2} s^{-1} visible light (squares), 6 μE m^{-2} s^{-1} UV-B light (circles), and the combination of the above visible and UV-B light intensities (up triangles). For comparison the calculated inactivation by assuming independent damage mechanisms is also shown (down triangles).

The semilogarithmic inactivation curves gave straight lines with intercepts on the Y axis at zero time being close to one (1.06 for visible and 1.04 for UV-B illumination, respectively). According to the target theory, this indicates that inactivation of a PSII unit is induced by absorption of a single photon in a single target. Dose-response curves created from measurements with different light intensities gave essentially the same results (not shown).

	Visible light intensity	*Illumination protocol*			**UV+ Visible calculated**
		Visible alone	**UV alone**	**UV+ Visible in combination**	
Synechocystis PCC6803 cells	130μE	$0.3*10^{-3}$	$1.6*10^{-3}$	$-0.5*10^{-3}$	$2.0*10^{-3}$
	650μE	$1.8*10^{-3}$	$1.7*10^{-3}$	$1.7*10^{-3}$	$3.5*10^{-3}$
	1300μE	$3.4*10^{-3}$	$1.7*10^{-3}$	$4.0*10^{-3}$	$4.4*10^{-3}$
Synechocystis PCC6803 cells+ lincomycin	130μE	$1.4*10^{-3}$	$4.2*10^{-3}$	$5.0*10^{-3}$	$5.6*10^{-3}$
	325μE	$2.3*10^{-3}$	$2.3*10^{-3}$	$4.5*10^{-3}$	$4.7*10^{-3}$
	1300μE	$3.5*10^{-3}$	$2.8*10^{-3}$	$5.3*10^{-3}$	$6.4*10^{-3}$
Tylakoids	130μE	$0.7*10^{-3}$	$2.9*10^{-3}$	$3.6*10^{-3}$	$3.6*10^{-3}$
	325μE	$1.7*10^{-3}$	$3.2*10^{-3}$	$4.5*10^{-3}$	$4.8*10^{-3}$
	625μE	$3.4*10^{-3}$	$3.6*10^{-3}$	$6.5*10^{-3}$	$6.7*10^{-3}$

Table II. The slopes of the PSII inactivation curves under separate and simultaneous UV-B abd visible illumination. Experiments were performed by using isolated thylakoids and Synechocystis 6803 cells. UV-B intensity was 6 μE m^{-2} s^{-1} in all experiments. In the last column the values show predicted slopes of the inactivation curves as calculated by the assumption of independent damaging mechanisms.

However, comparison of the separate and combined illumination protocols is straightforward only when the inactivation curves are plotted as a function of the illumination time.

It is important to note that the linear characteristic of the semi-logarithmic inactivation curves was retained in the combined UV-B plus visible illumination protocol, with 1.04 value for the Y axis intercept. From this result it follows that inactivation of the photosynthetic oxygen evolution in thylakoids exposed to visible and ultraviolet light simultaneously can be described by the single-photon-hit of single-target mechanisms, which act independently of each other. This conclusion is corroborated by simulation of the damage curve for the combined illumination protocol. In case of independent damaging events, the probability that PSII retains its activity under simultaneous UV-B and visible light exposure is given by the product of the probabilities that PSII survives the damage induced by the separately applied UV-B and visible illuminations. Thus, the damage curve of simultaneous illumination can be calculated by multiplication from the relative activities obtained for the separate illumination protocols. As shown in Fig. 22, this simulation gives a very good description of the measured values. On the semi-logarithmic plots, independent inactivation events are represented by additive slopes of the damage curves. According to the data presented in Table II, the additive behavior is valid for a wide range of light intensities.

The results obtained with isolated thylakoids show that UV-B and visible light impair PSII electron transport by non-interacting mechanisms, thus, there is no indication for a synergistic interaction between visible and UV-B light in damaging the PSII electron transport. The relative damaging efficiency of UV-B and visible photons can be estimated by converting the light exposure data to dose units. This calculation gives about 8.6×10^{-2} m^2/mol and 8.5 m^2/mol inactivation cross section for visible and UV-B photons, respectively, based on the incident photon flux. By taking into correction the attenuation of light in the optically thick samples as well as the concentration of PSII centers, the quantum yield of the photodamage can be estimated as 10^{-7} and 10^{-5} damaged PSII center per visible and UV-B photons respectively. The 100-fold difference in the quantum yield of photodamage by UV-B and visible photons is consistent with the different mechanism of these damaging events.

4.2.2 Loss of Flash-Induced Fluorescence in Thylakoids

The rate of oxygen evolution measured in the presence of artificial acceptors reflects the integrity of the whole electron transport chain from the water-oxidizing complex to Q_B. In order to probe the target sites of visible and UV photons within the PSII complex, we applied

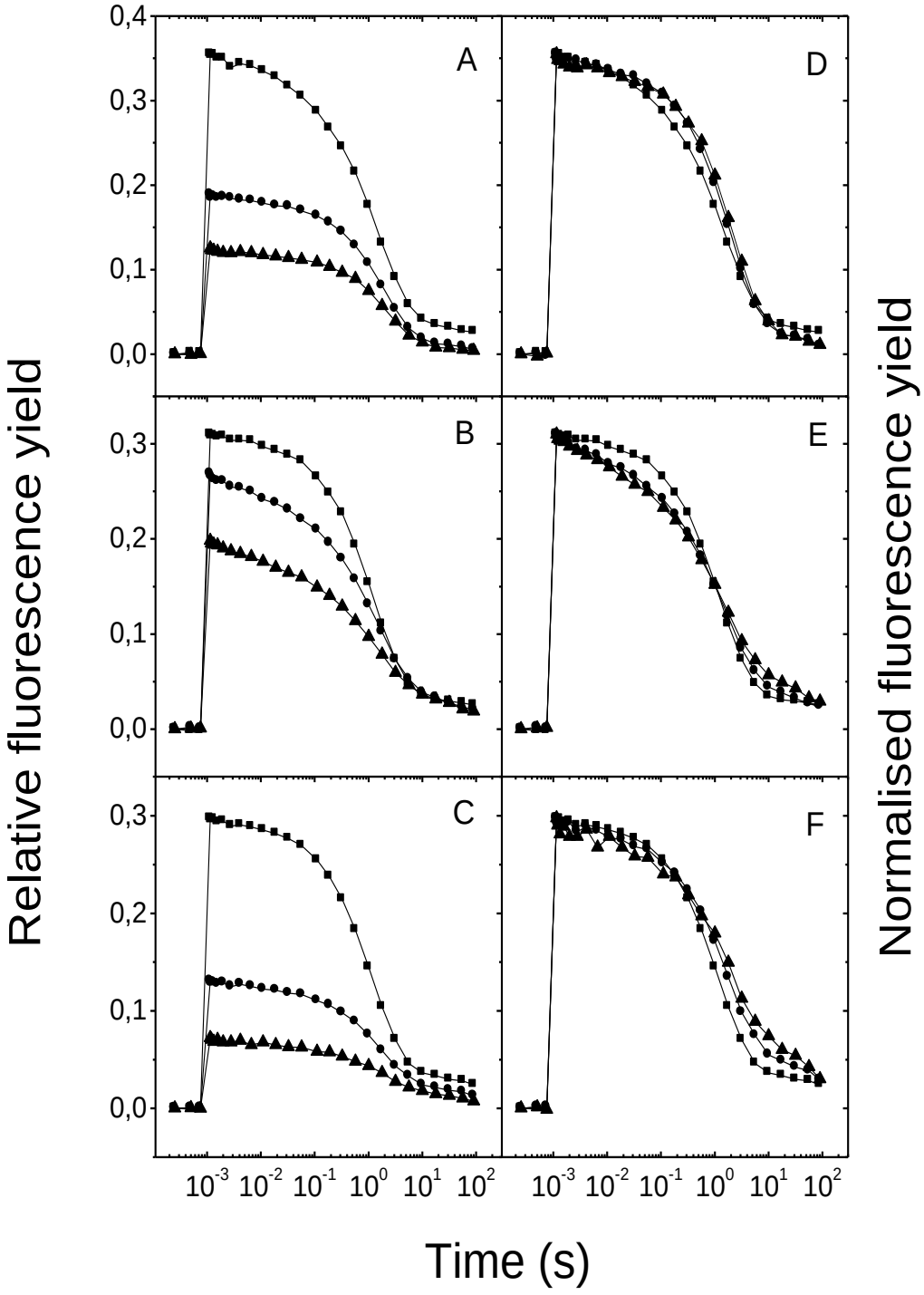

Figure 23. Effect of visible and UV-B light on flash-induced chlorophyll fluorescence in isolated thylakoids. Samples were illuminated by 325 μE m^{-2} s^{-1} visible light (A, D), 6 μE $m^{-2}s^{-1}$ UV-B light (B, E) and by the combination of the above visible and UV-B intensities (C, F). The applied illumination times were 0 (squares), 60 (circles) and 120 minutes (up triangles). On panels A, B and C the curves are shown without normalization, whereas on panels D, E and F the curves are normalized to the same initial amplitude.

flash-induced chlorophyll fluorescence measurements. The initial amplitude of the fluorescence signal is proportional to the number of PSII centers capable of reducing Q_A, whereas the relaxation kinetics reflect the reoxidation of Q_A^- via various pathways in the dark. When the Q_A to Q_B electron transfer step is blocked by DCMU, the fluorescence relaxation kinetics arises from the reoxidation of Q_A^- with donor side components. In non irradiated control thylakoids, the recombination partner of Q_A^- is the S_2 state of the water-oxidizing complex, which has apparent second order decay kinetics showing a sigmoidal shape on logarithmic time scale (Fig. 23). As a result of illumination with 325 μE m^{-2} s^{-1} visible light the initial amplitude of the fluorescence rise decreased, but the kinetics of the decay remained unaffected. In contrast to this, illumination with UV-B light induced not only the loss of amplitude, but led also to the appearance of fast decaying phase(s) in the relaxation kinetics.

Fast fluorescence decay of UV-B and UV-A irradiated samples in the presence of DCMU has been observed previously, and was assigned to recombination of Q_A^- with Tyr-Z^+ (P_{680}^+) in centers in which the Manganese cluster of water oxidation is inactivated (Vass et al. 1999, 2002). When UV-B and visible light was applied simultaneously, the amplitude decrease of the fluorescence signal was larger than those observed after the separate UV-B and visible light treatments. However, the fast decaying phase was not observed. In agreement with previous data, the present results show that irradiation by UV-B leads to the accumulation of some PSII centers in which electron transport is impaired at the Mn cluster of water oxidation. Donor side inhibited PSII centers are highly sensitive to visible light through the so called donor-side induced photoinhibitory mechanism (Blaubaugh and Cheniae 1990; Eckert et al. 1991). Rapid photoinactivation of these centers can explain the loss of the fast phase of fluorescence decay when UV-B is supplemented with visible light.

Semilogarithmic plots of the fluorescence amplitudes give straight lines with Y axis intercepts close to one (Fig. 24). The loss of initial amplitude under the combined visible plus UV-B illumination protocol could be simulated well by assuming independent damaging events. It is interesting to compare the time course of oxygen evolution and flash-induced variable fluorescence amplitude under the different illumination protocols. When thylakoids were illuminated with 325 μE m^{-2} s^{-1} visible light, oxygen evolution was inhibited only by 35-40% in 2h. However, the initial amplitude of flash-induced fluorescence decreased by 80% (Fig. 25). In the case of UV-B irradiation the time course of oxygen evolution and fluorescence amplitude changed in a parallel fashion. Under simultaneous exposure to UV-B and visible light, oxygen evolution was inhibited by 70% in 2h, but the fluorescence amplitude was decreased by more than 90% (Fig. 25). The more pronounced inhibition of

steady-state variable chlorophyll fluorescence as compared to oxygen evolution is well documented under visible light (see Maenpaa et al. 1995), and explained by the formation of a quencher state of PSII (Krause 1988; Critchley et al. 1999). Here we demonstrate that the same phenomenon is observed both under visible and visible plus UV-B illumination for the case of flash-induced variable chlorophyll fluorescence. In contrast to this, steady-state variable fluorescence underestimates the loss of oxygen evolution under UV-B treatment (Vass et al. 2000). Formation of a non-photochemical quencher, which affects the initial fluorescence amplitude has also been proposed (Larkum et al. 2001). However, according to the data show in the present work, the amplitude of flash-induced variable fluorescence gives a reliable estimation of PSII activity, in the 400-100% range, when UV-B radiation is applied without visible light.

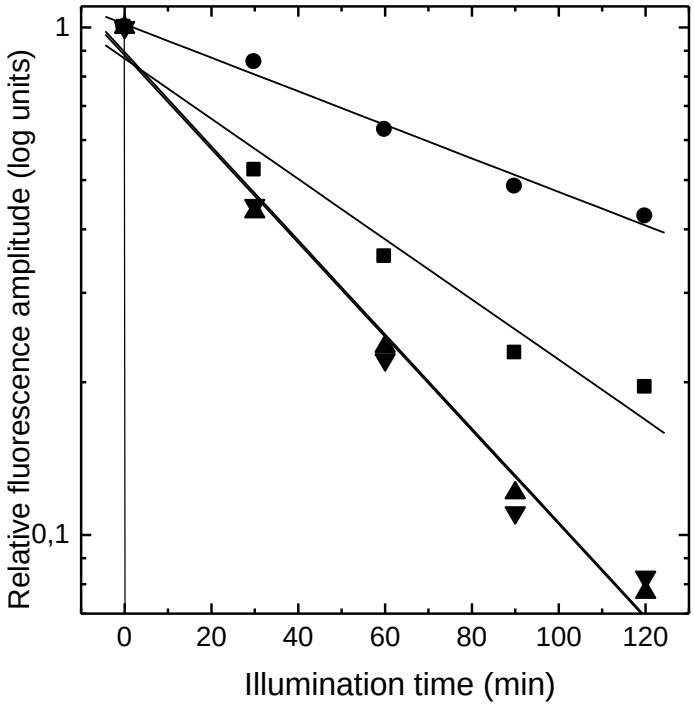

Figure 24. Semilogarithmic inactivation curves of flash-induced chlorophyll fluorescence amplitudes in isolated thylakoids. Samples were illuminated by 325 μE m^{-2} s^{-1} visible light (squares), 6 μE m^{-2} s^{-1} UV-B light (circles), and by the combination of the above visible and UV-B intensities (up triangles). For comparison the calculated inactivation by assuming independent damage mechanisms is also shown (down triangles).

Our data obtained with isolated thylakoids and *Synechocystis* cells, demonstrate that UV-B and visible photons inactivate PSII via single-hit of single-target mechanisms. In other words the inactivation of PSII does not need multiple photon hits of the same target, or cooperative single hits of more then one target. Importantly, in the absence of protein repair

capacity photodamage induced by visible and UV-B light proceed independently of each other, as shown by the good simulation of the inactivation curves obtained for the simultaneous illumination protocols using the assumption of independent damaging events. It has to be noted that although this feature is an important characteristic of the photo inactivation mechanisms, alone it does not permit the identification of the actual target species. However, our results are consistent with previous literature data showing that UV and visible photons have different target sites in PSII. Moreover it is also shown that the sensitivity of the important UV targets is not influenced significantly by the presence of visible light, and *vice versa*. Some earlier data in the literature suggested that the Q_A and/or Q_B acceptors could be more sensitive to UV-B in the semireduced then in the oxidized form (Greenberg et al. 1989; Melis et al. 1992). However more recent data shows that Q_A and Q_B are unlikely to represent major UV-B targets in PSII (Vass et al. 1996, 1999; Larkum et al. 2001). The lack of synergistic enhancement of photodamage in the presence of visible light, which can fully reduce Q_A and Q_B in thylakoids, supports this conclusion.

The appearance of the fast phase in the decay of flash induced chlorophyll fluorescence in the presence of DCMU is a very important feature, since it demonstrates the accumulation of PSII centers in which the Mn cluster is inactivated (Vass et al. 1999, 2002). This phenomenon was observed when UV-B illumination was applied alone and also under non-filtered sunlight, in agreement with the primary UV target being the water-oxidizing complex (Vass et al. 1999; Larkum et al. 2001). The lack of the fast phase of fluorescence relaxation under UV-B plus visible illumination in the thylakoids and lincomycin-treated cells most likely indicates that PSII centers whose donor side is damaged by UV-B are destroyed by visible light. Thus, a clear manifestation of donor side induced photoinhibition could occur under mixed UV-B and visible illumination. However, the small amplitude of the fast fluorescence decay seen under UV-B illumination alone indicates that sequential destruction of UV-B damaged PSII centers by visible light is unlikely to be the photodamage by mixed illumination.

It is important to note that fast phase in the fluorescence decay was not seen when photoinhibition was induced by visible light. Consequently, donor side damaged PSII centers do not accumulate in visible light, which is consistent with the acceptor side mechanism of photoinhibition.

4.2.3 Photodamage in Synechocystis 6803 Cells

Photodamage of PSII can be repaired via *de novo* synthesis of the D1 and D2 reaction center proteins and the net loss of activity is determined by the balance of the damage and the repair processes.

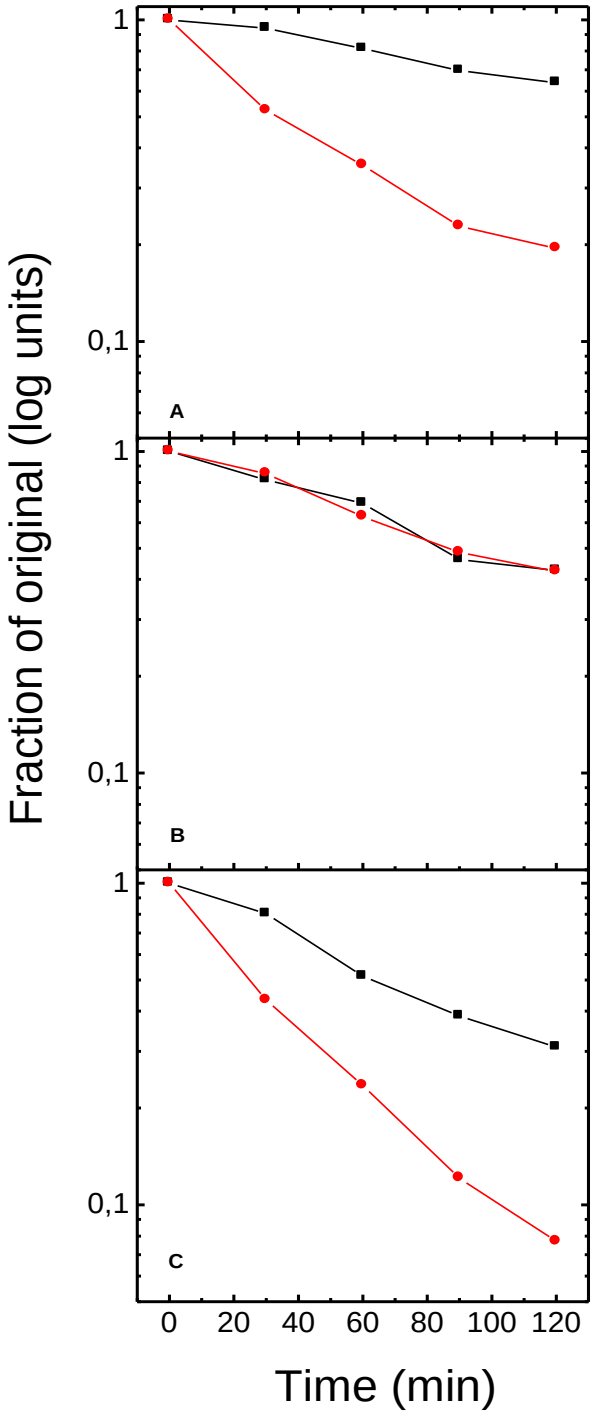

Figure 25. Comparison of oxygen rate and flash-induced chlorophyll fluorescence inactivation curves in isolated thylakoids. The oxygen evolution rates (squares) and the initial fluorescence amplitudes (circles) were plotted on a semilogarithmic scale for visible (A) and UV-B illumination (B) as well as for combined UV-B plus visible exposure (C).

Protein repair is regulated in a complex way by both visible and ultraviolet light (Aro et al. 1993; Sass et al. 1997; Mate et al. 1998; Vass et al. 2000; Sippola and Aro 2000). Therefore, it is important to understand the interaction of the two spectral regions in intact organisms. In order to verify if the results obtained for PSII photodamage with isolated tylakoids are applicable to intact cells or not, the effect of UV-B and visible light was first studied in *Synechocystis sp.* 6803 cells in which *de novo* protein synthesis was inhibited by lincomycin.

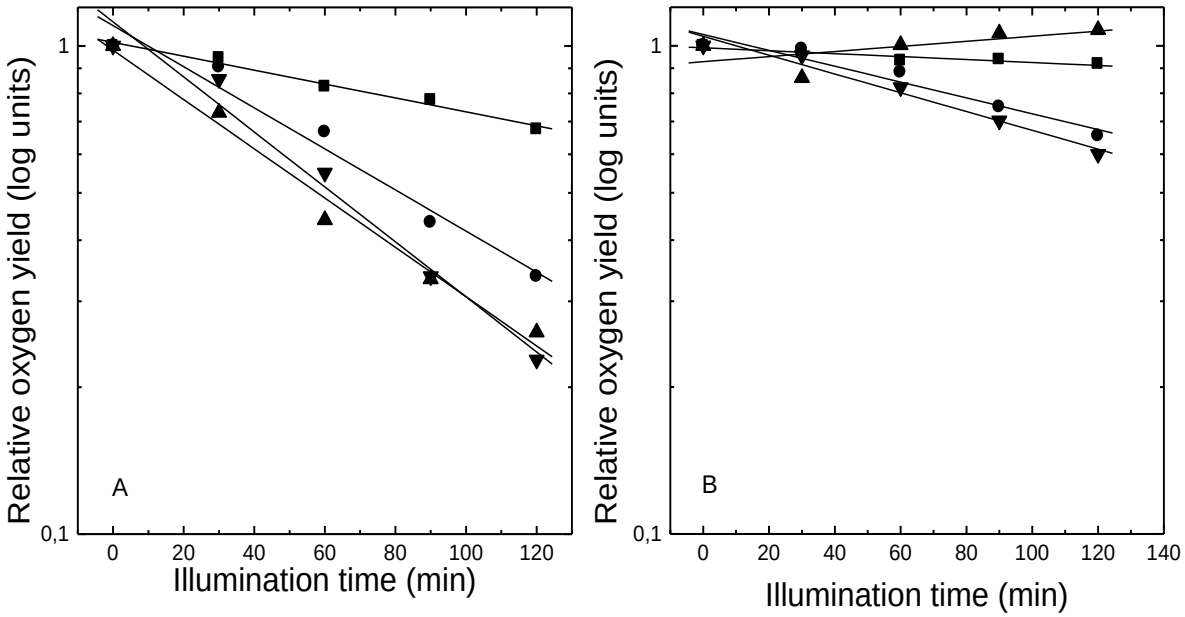

Figure 26. Semilogarithmic inactivation curves of oxygen evolution measured in Synechocystis 6803 cells. Samples in the presence (A) and absence of 300 μg/ml lincomycin (B) were illuminated by 130 μE m^{-2} s^{-1} visible light (squares), 6 μE m^{-2} s^{-1} UV-B light (circles), and the combination of the above visible and UV-B intensities (up triangles). For comparison the calculated inactivation obtained by assuming independent damage mechanisms is also shown (down triangles)

The semilogarithmic inactivation curves of oxygen evolution could be well described by straight lines with Y axis intercepts close to one (Fig. 26). In addition, the measured extent of oxygen evolution loss under UV-B plus visible light could be simulated by independent damage mechanisms in the 130-1300 μE m^{-2} s^{-1} intensity range (Fig. 26.A and Table II). However, the situation was quite different in intact cells, which are capable of *de novo* protein synthesis. On the first place, the damage induced by either visible or UV-B illumination applied separately is significantly smaller then that observed in the presence of protein synthesis inhibitor (Fig. 26.B). This is in agreement with previous findings showing that repair of damaged PSII centers is a constitutive process during visible and UV-B illumination (Aro et al. 1993; Sass et al. 1997). Even more important is the observation that the presence of low intensity visible light prevents the UV-induced loss of PSII activity. At higher light

intensities, the UV-induced damage was not prevented, or even got enhanced, and the difference between the slope of the measured and calculated inactivation curves became smaller or disappeared as observed at 650 or 1300 μE m^{-2} s^{-1} intensity, respectively (Table II). Measurements of flash induced fluorescence relaxation demonstrate the appearance of the fast

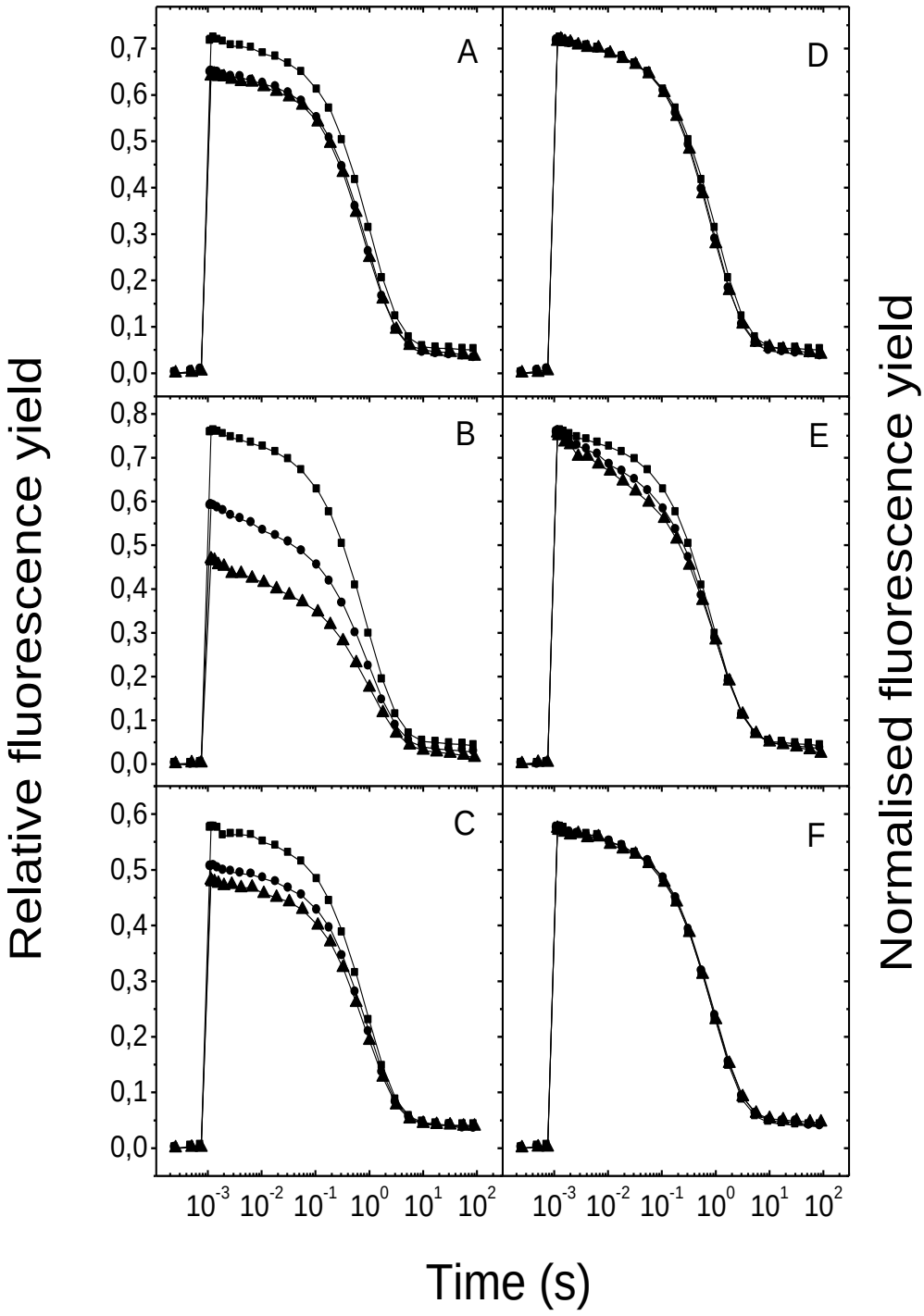

Figure 27. Effects of visible and UV-B illumination on flash-induced chlorophyll fluorescence in Synechocystis 6803 cells. Cells were illuminated with 130 μE m^{-2} s^{-1} visible light (A, D), 6 μE m^{-2} s^{-1} UV-B light (B, E) and by the combination of the above visible and UV-B intensities (C, F). The applied illumination times were 0 (squares, 60 (circles) and 120 min (up triangles). Panel A, B and C show curves without normalization, whereas on panels D, E and F the curves are normalized to the same initial amplitude.

phase when the cells were illuminated with UV-B alone, which was not observed when the cells were illuminated by visible light or by the mixture or visible and UV-B light (Fig. 27). Similar results were obtained in the presence of lincomycin, with even more pronounced fast phase in the UV-B irradiated cells (not shown). Comparison of the time course of the oxygen evolution and fluorescence amplitudes confirmed also the results obtained with thylakoids, namely the faster loss of fluorescence amplitude under visible and visible plus UV-B illumination as well as the close match of oxygen evolution and fluorescence loss under UV-B exposure (not shown).

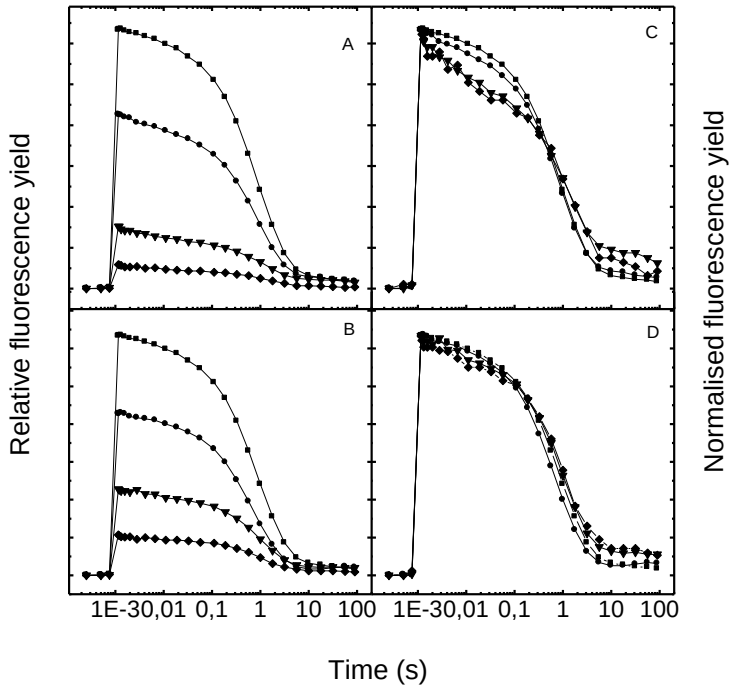

Fig.28. The effect of natural sunlight on flash-induced chlorophyll fluorescence in Synechocystis 6803 cells. Cells were exposed to natural sunlight of 1400 μE m^{-2} s^{-1} visible light, 10.4 Wm^{-2} ≈ 50 μE m^{-2} s^{-1} UV-A and 5.6 Wm^{-2} ≈ 14 μE m^{-2} s^{-1} UV-B intensity (A, C) or after filtering out the UV-A and UV-B components (B, D). The applied exposure times were 0 (squares), 5 (circles), 15 (down triangles) and 40 min (diamonds). Panels A, B show the curves without normalization, whereas on panels C, D the curves are normalized to the same initial amplitude.

Under natural conditions, the intensity of visible light is much higher than that of the UV-B. In direct sunshine under clear sky typically 1500-2000 μE m^{-2} s^{-1} visible intensity is accompanied by 8-10 μE m^{-2} s^{-1} UV-B. These conditions are best approximated by our mixed

protocol of 1300 μE m^{-2} s^{-1} visible light supplemented with 6 μE m^{-2} s^{-1} UV-B, in which the protective effect of visible light against UV-B damage was negligible. In order to characterize photodamage under natural conditions, *Synechocystis* cells were exposed to direct sunshine, and the activity loss was followed by flash fluorescence measurements. Exposure to unfiltered sunlight, containing UV-B and (UV-A) radiation led to a rapid loss of initial fluorescence amplitude, and induced the appearance of a fast decaying phase (Fig. 28). When the ultraviolet spectral range was filtered out the amplitude loss was decreased and the fast decaying phase disappeared.

In contrast to the lack of interference of UV-B and visible photons in damaging PSII function and structure there is clear interaction at the level of PSII repair. In intact *Synechocystis* 6803 cells protein repair is initiated by both UV-B and visible light when applied separately as shown by the different slopes of inactivation curves measured in the presence and absence of lincomycin. However, illumination with low intensity visible light (130 μE m^{-2} s^{-1}), which alone does not cause net loss of photosynthetic oxygen evolution, provides protection against UV-B damage. Since this protective effect is prevented by inhibition of protein synthesis it can be assigned to enhanced protein repair capacity. At higher visible light intensities, which alone induce inactivation of PSII activity, absolute protection against UV-B damage does not occur, i.e., the activity loss induced by combined illumination protocol is larger than that caused by UV-B or visible light alone. However, relative protection, i.e., smaller damage than calculated from assuming non-interacting damage mechanisms, was observed up to 1300 μE m^{-2} s^{-1} intensity. It is also of note that the extent of the relative protective effect decreased with increasing light intensities. This phenomenon might be related to the partial inhibition of protein repair by the reactive oxygen species induced by the strong illumination (Nishiyama et al. 2001). The experiments performed under natural sunlight show that even the enhanced repair capacity was not sufficient to completely abolish or repair all of the donor side UV damaged PSII centers in *Synechocystis* 6803 cells, which were grown under low light intensity. Long term acclimation to high light intensity may induce other defense mechanisms, which could help to ameliorate the consequences of the photodamage.

4.2.4 D1 Protein Degradation in Thylakoids

Photodamage not only inactivates the electron transport of PSII, but also destroys the protein backbone of the reaction center complex (Greenberg et al. 1989; Trebst and Depka

1990; Friso et al. 1993). We followed this protein degradation process by measuring the amount of the D1 protein. The protein loss was more pronounced for UV-B than for visible light, and was enhanced under the combination of visible and UV-B (Fig. 29). Simulation of the fraction of the PSII centers with degraded D1 protein by assuming independent damaging events for UV-B and visible photons yielded values close to the measured ones. This indicates that there is no significant interaction of UV-B and visible light at the level of damaging the protein structure of PSII reaction center either.

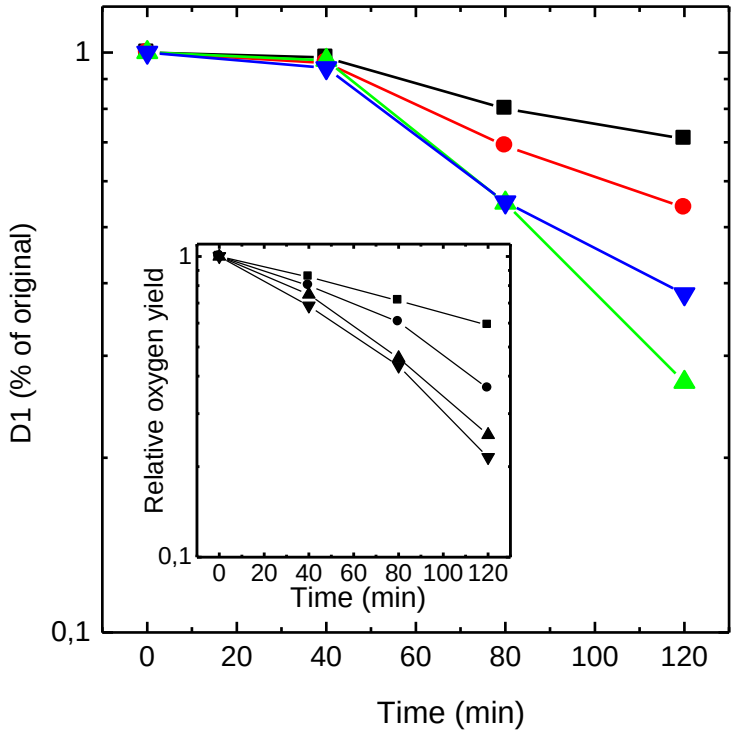

Figure 29. The effect of photodamage on the D1 protein amount in isolated thylakoids. Samples were illuminated by 325 μE m^{-2} s^{-1} visible light (squares), 6 μE m^{-2} s^{-1} UV-B light (circles) and by the combination of the above visible and UV-B intensities (up triangles). The amount of D1 protein was determined by imunoblotting. For comparison the calculated D1 protein loss is also shown by assuming independent damage mechanisms (down triangles). The inset shows the rates of oxygen evolution values measured in the same samples.

The data from this experiment can not be used for target theory analysis since aliquots for parallel protein determination and oxygen measurements were withdrawn from the same sample after different illumination times. This procedure decreased the sample volume significantly and therefore enhanced the average intensity during the course of the experiment, leading to continuous bending of the inactivation curves (see corresponding oxygen evolution data in the inset).

4.3 The Characterization of Different D1 Protein Forms from *Synecoccocus* PCC7942

As mentioned in the previous paragraph, the D1 protein subunit of the PSII is one of the main targets of UV-B radiation in photosynthetic cyanobacteria. However, the D1 protein is not fully conserved in plant the kingdom and different forms are present in different or even in the same species forming a small multigene family. The different forms of D1 are thought to have different characteristics in their resistance to UV-B irradiation stress. The D1 proteins subunit are encoded by the psbA genes. In contrast to higher plants that have just one type of psbA gene, in cyanobacteria there is a small gene family (Golden 1995). In Synechococcus PCC7942, a photosynthetic aquatic cynacobacteria, three form of psbA genes exists coding two different forms of D1 proteins (D1:1 and D1:2) (Golden 1986). Those two different forms are believed to have different responses to stress factors. The form D1:1 that is encoded by two psbA genes (psbA1 and psbA2) is the constitutive form of the protein that is present in the PSII protein complex under low light conditions. The D1:2 form is considered as a "stress" form, that replaces the D1:1 form when the cells are exposed to certain stress factors, such as high light and/or low temperature. (Schaefer and Golden 1989; Clarke et al. 1993).

In Synechocystis PCC6803 three forms of psbA genes are also present. However, their functions are different. The psbA1 gene is silent and have not been proved to be expressed under any experimental or natural conditions. The other two forms, psbA2 and psbA3 code the same form of D1 protein subunit but have different UV-B expression patterns (Jansson et al. 1987).

Considering these, we wanted to characterize the two different forms of D1 from Synechococcus PCC7942 and to elucidate their individual resistance to UV-B damage. For achieving this, we used three different genetically engineered strains (D1:1, D1:2, and D1:1,2) as well as the control wild types of Synecococcus PCC7942 and Synecocystis PCC6803. The D1:1 strain contains the D1:1 form of the D1 protein from Synecococcus PCC7942 expressed in Synechocystis PCC6803. The D1:2 strain contains the D1:2 form of the D1 protein from Synecococcus PCC7942 expressed in Synechocystis PCC6803. The strain D1:1,2 contains a chimeric form of the D1 protein obtained by the expression of a gene engineered to contain part of the D1:1 and the part of the D1:2 protein forms expressed also in Synechocystis PCC6803. The extraction of the D1 protein from their natural environment and their repression on another organism allows the reliable characterization of the proteins without influences from their genetic or structural background.

4.3.1 Thermoluminescence Characteristics

In frozen cells the position of the B band is practically not affected by mutations. However, the TL intensity is only 40-60 % of that in the WT cells (Fig. 30A.). The Q band measured in the presence of DCMU (Fig. 30B) is somewhat downshifted in the D1: 1 and D1:2 mutants and up shifted in D1:1, 2 which can indicate a slight destabilization of the $S_2Q_A^-$ redox pair due to modifications of the water oxidation complex or of Q_A site. The intensity was decreased to a similar extent as in the absence of DCMU.

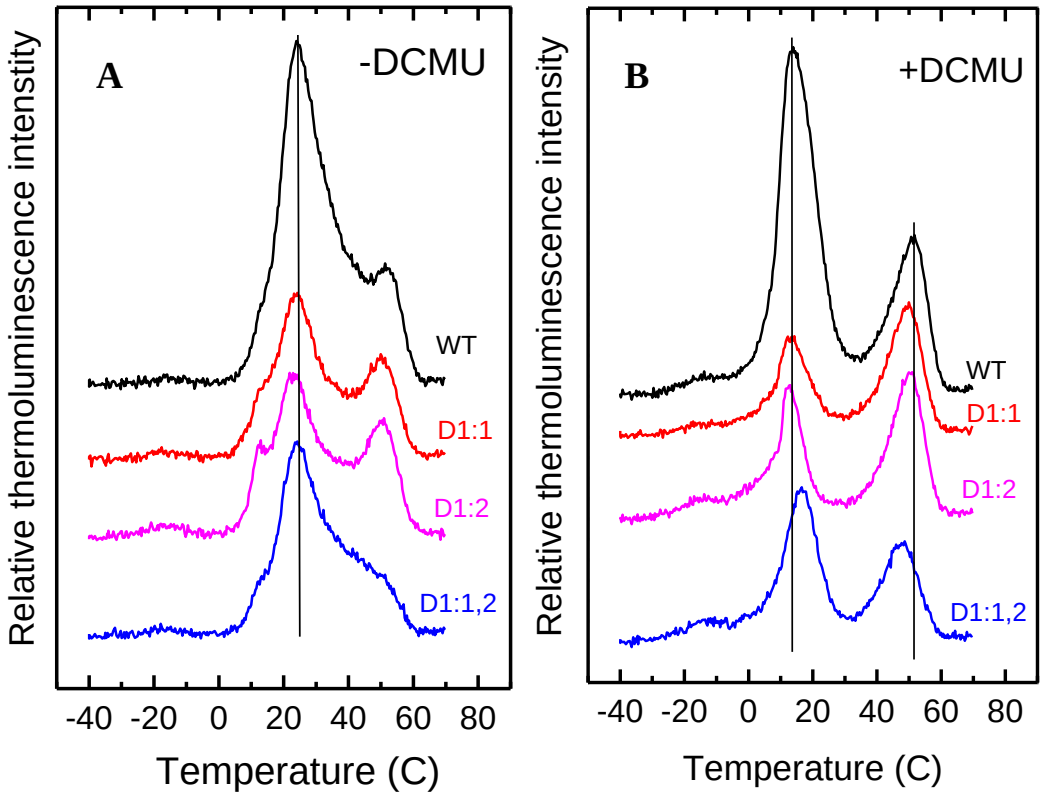

Figure 30. Relative TL profiles measured in the absence (A) and presence (B) of DCMU. For measurements we used 400 µl samples of whole Synechocystis 6803 cells containing 50 µg chl. Excitation was done by one flash at –20 C. Luminescence emission was then recorded from –40 C to 70 C.

The measurements in unfrozen cells show that the B band is up shifted from 25 °C in the WT *Synechocystis* 6803 in the D1: 1, D1:2 and D1: 1, 2 mutants (Fig. 31.A.). Comparative measurements on the *Synechococcus* 7942 cells, which produce mainly the D1: 1 form under the studied conditions show the position of the B band at around 35 °C. This is about 10 °C higher than in WT *Synechocystis* 6803 or in the D1: 1 mutant. The higher peak position of the B band is *Synechococcus* is consistent with its more mesophyllic nature, i.e.

higher optimal growth temperature under natural conditions, as compared with *Synechocystis*. This feature shows the higher stability of the $S_2Q_B^-$ charge pair. It is interesting to note that the presence of the D1: 1, 2 protein in the *Synechocystis* background induces only a small increase in the peak temperature. This indicates that the stability of the $S_2Q_B^-$ charge pair is determined not only by the D1 protein, but also the other subunits of the reaction center complex. In unfrozen cells the Q band is located below 0 °C in *Synechocystis* and can't be detected in the measurements starting from 0 °C (Fig. 31.B.). However, the high temperature tail

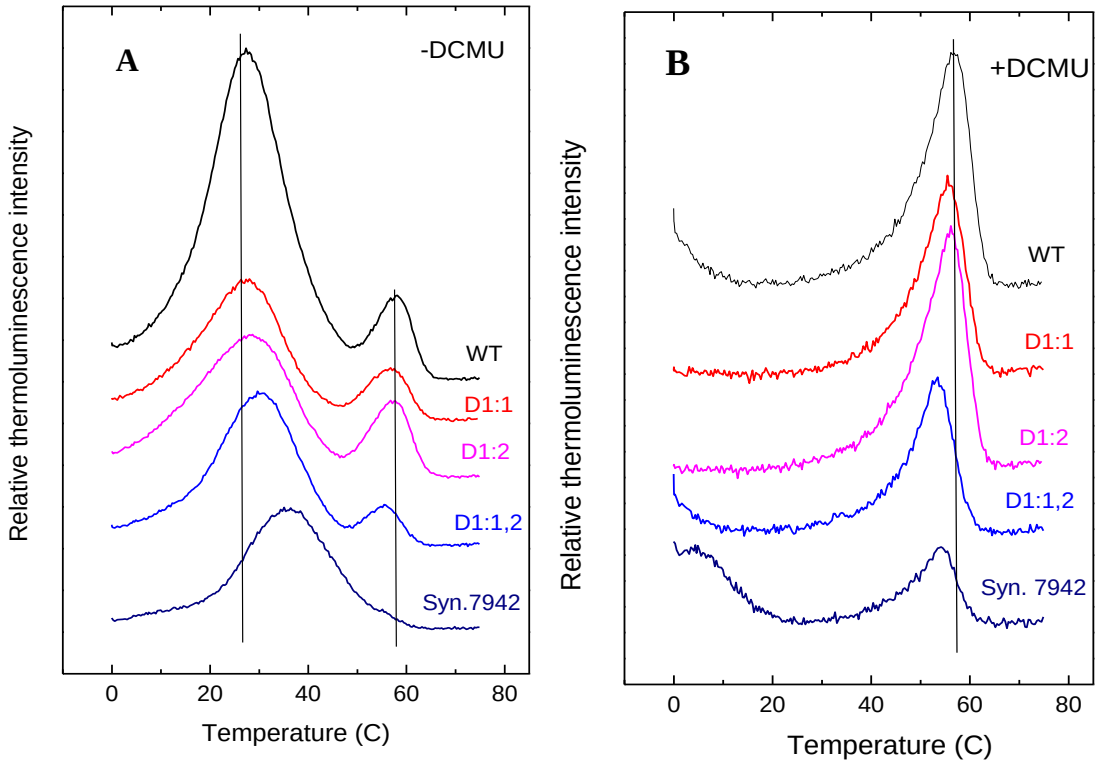

Figure 31. Relative TL profiles measured in the absence (A) and presence (B) of DCMU. For measurements we used 400 µl samples of whole Synechocystis 6803 cells containing 50 µg chl. Excitation was done by one flash at 0 C. Luminescence emission was then recorded from 0 C to 70 C.

of the Q band can be seen in the WT cells, but not in the D1:1, and D1:2 mutants. This indicates that the $S_2Q_A^-$ charge recombination is destabilized in these strains. The high temperature tail of the Q band can be seen in the D1: 1, 2 strain, and the peak itself in *Synecococcus* 7942. These data show that the $S_2Q_A^-$ recombination has about the same stability in D1: 1, 2 as in WT, and increased in *Synechococcus* 7942.

4.3.2 Flash Oxygen Characteristics

The pattern of flash-induced oxygen evolution was modified by all of the mutations (Fig. 32.). If in the case of *Synechocystis* PCC6803 WT the pattern is normal showing at least three, maybe four oscillations with the maximum of the oxygen released on the third flash, in the case of the mutants not more that two oscillations are observed with the maximum on the flashes 3 and 4. This led to decreased steady-state values compared to the wild type, the mutants D1:1 and D1:2 showing similar values but higher then D1:1,2. The miss parameters, were increased in all the mutants with no significant differences between them, as well as increased proportion of the S_0 state in the dark except for the chimeric D1:1,2 mutant as seen in Table III. Unfortunately we could not measure flash-induced oxygen signals from *Synechococcus* cells, thus direct comparison of the mutants and *Synechococcus* is missing so far. However, it is possible that insertion of *Synechococcus* D1 protein into the reaction center of *Synechocystis* modifies the functioning of the water-oxidizing complex.

Parameter/strain	**050/Wt**	**011/D1:1**	**012/D1:2**	**M13/D1:1,2**
S_0	33.3	37.6	37.7	27.5
Miss	19.3	24.1	27.1	27.9
D hit	4.1	4.8	4.4	5.9

Table III. Parameters of flash-induced oxygen evolution

4.3.3 Flash Fluorescence Characteristics

In the absence of DCMU the relaxation of flash-induced fluorescence is not affected by the mutations (Fig. 33.). This shows that the $Q_A \rightarrow Q_B$ electron transfer step is not modified by the replacement of native *Synechocystis* 6803 protein with D1: 1 or D1:2 of *Synechococcus*. Although the relaxation kinetics is somewhat slower in the *Synechococcus* 7942 than in the WT *Synechocystis* 6803 cells, all the mutants follow in this case the trend of the *Synechocystis* 6803. From this we can conclude that the donor side of the mutated PSII complexes does not significantly differ from that of *Synechocystis* 6803 (Table IV.a)

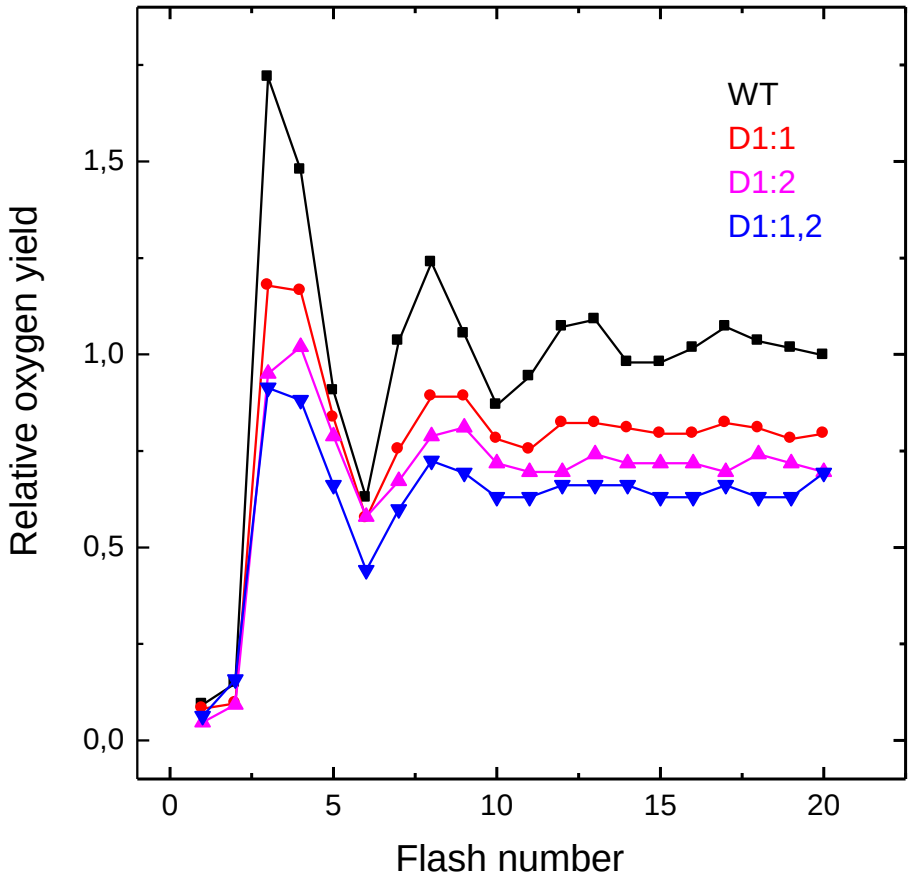

Fig. 32. Flash-induced oscillation of oxygen evolution from cells of the Synechocystis 6803 wild-type (WT) and mutants. Oxygen yield was obtained from a sequence of 20 exciting flashes of frequency 1 Hz in the 40 µg chl of cells.

When the relaxation was measured in the presence of DCMU a marked difference was observed between the WT and mutant *Synechocystis* 6803 cells (Fig. 33.). The fluorescence decay was accelerated in the D1:1 and even more in the D1:2 mutant, indicating that the stability of the $S_2Q_A^-$ charge pair was decreased. In the chimeric D1: 1, 2 mutant a fast phase was also observed (Table IV.b). The relaxation in the *Synechococcus* 7942 cells is slower than in the *Synechocystis* 6803, which shows the increased stability of $S_2Q_A^-$ recombination in agreement with the TL data.

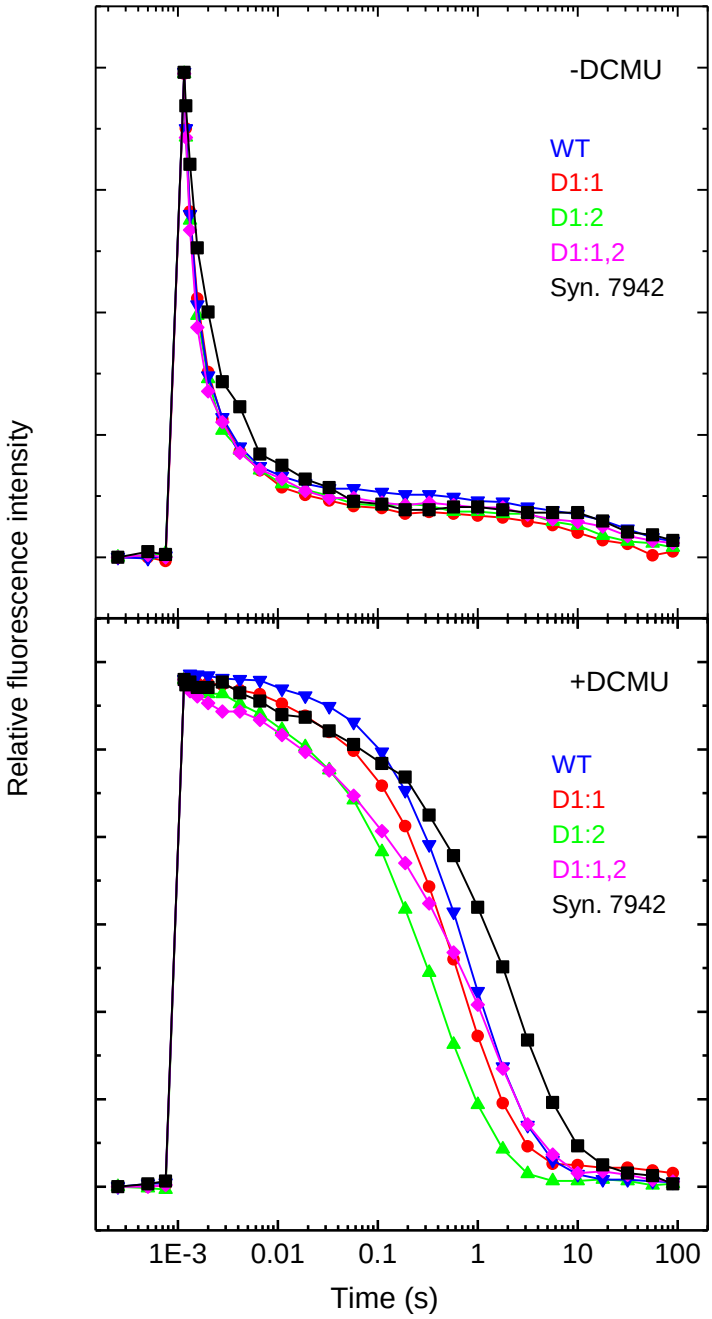

Fig. 33. Relaxation of the variable fluorescence after a single saturating flash given to cells of the Synechocystis mutants. Cells were dark adapted for 10 min and then the variable fluorescence was followed before and after a saturating flash using the P.S.I. double modulated fluorometer in the absence (upper panel) or presence of 10 μM DCMU (lower panel)

a)
- DCMU

Strain	Fast phase		Middle phase		Slow phase	
	A1 (%)	**T1 (ms)**	**A2 (%)**	**T2 (ms)**	**A3 (%)**	**T3 (s)**
050/Wt	72.4	0.29	19.66	3.15	7.88	9.15
011/D1:1	72	0.33	20.22	4.29	7.8	8.02
012/D1:2	74.7	0.31	17.1	4.56	8.13	12.16
M13/D1:1,2	76.5	0.26	17.06	3.85	6.48	7.73
S7942	67.5	0.55	23.2	8.1	9.2	35.54

b)
+DCMU

Strain	Fast phase		Slow Phase	
	A1 (%)	**T1 (ms)**	**A2(%)**	**T2(s)**
050/WT	0	5	100	0.64
011/D1:1	3	1.94	97	0.45
012/D1:2	7.5	2.45	92.5	0.25
M13/D1:1,2	18.6	11.6	81.4	0.74
S7942	10.7	10.9	98.3	1.63

Table IV. Parameters of fluorescence relaxation

4.3.4 The Effect of UV-B Radiation

To asses the stress resistance of the mutants we exposed them to UV-B radiation in the presence and absence of protein synthesis inhibitor lincomycin. In the presence of lincomycin UV-B induced loss of PSII activity was larger in both mutants than in the WT *Synechocystis* cells (Fig. 34 upper panel). The damage was more pronounced in the D1:1 than in the D1:2 construct. This can lead us to conclude that the two forms of D1 from *Synecococcus* 7942 have different resistance capability to stress, at least to UV-B damage. Fig. 34 clearly shows that D1:1 is more sensible than the D1:2 form after 90 minutes of UV-B stress in the absence of any protein repair. Also the fact that both the D1:1 and the D1:2 forms are more sensible to UV-B damage than the wild-type *Synecoscystis* 6803 cells suggests that the form of D1 present in *Synechocystis* 6803 may be the most resistant to this stress from those we tested. However, this conclusion can be misled by the fact that the overall stress resistance of the *Synecococcus* 7942 forms may be decreased by the less than perfect interactions between the proteins and its molecular environment.

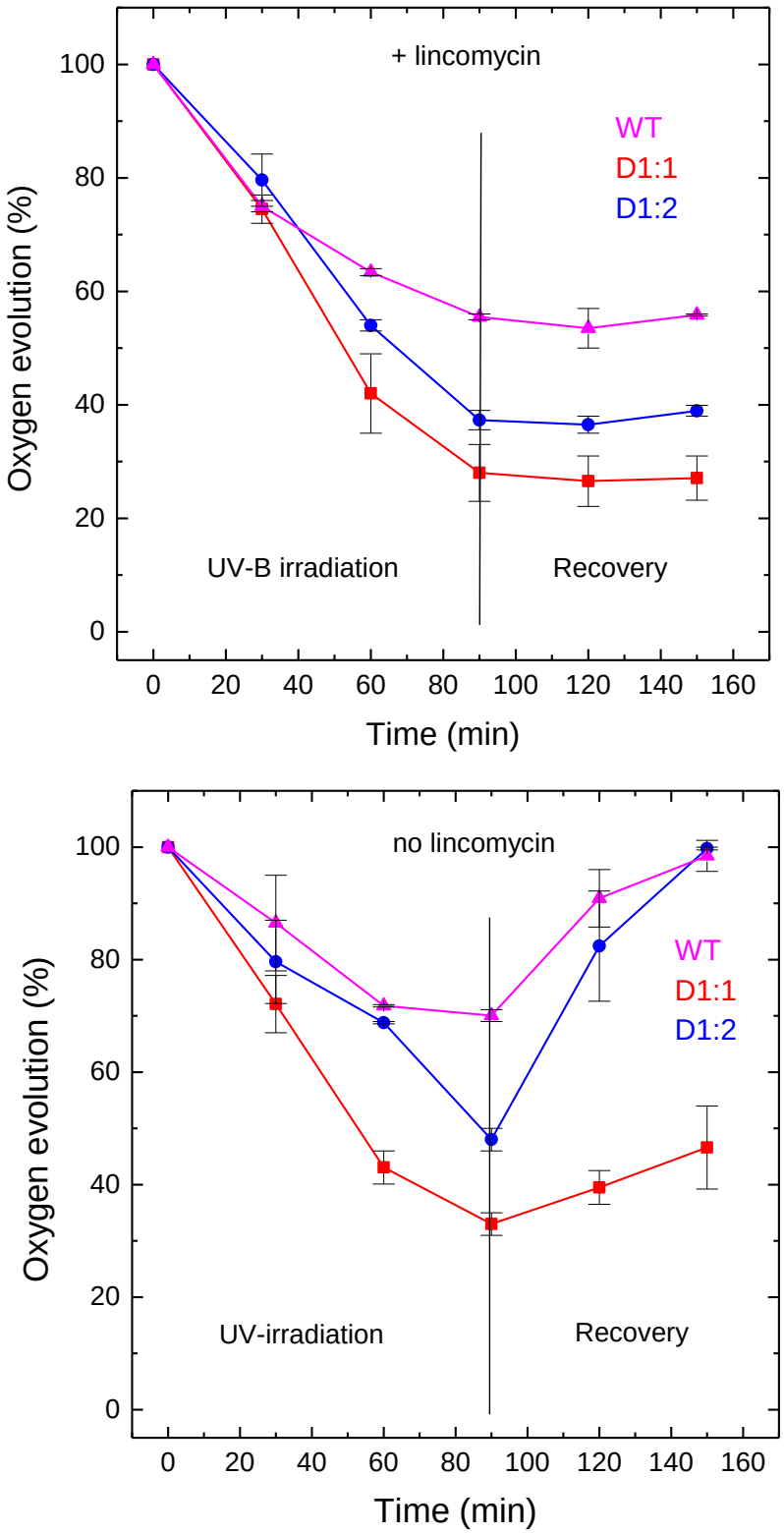

Fig. 34. Time course of the PSII inactivation in the mutant cells under increased UV-B radiation in the absence (lower panel) or presence of lincomycin (upper panel). Samples were covered with a 0.1mm cellulose acetate filter. UV-B light intensity was 6μE m^{-2} s^{-1} in the sample. Recovery was done at 30^0C in 100 μE m^{-2} s^{-1} visible light.

In the absence of lincomycin the sensitivities of the mutants, especially D1:1 to UV-B radiation was higher than that of the WT (Fig. 34 lower panel). Also, except for D1:1 the cells were less damaged than in the presence of lincomycin.

There was also a marked difference in the ability of the mutant cells for recovery after the UV-induced damage. The D1:2 containing cells recovered fully, but the recovery process was largely retarded in the D1:1 containing cells (Fig. 34 lower panel). These data are in agreement with the previous results of Campbell et al. showing that the *Synechococcus 7942* show higher UV-B tolerance when the D1:1 form is replaced by the D1:2 form.

From these data we can conclude that the ability of the different D1 forms to cope with the UV-B damage, as well as their ability to recover after the damage, is different. D1:1 form seems to be more sensible to the damage and shows a depleted capacity to recover after damage as compared to D1:2 form. This difference can come from structural differences induced by the difference in the aminoacid composition between the two forms. Also both forms seem to show a higher sensibility to UV-B damage as compared to WT *Synechocystis* 6803. This may be caused by the imperfect fit of the foreign D1 proteins in the existing PSII centers.

4.4 The Role of DNA Damage in PSII Repair

DNA is a well known target of UV-B irradiation not only in non-pigmented cells, but also in photosynthetic organisms. The repair of UV damaged PSII proceeds via *de novo* protein synthesis, which requires the transcription of DNA encoding the D1 and D2 reaction center subunits. Accumulation of damaged DNA could obviously hamper the transcription process and retard the protein-synthesis-dependent repair of PSII. The repair proceeds via *de novo* protein synthesis. In order to create a suitable experimental system to study this question a mutant that have the *slr0854* gene inactivated was used. These gene encodes a type-I photolyase enzyme in S*ynechocystis* 6803 This mutant was unable to grow under UV-B light complemented with visible illumination, which conditions did not affect the wild-type cells (data not shown). However, the photolyase deficient cells could grow as wild type under visible light, in the absence of UV-B irradiation. This results shows that the photolyase encoded by the *slr0854* gene is indispensable for the growth in the presence of UV-B irradiation, but DNA damage which could occur under normal growth conditions is either not significant or can be repaired by systems other than photolyase.

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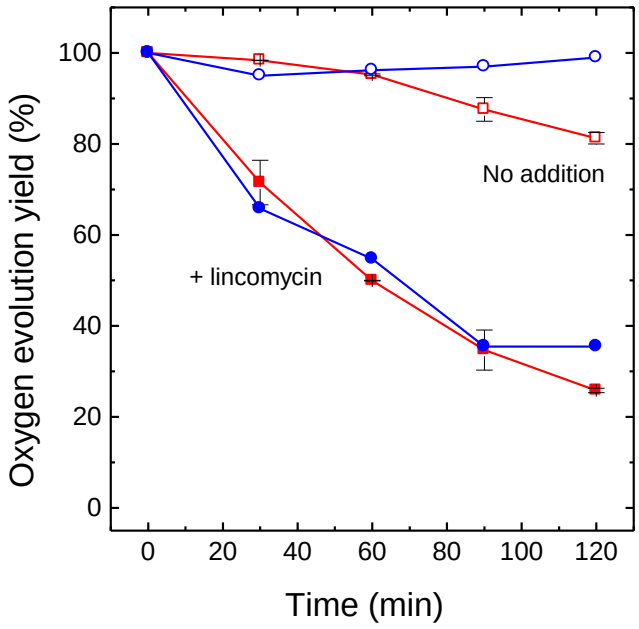

Fig. 35. The effect of potolyase (slr0854) inactivation on the UV-B induced loss of PSII activity. Wild-type (circles) and Δslr0854 cells (squares were cultured under visible light. The cultures were exposed to UV-B radiation complemented with visible light in the absence(open symbols) and presence of lincomycin (closed symbols).

To study the role of DNA damage in the protein-synthesis-dependent repair of PSII the photolyase deficient cell cultures, which were grown under visible light, were exposed to UV-B irradiation. When protein synthesis was blocked by lincomycin, the oxygen evolution activity was inhibited with the same rate in the mutant as in the wild type. In the presence of lincomycin, UV-B induced loss of oxygen evolution was not significantly different in the mutant and wild type cell up to one hour, but after longer illumination the activity of the mutant was lost somewhat faster than that of the wild type cells (Fig. 35). According to these data the lack of photolyase activity does not influence the damage of PSII complex by UV-B radiation. However, the efficiency of protein repair is somewhat slowed down after relatively short (few hours) exposure times, showing that unrepaired DNA accumulates and retards *de novo* synthesis of the D1 and D2 reaction center subunits required for the restoration of PSII activity.

4.5 Damage of the Phycobilisomes by UV-B

UV-B radiation affects not only the electron transport processes mediated by the PSII complex, but also the light harvesting antenna systems. In cyanobacteria light capture is performed mainly by phycobiliproteins which form large rod-like structures, the so called phycobilisomes. Prolonged exposure to UV-B radiation damages the phycobilisomes, leading to the loss of their characteristic absorption at 600-650nm (Fig. 36) and inhibits the transfer of energy to the photosynthetic reaction centers (Lao and Glazer 1996; Sinha et al. 1995; Pandey et al. 1997). However, the UV-sensitivity of phycobilisomes relative to PSII has not been studied previously. In order to clarify this point we monitored the activity of PSII in parallel with the integrity of phycobilisomes, by measuring their absorption peak at 626 nm in *Synechocystis* 6803 cells. Under illumination with 6 μE m^{-2} s^{-1} UV-B complemented with 250 μE m^{-2} s^{-1} visible light oxygen evolution was stable during the course of the experiment

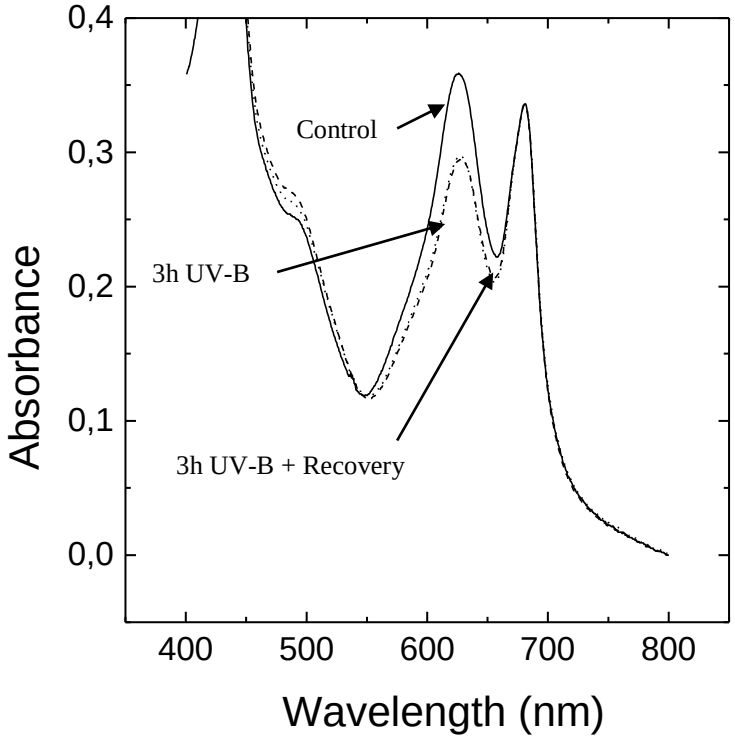

Fig. 36. The effect of UV-B radiation on the absorption of phycobilisomes. Synechocystis 6803 cells were irradiated with 6μE m^{-2} s^{-1} complemented with 250 μE m^{-2} s^{-1} visible light and the absorption spectrum of the cell suspension was measured after the indicated exposure times. After the UV-B treatment the samples were transferred to visible light for a 3 hours recovery period.

(Fig. 37 upper panel). Under these conditions oxygen evolution was completely inactivated within one hour, but the time course of the phycobilisome damage was the same as in the absence of lincomycin.

These results demonstrate that in the absence of protein reapir UV-B induced damage of phycobilisomes occurs much slower than that of PSII. However, in cells which are capable of *de novo* protein synthesis, PSII is efficiently repaired when the UV-B radiation is removed from the illumination protocol. In contrast, the restoration of phycobilisome was observed only after 24h (Fig. 37 shows only the first three hours of the recovery). Since under our conditions the doubling time of the cells is 10-12 hours, it is quite likely that recovery of phycobilisome function requires the development of new cells, via cell divison. Comparison of PSII and phycobilisomes provide an interesting example for a highly UV-sensitive, but well repaired component in contrast to a low sensitivity, but inefficiently repaired component.

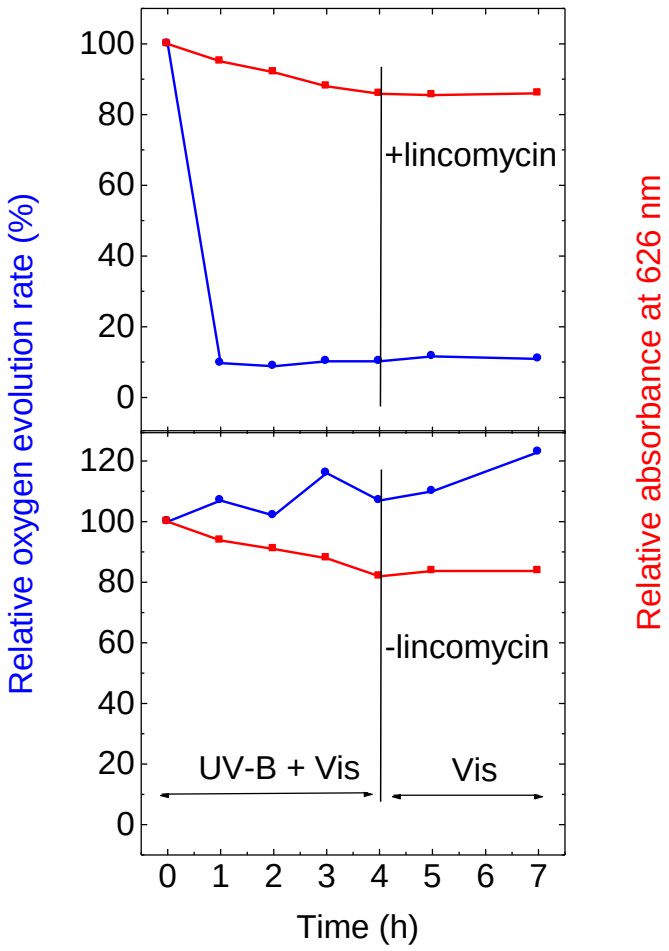

Fig. 37. UV-B induced damage of phycobilisomes and oxygen evolution. Synechocystis 6803 cells were irradiated in the presence (upper panel) and absence of lincomycin (lower panel). The integrity of phycobilisomes was quantified by measuring absorbance ar 626 nm (squares), and PSII activity was followed by measuring the rate of oxygen evolution (circles).

5. Conclusions

From our present work we can conclude that:

- UV-B radiation is an important damaging factor for the photosynthetic apparatus.
- The UV-B sensitivity of cyanobacterial cells depends on environmental factors such as temperature.
- UV-B and visible light damages the photosynthetic apparatus via independent mechanisms and the effect is additive in experimental systems that are not able of protein synthesis such as isolated tylakoid membranes or cyanobacterial cells in the presence of lincomycin.
- In intact cells, the visible lights interacts with UV-B damage by enhancing the protein synthesis capacity of the cells and speeding up the D1 protein turnover. However, this effect that is evident at low visible light intensities becomes less and less important as the intensity of the visible light increases and becomes itself a damaging factor inducing photoinhibition. At very high visible light intensities the stimulation of protein synthesis becomes insignificant and the UV-B damage and the photoinhibition effect show additive feature
- The mutants expressing different D1 forms (D1:1 and D1:2) have shown different sensitivities to UV-B. The rate of damage is different regardless of protein synthesis with the mutant expressing the D1:2 form being more resistant to damage then the one eexpressing D1:1. However, both of them were more sensible then the WT. In addition the rate of repair was affected especially in the cells containing the D1:1 form.
- The absence of photolyase dependent DNA-repair does not affect the rate of damage of PSII. PSII repair is affected after longer times as a consequence of the accumulation of DNA-damage. However, the photolyase is needed for the cells growth in the presence of low UV-B intensities added to the normal visible light illumination.
- The UV-B damage of the phycobilisomes is slow and the repair capacity is low as compared to the fast rate of damage and efficient repair of PSII.

6 List of Publications

1. **Sicora, C.** and Vass, I. (2000) "The interaction of visible and UV-B light in damaging the electron transport of Photosystem II in the cyanobacterium *Synechocystis* 6803" Plant Physiol. Biochem. 38 (suppl.): 110
2. **Cosmin Ionel Sicora,** Mihail Dragan-Bularda and Imre Vass (2000) UV-B-Induced Damage and Recovery of Photosynthetic Activity in the Cyanobacterium *Synechocystis* sp. PCC 6803. Studia Universitatis Babes-Bolyai, Biologia, XLV, 2, 2000, pp 82-90.
3. I Vass, E Turcsányi, L Sass, A Szilárd, **C Sicora**, Z Máté, É Hideg, F. Nagy, A Viczián (2001) Damage and repair of Photosystem II under exposure to UV radiation. Proc. 12th Int. Congress on Photosynthesis, Brisbane, Australia
4. **Cosmin Sicora** and Imre Vass (2001) The Interaction of Visible and UV-B Light in Damaging the Electron Transport of Photosystem II in the Cyanobacterium *Synechocystis 6803***.** Satellite Meeting of the 12th International Congress on Photosynthesis, Aug 13-17, Heron Island, Australia (2001) pp. 15**4.**
5. **Cosmin Sicora,** András Szilárd, László Sass, Enikő Turcsányi, Zoltán Máté and Imre Vass (2003) UV-B and UV-A radiation effects on photosynthesis at the molecular level. NATO ASI Series Environmental UV radiation: Impact on Ecosystems and Human Health and Predictive Models. (Book chapter-in press)
6. **Cosmin Sicora,** Zoltan Mate and Imre Vass (2003) The interaction of visible and UV-B light during photodamage and repair of photosystem II. Photosynthesis Research 75: 127-137.
7. M. Tichy, L. Lupinkova, **C. Sicora**, I. Vass, O. Prasil and Josef Komenda (2003) *Synechocystis* 6803 mutants expressing distinct forms of the photosystem II D1 protein from *Synechococcus* PCC 7942: Relationship between the D1 sequence and sensitivity to visible and UV-B radiation. Submitted to Biochem, Biophys. Acta.

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8 Summary

The aims of the present study were:

- Investigation of the UV-B damaging effect on PSII and the recovery of the activity under visible light conditions.
- Investigation of the temperature influence on the kinetics of UV-B stress on cyanobacterial cells.
- Elucidation of the relationship between visible light and UV-B light in damaging the PSII activity.
- Determination of the importance of UV-B induced DNA-damage, in relation to the damage at the photosynthetic electron transport level.
- Comparison of the two different cyanobacterian response strategies to UV-B damage from *Synechocystis* sp and *Svnechococcus* sp.

From our investigations we obtained the following results:

1. UV-B induced damage and recovery of the photosynthetic activity in Synechocystis sp. PCC 6803. In intact Synechocystis cells UV-B induces a decrease in oxygen evolution as a consequence of decrease in the photosynthetic activity. Under our experimental conditions a decrease of about 50 % of the initial oxygen evolution rate was obtained in about 120 minutes. The Synechocystis cells have the capacity of recovery after UV-B damage if exposed to visible light.
2. From our study it is evident that temperature is also one factor which influences the extent of the damage caused by UV-B and the rate of recovery. To prove that we used a chemical agent, benzyl-alcohol, that is known to affect the fluidity of cell membranes in order to induce physical changes that will be similar by those of temperature. During these experiments we observed that BA has an inhibitory effect on the function of the electron transport chain that may be caused by the inhibition of *de novo* protein synthesis.
3. UV-B and visible light interaction in damaging the PSII activity. In our experiments we used three experimental models: intact Synechocystis cells, Synechocystis cells treated with lincomycin, and tylakoid membranes isolated from spinach.The experimental protocols used were treatment with visible light of different intensities, UV-B light, and the combination of the two. When there is no repair, in lincomycin treated cells and isolated thylakoids, the inhibition of oxygen evolution by visible and UV-B light show an additive feature when applied together. This suggests that UV-B light and visible light damages the photosynthetic apparatus independently. When the repair mechanisms are intact this effect is

shadowed by the fact that visible light is triggering the protein synthesis dependent repair mechanisms.

4. Role of DNA repair. We used in our experiments a mutant which had the photolyase gene inactivated and consequently lacks the light induced repair of DNA damage. In contrast to WT cells this mutant is not able to grow when visible light is supplemented by UV-B due to accumulation of DNA damage. However on a short time scale (up to a few hours) there is no significant difference on the rate of damage induced by UV-B and visible at the PSII level.

5. The difference between the two D1 forms from Svnechococcus regarding the UV-B sensitivity. In Svnechococcus there are three different genes coding for two forms of D1 protein (D1:1 and D1:2). In order to see the difference between the two forms of D1 in regard to UV-B sensitivity we used mutants of Synechocystis which contain the gene for D1:1 and D1:2 respectively from Svnechococcus. The mutant with D1:1 was more sensible than the mutant with D1:2. However both the mutants containing D1:1 and D1:2 showed a higher rate of damage and a lower capacity of recovery than WT.

Összefoglaló

A Földet érő napsugárzást a fotoszintézis szempontjából három nagy spektrális régióra lehet osztani: UV-B (280-320 nm), UV-A (320-400 nm) és a fotoszintézis szempontjából aktív PAR (400-700 nm). Az UV-B sugárzást a sztratoszférában található ózon réteg elnyeli. Sztratoszférikus ózon réteg az elmúlt 30 év során az emberi tevékenységnek köszönthetően fokozatosan csökken, így egyre több UV-B sugárzás éri el a Föld felszínét és az óceánok élettanilag fontos 10-15 méteres mélységét. Az UV-B sugárzás fontos célpontja a növényekben a fotoszintetikus apparátus, amelynek a legérzékenyebb része a fényenegia átalakító második fotokémiai rendszer. Az UV-B sugárzás a második fotokémiai rendszer D1 és D2 fehérje alegységeinek lebomlását okozza, amely *de novo* fehérje szintézissel állítódhat helyre.

A dolgozat célja:

- az UV-B sugárzás által a második fotokémiai rendszerben okozott károsodás és az azt követő helyreállás mechanizmusának vizsgálata látható fény jelenlétében.
- Az UV-B és a látható fény kölcsönhatásának értelmezése a második fotokémiai rendszer károsítása folyamán.
- A UV-B okozta DNS szerepének tisztázása a fotoszintetikus elektron transzportlánc működésének gátlásában.
- A *Synechocystis* 6803 és *Synechococcus* 7942 cianobaktériumok UV-B elleni védekezési stratégiáinak összehasonlítása.

Eredmények és megvitatásuk:

A fotoszintetikus aktivitás UV-B sugárzás okozta károsodása és helyreállása a Synechocystis PCC 6803-ban

Az UV-B sugárzás gátolja az oxigén fejlesztő képességet az intakt *Synechocystis* sejtekben. A kísérleteikben az kezdeti oxigén fejlesztés 50%-os károsodását lehetett megfigyelni körülbelül 120 perc után. A S*ynechocystis* sejtek képesek voltak regenerálódni az UV-B károsítás után, ha látható fénnyel megvilágítottuk őket. Munkánkban megmutattuk,

hogy a hőmérséklet befolyásolja az UV-B okozta károsodás és az azt követő helyreállás hatékonyságát.

Az UV-B sugárzás és a látható fény kapcsolata a második fotokémiai rendszer károsodása során

Kísérleteinkben háromféle modellt alkalmaztunk: intakt *Synechocystis* sejteket, linkomicin fehérje szintézis gátlóval kezelt *Synechocystis* sejteket és spenótból izolált tilakoid membránokat. A kezeléseknél különböző intenzitású látható fényt, UV-B sugárzást és ezek kombinációját alkalmaztuk. Izolált tilakoid membránok vagy linkomicinnel kezelt sejtek esetén összegzett hatást lehetett megfigyelni az oxigén fejlődés gátlásában, a látható fényt és az UV-B sugárzás együttes alkalmazása esetén. Ez arra utal, hogy az UV-B sugárzás és a látható fény egymástól független mechanizmusok szerint károsítja a második fotokémiai rendszert. Aktív fehérje szintézis esetén azonban a látható fény szinergikusan felerősíti a helyreállító mechanizmus hatékonyságát. Ez a hatás alacsony látható fényintenzitások esetén kifejezett, magas intenzitások esetén csökkenő mértékű.

A DNS repair szerepe

Ezen kísérleteinkben egy olyan mutánst alkalmaztunk, amelyben a fotoliáz enzimet kódoló gén inaktiválva volt, ezért nem képes az UV-indukált DNS károsodás kijavítására. A vad típusú sejtekkel szemben ez a mutáns UV-B sugárzás jelenlétében nem képes növekedni. A második fotokémiai rendszer károsodása azonban rövid idejű (néhány órás) UV-B kezelések esetén nem szignifikáns.

A Synechcoccus 7942 két D1 fehérje formájának eltérő UV-B érzékenysége

A *Synechococcus* 7942 cianobaktériumban három különböző gén a D1 fehérje két különböző formáját kódolja (D1:1 és D1:2). Ezen fehérje formákat tartalmazó sejtek UV-B sugárzással szemben mutatott érzékenységének kimutatására olyan *Synechocystis* 6803 mutánsokat alkalmaztunk, amelyek a *Synechoccoccus* D1:1 és a D2:1 fehérjéket termelik. Eredményeink szerint a D1:1-et tartalmazó mutáns érzékenyebb volt, mint a D2:1-et tartalmazó. Azonban, mindkét mutáns gyorsabb károsodást illetve lassabb helyreállást

mutatott, mint az eredeti *Synechocystis* 6803-ra jellemző D1 fehérjét tartalmazó vad típusú sejtek.