Reverse genetics of PsaA and PsaB to dissect their function in binding and electron transfer from plastocyanin or cytochrome c_6 to the core of photosystem 1

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1 Introduction

1.1 Energy conversion in photosynthesis

Photosynthesis is the basis of almost all life on earth. This process converts light energy to storable chemical energy. It is mastered by a variety of organisms. A major field of research is the gain of knowledge on oxygenic photosynthesis. Oxygenic photosynthetic organisms convert CO_2 (carbon dioxide) to organic compounds by reducing this gas to carbohydrates in a rather complex set of reactions. In this process electrons for reduction are extracted from water which is oxidized to oxygen and protons. In subsequent reactions the chemical energy equivalents NADPH and ATP are formed as a consequence of the so called light reaction of oxygenic photosynthesis. Energy for this process is provided by light which is absorbed by pigments that are embedded in the multiprotein complexes of photosystem 1 (PS1) and photosystem 2 (PS2).

The primary light reaction is comprised of three main steps which are light absorption, charge separation and its stabilization. In membrane bound antenna proteins light quanta are absorbed by pigments and transferred *via* different excited states to the reaction centre. After excitation a primary donor within the reaction centre transfers an electron to a nearby located acceptor molecule. The electron is then transferred inside the complex *via* a chain of suitable arranged cofactors across the membrane. Delocalisation of the charges on both sides of the reaction center to stabilizes this state of charge separation and prevents recombination. Soluble electron transporters can subsequently reduce / oxidize the photosynthetic complex. Electron transfer reactions are often coupled to proton transfer reactions, used to generate an electrochemical gradient across the membrane which in turn usually drives an ATP-synthase to generate ATP.

This principle of photosynthesis is already used by purple bacteria and green sulphur bacteria. In cyanobacteria, algae and vascular plants oxygenic photosynthesis is performed. Hereby a mechanism evolved which uses water as electron source. In these species two specialized photosystems are joined in series to generate one of the strongest cellular reductant ferredoxin (fd) (E_0~-420 mV) and NADPH (E_0=-320 mV) from the poor electron donor water which has an extremely high electrochemical potential (E_0=+820 mV at pH 7).

1.2 The electron transport in thylakoids

In algae and vascular plants the photosynthetic process occurs inside specialized organelles, the chloroplasts. Chloroplasts consist of three membranes: the outer and inner envelope membrane and an internal membrane system, the thylakoid membrane, which functions as a vesicle with an inner (lumenal) and an outer (stromal) water phase (see Fig. 2). This thylakoid membrane consists mostly of glyceral lipids and is heavily embedded with protein complexes that form the photosynthetic apparatus.

In chlorophyll (Chl) containing organisms, linear electron transfer from water to $NADP^+$ involves three integral protein complexes operating in accordance to the classical Z-scheme (textbooks e.g. Stryer, 1990), PS2, cytochrome b_6f (cyt b_6f) complex and PS1, which are linked by a membrane soluble electron carrier, plastoquinone (PQ), and one of the water soluble carriers plastocyanin (pc) or a c-type cytochrome c_6 (cyt c_6). Under certain conditions cells can switch to cyclic electron transport where light driven electron flow through PS1 and the cyt b_6f complex is used to generate a proton gradient not coupled to NADPH production. In addition to the main complexes two sets of light harvesting proteins Lhca and Lhcb, containing most of the Chl a, Chl b and carotenoids, are used for light harvesting and directing excitonic energy to PS1/2, respectively.

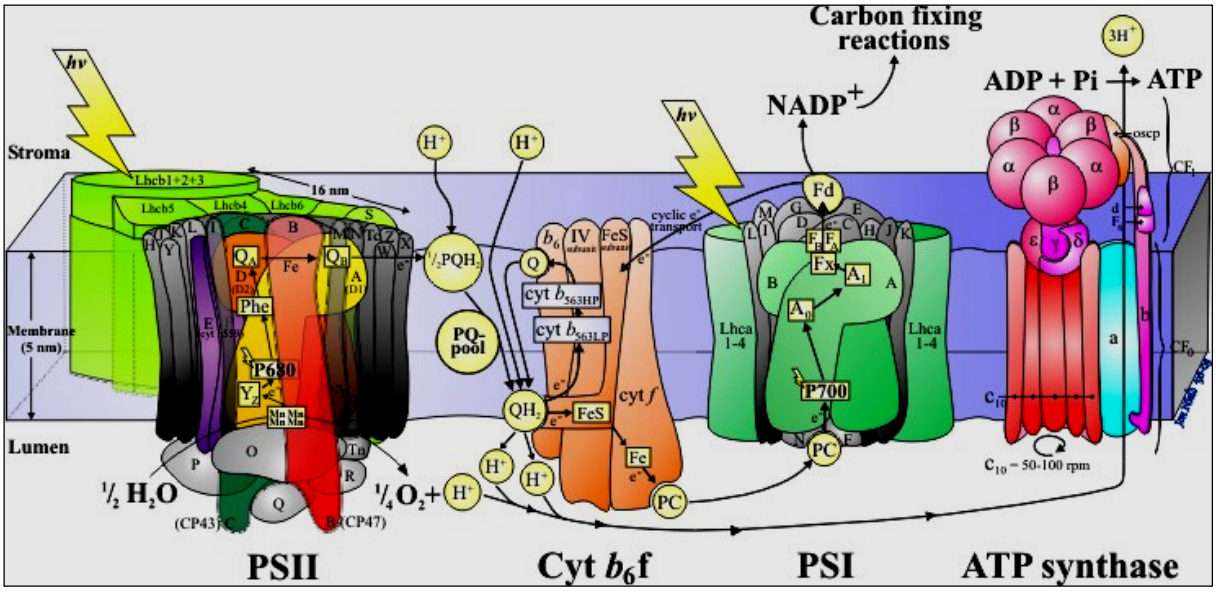

Figure 1: Schematic view of the main thylakoid complexes and their involvement in light absorption, electron transfer reactions and proton gradient formation / utilization. (Taken from John Nield, http://www.bio.ic.ac.uk/research).

1.2.1 Photosystem 2

PS2 uses light energy to drive the oxidation of water and reduction of PQ. It consists of over 20 nuclear as well as plastid encoded polypeptides and contains at least 9 different redox cofactors (Manganese, tyrosine, histidine, Chl, pheophytin (Phe), PQ, Iron, cyt b559, carotenoid) which have been shown to undergo light induced electron transfer. Only five of these redox components have been shown to be responsible for electron transfer from water to PQ (Xiong *et al.*, 1998; Rhee *et al.*, 1998; Barber 2002; Diner *et al.*, 2002). The most detailed insight in PS2 is provided by an X-ray crystal structure of PS2 isolated from *Synechococcus elongatus* (resolution of 3.8 Å) (Zouni *et al*., 2001) or from *Thermosynechococcus vulcanus* (resolution of 3.7 Å) (Kamyia *et al*., 2003). Due to a high degree of homology of proteins, arrangement of cofactors and reaction mechanism it provides a good model for eukaryotic reaction centers.

Photochemistry in PS2 (see also Fig. 1) is initiated by light induced excitation of the primary electron donor P680, a Chl a pair with a maximum photoinduced bleaching at 680 nm. Charge separation between P680 and pheophytin, creating $P680^+$/Phe takes only a few ps. The subsequent electron transfer step to covalently bound quinone A (Q_A) within 200 ps stabilizes the separated charge from recombining with $P680^+$. The electron on the Q_A side is then transferred to a loosely bound PQ on the Q_B side which differs from Q_A in that it works as a two electron carrier. PQ at the Q_B side becomes fully reduced after two photochemical turn overs of the reaction centre and gets electro neutral after taking up two protons from the stromal side. The PQH_2 then unbinds from the Q_B side and diffuses in the hydrophobic core of the membrane. It is replaced by an oxidized PQ of the PQ pool in the membrane which consists of approximately 6 – 8 PQ per PS2 reaction center (Stiehl *et al.*, 1969). $P680^+$ gets reduced from a tyrosine, which in turn is reduced by a $[Mn]_4$ cluster that is able to store the oxidation equivalents of four repeated turn over to oxidize two water molecules producing one O_2 and four protons.

1.2.2 Cytochrome b_6f complex

The cyt b_6f complex is an integral component of the photosynthetic membranes of all oxygenic photosynthetic organisms. It transfers electrons from PS2 to PS1 by catalyzing the oxidation of PQH_2 and the reduction of pc (or cyt c_6

in some algae and cyanobacteria). These redox reactions are coupled to proton translocation across the photosynthetic membrane. The cyt b_6f complex isolated from *C. reinhadtii* (Popot, conference data 2003) as well as from *Mastigocladus laminosusis* (Kurisu *et al.*, 2003) has been determined by X-ray diffraction to a resolution of about 3 Å and displays close analogies to the cyt bc_1 complex of mitochondria (Yu *et al*., 1996) performing a similar reaction.

The cyt b_6f complex is composed of at least eight components encoded by the nucleus and the plastid genome. The four large subunits with a well known function carry the cofactors for the redox reactions: A 2Fe2S center (the Rieske FeS centre), two b-hemes (cyt b_6), and a c-type heme (cyt f). The functions of the four small hydrophobic subunits are not yet clear but they are assumed to stabilize the complex and to play a role in phosphorylation-reactions (Hamel *et al.*, 2000). From the crystal structure a dimerisation promoted by the major subunits can be assumed. Two complexes formed a cavity with the PQH_2 binding site Qo while the smaller subunits are located on the edges.

The electron transfer pathways and the sites of proton uptake and release during operation of the Q-cycle are shown in Fig 1. Initially, PQH_2 binds to the Q_O-site (lumenal) and PQ binds to the Q_R-site (stromal). In the dark, the Rieske FeS centre and cyt f are reduced, and cyt bL and cyt bH (see Fig. 1) are oxidized. The oxidation of PQH_2 at the Q_O-site is initiated by oxidation of the Rieske FeS centre by PS1 turnover. The oxidized FeS-cluster performs a concerted reaction in which two electrons are transferred from PQH_2, the first electron going to the Rieske centre, and the second electron going to cyt b_L. This reaction with a half life of about 1.2 – 15 ms is assumed to be the rate limiting step in linear electron transport (Stiehl *et al.*, 1969; Joliot *et al*., 1986; 2002). The removal of electrons from PQH_2 leads to the release of two protons into the lumenal aqueous phase. The electron transferred to the Rieske centre is subsequently transferred to cyt f. The electron transferred to cyt b_L is subsequently transferred to cyt b_H, which in turn transfers the electron to PQ at the Q_R-site. Once PQH_2 is oxidized, it detaches from the complex and is thereafter replaced by a new PQH_2 from the PQ pool. The complete Q-cycle requires another turnover of the cytochrome b_6f complex. The second round of the cycle leads to the full reduction of PQ at the Q_R-site including the uptake of two protons from the stromal aqueous phase. Taken together the cyt b_6f complex transfers four protons across the membrane upon two subsequent PS1 turnovers.

Interestingly, a c-type cyt as a putative additional redox cofactor as well as a Chl a and a carotenoid with unknown function have been found in the crystal structure.

1.2.3 Photosystem 1

The PS1 complex catalyzes the oxidation of pc or cyt c_6 and the reduction of ferredoxin (fd). The reaction centre is composed of more than eleven polypeptides, encoded by the nuclear and the plastid genome and shows considerable similarity to the PS2 reaction center. The three-dimensional structure of PS1 from the cyanobacteria *Synechococcus elongatus* has recently been solved to a resolution of 2.5 Å (Jordan *et al*. 2001). Two polypeptides, PsaA and PsaB, which form a heterodimer bind the primary electron donor P700 and two sets of cofactors, to carry electrons across the membrane.

The primary donor, P700 with a maximum photoinduced bleaching at 700 nm, a Chl a dimer, and the primary acceptor, AO, a Chl a monomer act in the initial steps of charge separation similar to P680, whereas the photo-generated positive charge of $P700^+$ is mainly localized on one of the Chl a molecules (Webber *et al*., 2001). As in PS2 a phylloquinone operates as a further single electron acceptor (A_1) (Fig. 1). Electrons are transferred from A1 to a 4Fe4S cluster (F_X) that is ligated to both polypeptides of the heterodimer. Recently, it has been shown that both sets of cofactors bound to either PsaA or PsaB contribute to the electron transfer trough the PS1 complex with different rates (Joliot *et al.*, 1999; Guergova-Kuras *et al.*, 2002). An extrinsic protein, PsaC, containing two 4Fe4S clusters, FA and FB, is located on the stromal side of the reaction centre close to FX. The reduced F_x donates the electron to F_A and subsequently to F_B which in turn reduces fd on the stromal side of PS1. On the lumenal side PS1 is reduced by pc (or cyt c_6 in some algae and cyanobacteria).

1.3 Balancing the power

1.3.1 Heterogeneity

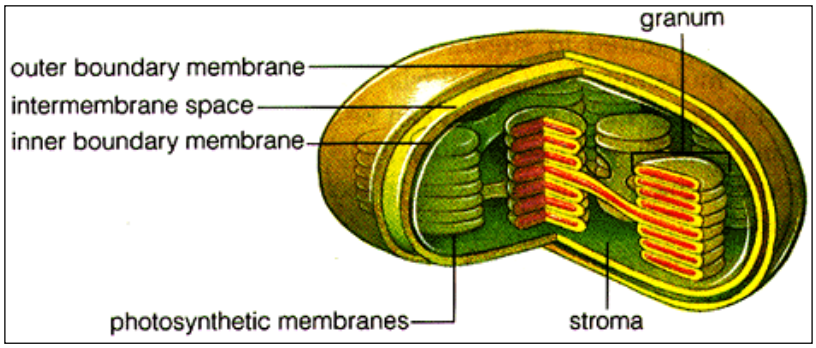

Figure 2: Schematic view of a chloroplast. Three types of membranes are present, the inner photosynthetic or thylakoid membrane is organized in grana stacks connected by the stroma lamellae.

To further complicate the picture, eukaryotic photosynthetic membranes have been shown by electron micrographs to display a lateral heterogeneity. Thylakoid membranes are organized inside the chloroplast in regions of appressed stacks (the grana stacks) which are connected by the stromal lamellae. Photosynthetic complexes of the thylakoid membrane are differentially distributed amongst thylakoid grana and lamella. While the appressed regions of grana have been shown to contain mainly PS2 complexes, the stromal lamellae contain PS1 and ATP-synthase. The cyt b_6f complexes however are distributed homogeneous in both grana and lamellae. This unequal distribution is closely related to the 3D-structure of the different components. PS1 protrudes on the acceptor side, consisting of the extrinsic peptide PsaC, PsaD and PsaE where fd binding takes place, to about 50 Å into the stromal side and the ATP-synthase even to about 100 Å. In contrast PS2 has a rather flat acceptor side. In this way it is rather impossible for a PS1 to get between the grana stacks which are separated by no more than 40 Å. The opposite appears on the donor side where PS2 displays an extension of about 45 Å and is much bulkier than PS1 with only about 20 Å. Also the nature of the soluble electron donors / acceptors for PS1 and their requirements to find a relatively large surface for recognition and binding might count for this structure. Not fully understood is the way the topology of the stacked grana is held and/or modified (Olive *et al.*, 1991; Andersson *et al.*, 1980; Allen *et al.*, 2001).

1.3.2 Reactive oxygen species generation

Photosynthesis under aerobic conditions may be deleterious for the performing organisms if light energy, required for the photosynthetic activity, is in excess. Over-reduction of the electron carriers can lead to a series of reactive oxygen species (ROS) such as superoxide anion radical, hydrogen peroxide, hydroxyl radicals or singlet oxygen by transferring excitation energy or electrons to the thermodynamically active but kinetically hindered ground state $^3O_2$ (Asada, 1994; 1999). These ROS can damage pigments, proteins and lipids leading to a impairment of photosynthetic capacity called photoinhibition.

The main target of photoinhibition is assumed to be PS2 which can be damaged at the acceptor or donor side. A lot of data is available on the mechanisms leading to ROS generated by PS2: At the acceptor side O_2 can get reduced by pheophytin$^-$ or Q_A^-, at the acceptor side the tyrosine radical can reduce O_2 and $^1O_2$ can be generated upon recombination and spin conversion of P680 (Vass *et al.*, 1992; Asada, 1999). These reactions occur mainly when the PQ pool is fully reduced and the Q_B site is functional but empty. Photoinhibition on PS1 is less well investigated but there is evidence that O_2 can be directly reduced by PS1 generating superoxide anion radical and other reactive species (Mehler, 1951; Asada, 1999). In addition metal ions, mainly iron, from destroyed redox centers represent another class of ROS generators reacting mainly in Fenton type reactions (Tyrrell, 1997).

1.3.3 Scavenging mechanisms

To prevent photoinhibition within photosynthetic organisms several mechanisms to prevent oxidative stress evolved. On one hand direct elimination of the ROS is performed and on the other hand mechanisms to redirect the excess energy to an unperilous form or balance the light energy between the two PS in a way that over-reduction of the electron transport chain and electron leakages are prevented.

To decompose ROS a set of small molecules, the so called scavengers, and other specialized proteins exist. In aqueous phase the best known scavengers are ascorbate and glutathione whereas in membranes tocopherols perform direct decomposition of radicals. Furthermore carotenoids and quinones act as quenchers for $^1O_2$ (Niyogi *et al.*, 1997; Asada, 1999). Enzyme based

detoxifications are performed by a set of different SOD (superoxide dismutase), GPX (glutathion peroxidase), APX (ascorbate peroxidase) and catalase, which in part are closely associated to the ROS generation sites (Ogawa *et al*., 1995; Asada, 1999). Scavengers can also display highly reactive species e.g. MDA (mono dehydro ascorbate) and require an efficient recycling. As a special form of scavengers a protein itself can intercept ROS. This mechanism is realized and extensively studied in PS2 where the D1 subunit reacts in a controlled way with ROS subsequently leading to defined D1 degradation, disassembly of PS2 and reassembly with newly synthesized D1 (Barber *et al.*, 1992; Silva *et al.*, 2003).

Mechanisms that balance energy input and consumption can be divided into short term and long term responses. Long term responses take several hours and mainly change the nuclear and the plastid expression patterns of distinct subunits from the photosynthetic complexes or scavengers and involve signal sensing and transduction (Yoshida *et al*., 2003). Short term responses, for example nonphotochemical dissipation of excess energy react within several minutes (Gilmore *et al.*, 1994). Another short term response called state transitions leads to a redistribution of excitation energy to PS1 by reorganization of the antennae and thylakoid structure (Allen *et al.*, 2001; Wollman, 2001) and is assumed to be accompanied by a switch from linear to PS1 driven cyclic electron transfer in *C. reinhardtii* (Finazzi *et al*., 2002). This process involves several kinases for phosphorylation of Lhcb proteins, PS2 and cyt b_6f subunits and requires PsaH for Lhcb attachment (Haldrup *et al.*, 2001). A functional Q_o site, the slowest step in linear electron transfer, is essential for signal initiation inducing phosphorylation of Lhcb subunits (Vener *et al.*, 1995).

Interestingly in *C. reinhardtii* mutants hindered in electron transfer from pc to the donor side of PS1 became strongly light sensitive. A genetic suppressor screen identified suppressor strains in which this light sensitivity was overcome. In those strains almost no functional Lhc assembled within the thylakoid membrane, indicating that electron transfer and light harvesting must be in balance to avoid photo-oxidative stress. (Hippler *et al.,* 2000).

1.4 Reactions at the donor side of PS1

1.4.1 Electron transfer in proteins

Redox processes between redox centers, that are separated by no more than 20 Å, display an important role in photosynthesis (Kryshtalik *et al.*, 1996). The theory of Marcus (Marcus *et al.*, 1985) describes a system where electron transfer is coupled to a nuclear reorganization (induced upon charge allocation) of the reaction partners as well as the surrounding medium which can be more or less polar (Fig. 3). Most photosynthetic electron transfer reactions are therefore in a rather apolar surrounding with low dielectric constants resulting in a small reorganization energy λ. The rate of electron transfer k therefore depends not mainly on the free energy ($-\Delta G^0$) but on its relation to λ:

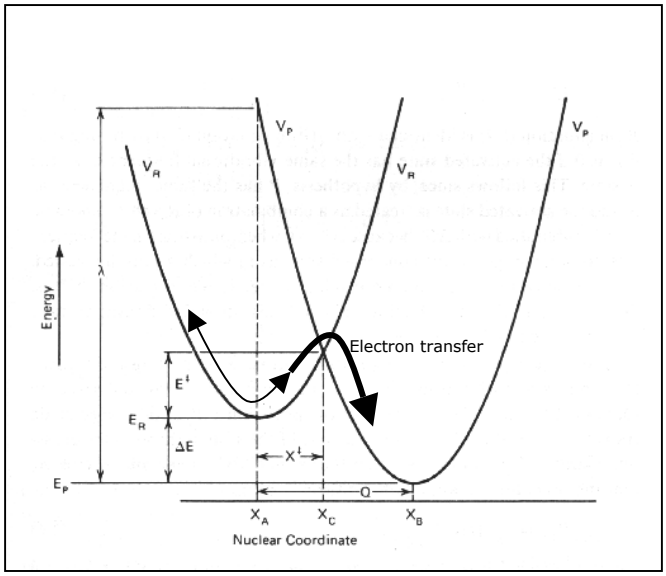

Figure 3: Energy diagram referring to the Marcus theory concerning the coupling of the generalized nuclear movement and electron transfer. Depicted are Activation energy $E^{\ddagger}$, free reaction enthalpy ΔE and the reorganization energy λ which is needed to bring the nuclear coordinates of the reactants V_R from the initial equilibrium E_R to the upon charge allocation changed equilibrium E_P of the products V_P.

$$k = \frac{2\pi}{\hbar} * H_{AB}^2 * \frac{1}{\sqrt{4\pi RT}} * \exp\left[\frac{-(\Delta G^0 + \lambda)^2}{4\lambda RT}\right] \qquad \text{Equation 1}$$

where $\hbar$ is the Planck´s constant, T the temperature, R the gas constant and H^2_{AB} the electronic coupling of the initial and the final state. The parabolic shape of the curve, derived from eq. 1 for ΔG^0 versus log k, implies an increase of k with $-\Delta G^0$ only for a small ΔG^0 compared to λ (realized in most inter protein transfer reactions (Ramesh *et al*., 2003). A maximum rate constant for electron transfer is realized when $-\Delta G^0 = \lambda$ (for primary charge separation of the PS1 or PS2). In the "inverted region" (when $-\Delta G^0 > \lambda$) k decreases with increasing $-\Delta G^0$ (realized in recombination reactions within the PS (Moser *et al.*, 1992)).

A further important factor regarding the electron transfer rate for electronically weakly coupled reactions, as it is true for electron transfer over long distances, is the element H^2_{AB} which has an impact on the probability of electron transfer and depends on cofactor orientation and the intervening medium. In model systems reduction factors β could be estimated for the electron transfer along different types of bounds (Bowler *et al.*, 1990) (see Fig. 4) but whether these are relevant in proteins is elusive, since a single exponential fit for the reaction center distance R versus k can account for most electron transfer reactions through a protein medium. From these findings Moser and Dutton (Moser *et al.*, 1995)) introduced an empirical formula where the electron transfer rate k is dependent only on the free energy $-\Delta G^0$ and the reorganization energy λ (both in the unit of eV) which allows an estimation of the edge-to-edge distance R between two intra-molecular redox-centers:

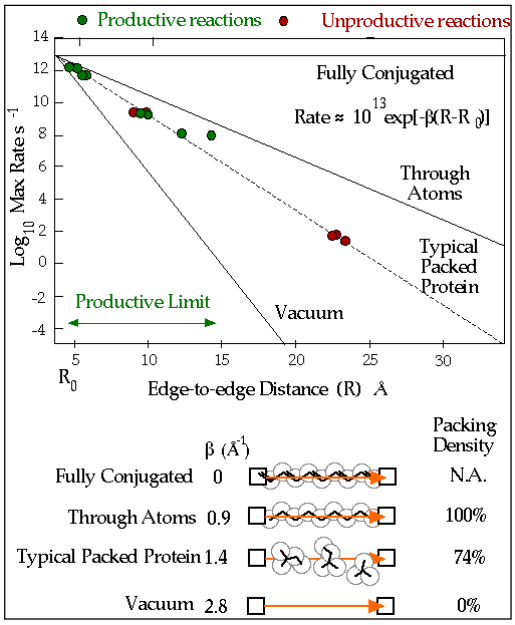

Figure 4: Empirical determination of reduction factor β for the rate constant of electron transfer within proteins (*Rp. viridis* and *Rb. sphaeroides* reaction centers) compared to the values found with model compounds (taken from: Johnson Research Foundation University of Pennsylvania)

$$\log_{10} k = 13 - 0.6 \times (R - 3.6) - 3.1 \times \frac{(\Delta G^0 + \lambda)^2}{\lambda} \qquad \text{Equation 2}$$

The initial constant 13 is the rate at van der Waals contact distance (R = 3.6 Å). The second term describes an approximately exponential fall-off in electron tunneling rate with distance through the insulating barrier; R is the edge-to-edge distance; the third term is the quantized Frank-Condon factor at room temperature.

1.4.2 Reaction mechanism of electron transfer to PS1

The mechanism of the reduction of photooxidized $P700^+$ by pc or cyt c_6 has been solved mainly by kinetic measurements using flash induced absorption changes induced upon P700 oxidation/reduction. Thereby an evolution for the

electron transfer mechanism has been proposed: (i) from a simple collision based mechanism found in cyanobacteria to a (ii) mechanism involving a transient complex formation sometimes (iii) accompanied by a rearrangement step of the donor/acceptor complex prior to electron transfer in eukaryotic cells (Bottin *et al.*, 1985; Hervas *et al.*, 1995). By studying the interactions of oxidized and reduced pc with PS1 in spinach a more simple model was introduced that takes a redox equilibrium for the electron transfer between the donor and PSI into account (Drepper *et al.*, 1996; Olesen *et al.*, 1999).

1.4.3 Electron donors to PS1

The electron transfer from cyt b_6f to PS1 is mediated by either cyt c_6 or pc. Until very recently, it was widely believed that plants produce only pc, whereas eukaryotic algae and cyanobacteria synthesize either cyt c_6 or pc, depending on copper bioavailability (Merchant *et al.*, 1986; Hill *et al.*, 1991).

The Protein Data Bank (http://www.rcsb.org/pdb/) contains a great number of 3D structures of cyt c_6 and pc from different photosynthetic organisms. Cyt c_6 is a typical class I c-type cyt, formed by four α-helices and a heme group in which the iron atom exhibits a His-Met axial coordination and the porphyrin ring is covalently bound to the polypeptide chain by two Cys. Pc consists of a β-barrel formed by eight β-strands, along with a small α-helix, and a redox center typical of type I blue copper proteins, in which the metal atom is coordinated by two His, one Met, and one Cys in a tetrahedral geometry (Harris, 1996).

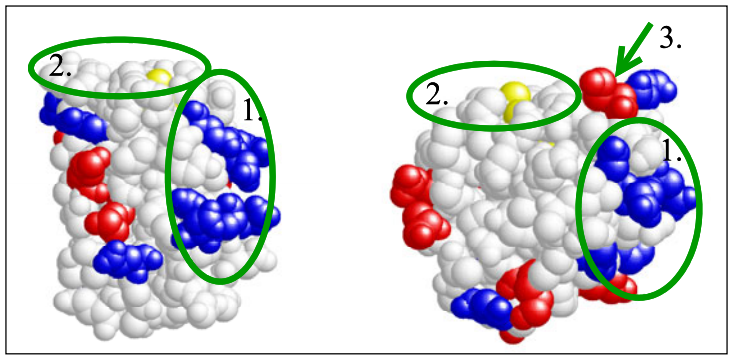

Figure 5: Crystal structure of plastocyanin at a resolution of 1.5 Å left (Redinbo *et al.*, 1993) and cytochrome c_6 at 1.9 Å right (Kerfeld *et al.*, 1995) isolated from *C. reinhadtii*. Acidic and basic residues in blueand red, resp., and the site oelectron transfer in yellow.Binding sites to PS1 depicted are:
1.) Acidic patches on the „eastern" side for electrostatic interaction with positive patches on PsaF.
2.) Flat "northern" side interacts with the PsaA/PsaB heterodimer, electron transfer occurs via this side.
3.) Cyanobacterial donors possess a positively charged amino acid at position of Arg66 in *C. reinhardtii*, which is missing in plastocyanin of vascular plants.

Most site directed mutagenesis and cross linking based investigations were targeted to establishing the structure-function relationships which allows cyt c_6 and pc to donate electrons to PS1. Despite their different structures and cofactors their physicochemical

properties are very similar: A molecular mass of about 10 kDa, a midpoint redox potential of about 370 mV at pH 7 and a practically identical isoelectric point within the same organism (about 4 for most algae and vascular plants). Fig. 5 shows the 3D structures of *C. reinhadtii* pc and cyt c_6. Fig. 5 further depicts sites 1 and 2 which have been found to be crucial for efficient interaction with PS1. Site 1 represents patches of negatively charged amino acids. These residues could be cross linked to the positively charged N-terminal of PsaF in eukaryotic PS1 (Hippler *et al*., 1996). Site 2 represents a flat hydrophobic surface around the copper ligating His in pc or the D ring of the porphyrin system in cyt c_6. This site has to be shown to interact directly with the core of PS1 (Nordling *et al*., 1991; Haehnel *et al*., 1994). A third site which is present in cyanobacterial pc and cyt c_6 as well as in *C. reinhardtii* cyt c_6 but absent in pc from *C. reinhardtii* and all vascular plants is the Arg66 which has been shown to play an important roll in interaction between PS1 with the cyanobacterial donors (Molina-Herida *et al.*, 2001; De la Cerda *et al*., 1999).

Recently a modified cyt c_6 was discovered in several plants. Physicochemical, structural and functional analysis of plant cyt c_6 however revealed that it can not replace pc in electron transport to PS1 (Gupta *et al.*, 2002; Molina-Herida *et al.*, 2003; Weigel *et al.*, 2003). The function of this plant cyt c_6 remains to be determined and may be related to an extra loop of 12 residues that is an unique feature of the plant heme protein.

1.4.4 Structural components of PS1

From the crystal structure of the cyanobacterial PS1 (for nomenclature see Sun *et* al., 1997) it can be deduced that PS1 protrudes about 20 Å into the lumenal space and forms a small cavity wherein the flat lumenal surface of the donor side of PS1 is located, mainly formed by the loop regions that connect the transmembrane domains of subunits PsaA and PsaB (Jordan *et al.*, 2001). Beside the lumenal loops of PsaA/B, subunit PsaF contributes significantly structural elements of this side which has been shown to provide an essential recognition site for efficient binding and electron transfer between PS1 and the electron donors only in eukaryotic organisms (see below).

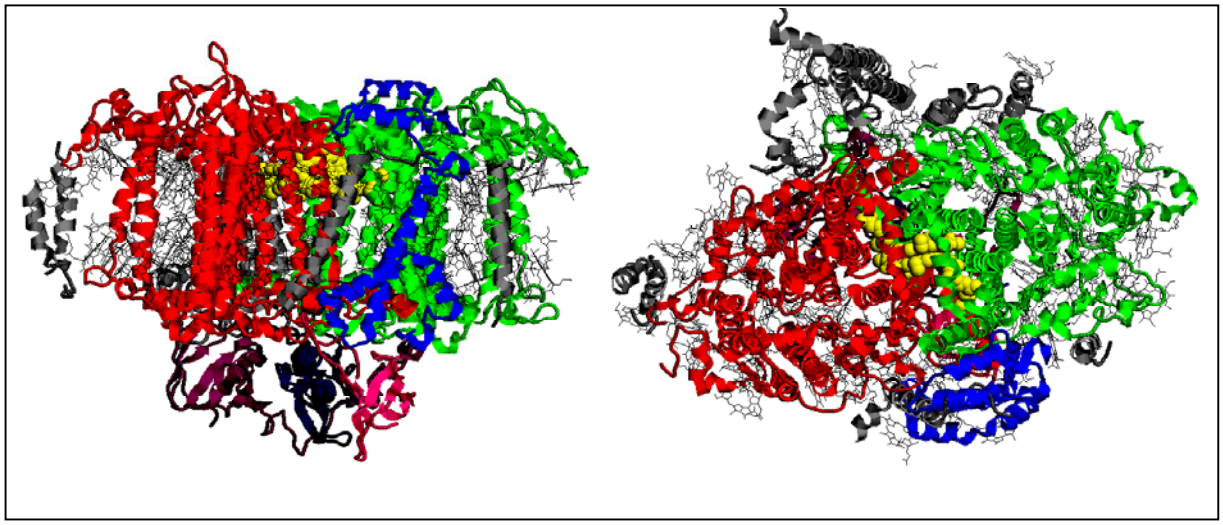

Figure 6: Crystal structure of *Synechococcus elongatus* PSI at a resolution of 2.5 Å (Jordan *et al.*, 2001) in a view along the membrane (left) and from the lumenal side (right). PsaF in blue, PsaB in green, PsaA in red, the special Chl a pair P700 in yellow and PsaC, PsaD, PsaE and 4Fe4S clusters F_A, F_B and F_X in purple. Two helices *I* and *I'* are placed on the lumenal side separating P700 from the lumenal space. The structure suggests *i)* interaction of the positively charged PsaF (more extended in *C. reinhadtii*) with the electron donors and *ii)* participation of helices *I* and *I'* of PsaB and PsaA respectively in binding.

1.4.4.1 PsaF

The PsaF subunit of PS1 differs greatly in cyanobacteria and algae/vascular plants concerning its molecular mass of about 15 kDa and 20 kDa and its isoelectric points in the neutral and basic region, respectively. These differences have been used to account for the different redox reaction mechanisms observed for the different organisms. In particular complex formation between the electron donors pc or cyt c_6 and PS1 is contributed to the function of PsaF in eukaryotic photosynthesis. While in cyanobacteria PsaF is dispensable for photoautotrophic growth (Chitnis *et al.*, 1991), deletion of this subunit in algae and vascular plants causes a severe light sensitive phenotype (Farah *et al.*, 1995; Haldrup *et al.*, 2000). Pc has been shown to cross link to eukaryotic PsaF in a 1:1stoichiometry (Wynn *et al.*, 1988; Hippler *et al.*, 1989). Analysis of the crosslinked product between pc and PsaF by tryptic digestion and mass spectrometry revealed that the conserved acidic patches on pc and the conserved Lys residues in the N-terminal eukaryotic domain are the sites of interaction (Hippler *et al.*, 1996). This view was substantiated by the fact that the intermolecular electron transfer within the crosslinked complex remained unchanged. A cyanobacterial PS1 with a chimeric PsaF consisting of the C-terminal part of PsaF from *S. eongatus* and the N-terminal part of *C. reinhardtii* was able to form a transient complex with the *C. reinhardtii*

cyt c_6 also indicating that this new site enabled an eukaryotic type of binding between both donors and the chimeric PSI (Hippler *et al*., 1999).

1.4.4.2 PsaJ

Crosslinking of PsaJ, a small hydrophobic subunit with a single transmembrane domain, to PsaF suggested an interaction between these two PS1 subunits (Jansson *et al.*, 1996). To further analyze this interaction the chloroplast *psaJ* gene of *C. reinhardtii* was inactivated (Fischer *et al.*, 1999). PsaJ-deficient cells grew photoautotrophically and accumulate normal levels of PS1. Measurements of electron transfer between PsaJ-deficient PS1 and the two donor proteins by flash absorption spectroscopy revealed that only 30% of the PS1 complexes oxidize pc or cyt c_6 with kinetics identical to the WT, while the remaining 70% were not able to form a transient electron transfer complex, although PsaF accumulated in normal amounts in the mutants. From these results it, was suggested that PsaJ may help to maintain PsaF in an orientation that its N-terminal domain can serve as a docking site for the soluble donor proteins mediating fast electron transfer to $P700^+$.

1.4.4.3 PsaN

The PsaN subunit of PS1, with a molecular mass of 9-10 kDa, is only present in eukaryotic organisms. The subunit is localized on the lumenal side of the thylakoids. Evidence for a contribution of PsaN in the interaction between PS1 and pc has been shown using an anti-sense strategy in *Arabidopsis* (Haldrup *et al.*, 1999). Anti-sense plants without detectable amounts of PsaN assemble functional PS1 complexes and grow photoautotrophically. The rate of electron transfer between the PsaN-deficient PS1 and pc as well as the steady-state $NADP^+$ reduction rate are two-fold lower as compared to wild type. However, it is unclear whether this effect means that PsaN is involved in the binding of pc or that the lack of PsaN causes a conformational change of PsaF which in turn has an impact on the binding of pc to PS1.

1.4.4.4 Recognition site at the core of PS1

For eukaryotic organisms site directed mutagenesis of pc and analysis of binding and electron transfer between the altered pc and PS1 (Lee *et al*., 1995;

Nordling *et al.*, 1991; Haehnel *et al.*, 1994; Hippler *et al*., 1996) suggested that beside the long-range electrostatic interaction of the positively charged PsaF and the negative patches of pc, a second recognition site is required which brings the flat hydrophobic surface of pc in close contact with the core of PS1 to allow efficient electron transfer from copper *via* the "northern face" of the molecule to $P700^+$ (see Fig. 5).

In *Synechocystis* sp. PCC6803 mutational analysis of loop H connecting transmembrane helices VII and VIII of PsaB was performed (Sun *et al.*, 1999; Navarro *et al.*, 2000). PS1 with deletions or duplications within this lumenal chain were generated and further analyzed in the view of complex stability and electron transfer. The data revealed that the margin parts of loop H are indispensable for stable accumulation of PS1 while a small middle part is dispensable. The effects on electron transfer were rather small, but led to the suggestion that loop H might provide residues that contribute to the over all negatively charged lumenal surface of PS1 and could be involved in repulsion of pc. No evidence has been found for a direct interaction of the electron donors to this site on PS1.

According to the three-dimensional structure of PS1, P700, the primary photosynthetic electron donor, is localized near the lumenal surface. Part of the loops j'/j of PsaA/B form two α-helices *l'/l* which are in parallel to the membrane plane. It is assumed that these loops could be involved in docking of the soluble donors pc and cyt c_6 (Jordan *et al.*, 2001; Schubert *et al.*, 1997; Sommer *et al.*, 2002). Sun and coworkers (1999) generated site-directed mutants within short stretches of the lumenal loop j of the PsaB protein from *Synechocystis sp.* PCC 6803 and consistent with previous hypothesis, the double mutant Trp622Cys/Ala632Arg was strongly affected in the interaction between the altered PS1 and the electron donors.

1.5 Aim of this work

Protein-protein interactions are an important field of research. In oxygenic photosynthesis electron transport and transfer needs to be performed in an exact way to avoid harmful side products, like reactive oxygen species. Electron transfer from pc or cyt c_6 to PS1 in eukaryotic organisms involves a well balanced transient inter-molecular complex which makes it an interesting model for the study of protein-protein interactions. In this study the hydrophobic binding site for pc and cyt c_6 formed by the core subunits PsaA and/or PsaB was aimed to be identified. As a strategy a thorough reverse genetics approach was applied in the green alga *C. reinhardtii* and combined with flash induced spectroscopy and cross-linking experiments for further functional characterization.

Since *C. reinhardtii* possesses pc and cyt c_6 which both function as electron donor to PS1, it was aimed to study the electron transfer between the altered PSI complexes and these both donors.

Changing the binding properties of the electron donors/acceptors to/from PS1 resulted in light sensitivity. Therefore it was aimed to elucidate the mechanisms that lead to photo-oxidative stress in these mutant strains.

2 Publications

2.1 Overview of the included publications

Publication 1:

Sommer, F., Drepper,F. and Hippler, M., (2002). The luminal helix *l* of PsaB is essential for recognition of plastocyanin or cytochrome c_6 and fast electron transfer to photosystem 1 in *Chlamydomonas reinhardtii*. *J. Biol. Chem*. **277** (8), 6573-6581.

describes a combinatorial site directed mutagenesis approach on helix *l* in PsaB for a screen of residues which are important for the interaction between the electron donors and PS1. Residue E613 is suggested to keep PsaF in a proper orientation for recognition, binding and fast electron transfer between pc or cyt c_6 and PS1. Residue W627 of PsaB forms one part of the hydrophobic recognition site for both donors but is more essential for binding of pc than cyt c_6.

The experiments were performed by F. Sommer.

Publication 2:

Sommer, F., Drepper, F. and Hippler, M., (2003). The hydrophobic recognition site formed by residues PsaA-W651 and PsaB-W627 of photosystem I in *Chlamydomonas reinhardtii* confers distinct selectivity for binding of plastocyanin and cytochrome c_6. (*manuscript for submission to: J Biol Chem*).

describes site directed mutagenesis analysis of PsaA-W651 that forms a sandwich complex together with PsaB-W627. The kinetic data indicate that W651 contributes to the hydrophobic recognition site formed by the core of PS1. Electron transfer from cyt c_6 to the altered PS1 is more perturbed than from pc suggesting that W651 is more important for binding of cyt c_6. The fact that the hydrophobic recognition site formed by residues PsaA-W651 and PsaB-W627 of photosystem I in *C. reinhardtii* confers distinct selectivity for binding of pc and cyt c_6 has not been described before.

The experiments were performed by F. Sommer.

Publication 3:

Sommer, F., Hippler, M., Biehler, K., Fischer, N. and Rochaix, J.-D., (2003). Comparative analysis of photosensitivity in photosystem 1 donor and acceptor side mutants of *Chlamydomonas reinhadtii*. *Plant, Cell and Environment*, (*in press, OnlineEarly: http://www.blackwell-synergy.com/links/doi/10.1046/j.1365-3040.2003.01105.x/full/*).

deals with the observed light sensitivity of PS1 donor and acceptor side mutants with emphasis on the relation between limitation in electron entry or exit to/from PS1 and the corresponding oxidative damage. This work clearly shows that a limitation of electron transfer at the donor or acceptor side has a strong impact on PS1 but also on PS2 *in vivo*.

the experiments herein were performed by F. Sommer, except:

- Growth tests for WT, 6a+, 3bF, PsaC-K35E-10, PsaC-K35E-9, PsaC FB1-7 and PsaC FB1-4 were done by N. Fischer.
- All $NADP^+$ reduction rates for WT and mutants PsaC and PsaF were measured by N. Fischer and M. Hippler.
- Gross oxygen evolution and oxygen uptake were determined by K. Biehler.

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The Luminal Helix *l* of PsaB Is Essential for Recognition of Plastocyanin or Cytochrome c_6 and Fast Electron Transfer to Photosystem I in *Chlamydomonas reinhardtii**

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At the lumenal side of photosystem I (PSI) in cyanobacteria, algae, and vascular plants, proper recognition and binding of the donor proteins plastocyanin (pc) and cytochrome (cyt) c_6 are crucial to allow subsequent efficient electron transfer to the photooxidized primary donor. To characterize the surface regions of PSI needed for the correct binding of both donors, loop *j* of PsaB of *Chlamydomonas reinhardtii* was modified using site-directed mutagenesis and chloroplast transformation. Mutant strains D624K, E613K/D624K, E613K/W627F, and D624K/W627F accumulated <20% of PSI as compared with wild type and were only able to grow photoautotrophically at low light intensities. Mutant strains E613N, E613K, and W627F accumulated >50% of PSI as compared with wild type. This was sufficient to isolate the altered PSI and perform a detailed analysis of the electron transfer between the modified PSI and the two algal donors using flash-induced spectroscopy. Such an analysis indicated that residue Glu^{613} of PsaB has two functions: (i) it is crucial for an improved unbinding of the two donors from PSI, and (ii) it orientates the positively charged N-terminal domain of PsaF in a way that allows efficient binding of pc or cyt c_6 to PSI. Mutation of Trp^{627} to Phe completely abolishes the formation of an intermolecular electron transfer complex between pc and PSI and also drastically diminishes the rate of electron transfer between the donor and PSI. This mutation also hinders binding and electron transfer between the altered PSI and cyt c_6. It causes a 10-fold increase of the half-time of electron transfer within the intermolecular complex of cyt c_6 and PSI. These data strongly suggest that Trp^{627} is a key residue of the recognition site formed by the core of PSI for binding and electron transfer between the two soluble electron donors and the photosystem.

In *Chlamydomonas reinhardtii*, the multiprotein complex photosystem I (PSI)[1] is a light-driven oxidoreductase that transfers electrons from the soluble lumenal donors plastocyanin (pc) and cytochrome (cyt) c_6 to the soluble stromal acceptor ferredoxin. The eukaryotic PSI reaction center is a membrane-bound complex consisting of 13–14 polypeptide subunits (1). Depending on the relative availability of copper in the culture medium, *Chlamydomonas* can replace the type I copper protein pc with a class I c-type cyt c_6 (2, 3). The two large PSI subunits, PsaA and PsaB, which carry the photochemical reaction center, each contain 11 transmembrane helices, of which helices *k* and *m* are connected by the lumenal loop *j* (Fig. 1). The 2.5 Å x-ray crystal structure of PSI from *Synechococcus elongatus* (4) reveals that a part of the loop forms an α-helix *l* that is oriented parallel to the membrane close to the primary donor, P700.

In eukaryotic organisms, docking of the soluble lumenal donors pc and cyt c_6 to PSI depends mainly on two different recognition sites, which are: (i) negative charges on the surface of the donor attracted by the positively charged N-terminal domain of the PsaF subunit of PSI (5–7), and (ii) the "northern part" of the donors interacting with an as yet undefined site of PSI (8).

The amino acid sequence of helix *l* is highly conserved among different species (Fig. 1). It is assumed that it could be involved in docking of the soluble donors (9). Sun *et al.* (10) generated site-directed mutants in the lumenal loop of the PsaB protein from *Synechocystis* sp. PCC 6803. Indeed, a double mutant (W622C/A623R) was strongly affected in the interaction between the altered PSI and the electron donors pc and cyt c_6. In contrast to eukaryotic organisms, in cyanobacteria, efficient binding and electron transfer between PSI and pc or cyt c_6 do not depend on the PsaF subunit because the specific deletion of the *psaF* gene in cyanobacteria did not affect photoautotrophic growth (11), and the *in vivo* measured electron transfer rate between cyt c_{553} and PSI was the same as that in wild type (12). *In vitro* measurements revealed that even at high concentrations of pc or cyt c_6 no difference in electron transfer rates could be measured between the donor proteins and PSI isolated from the wild type or the PsaF-deficient *Synechocystis* mutant (6). These differences can be explained by the absence of the specific eukaryotic N-terminal recognition site in the cyanobacterial PsaF protein that is required for the binding of pc and cyt c_6 to PSI (6, 7, 13).

To elucidate the role of loop *j* and especially that of helix *l* of the PsaB protein in a eukaryotic system we performed a combinatorial site-directed mutagenesis approach taking advantage of a PsaB-deficient mutant of *C. reinhardtii* (14). Of seven mutant strains, three allowed isolation of PSI sufficient for a further functional characterization. Electron transfer between the altered PSI particles and the donors pc and cyt c_6 has been investigated using flash-induced absorption spectroscopy. The results suggest features of the lumenal surface of PSI that are

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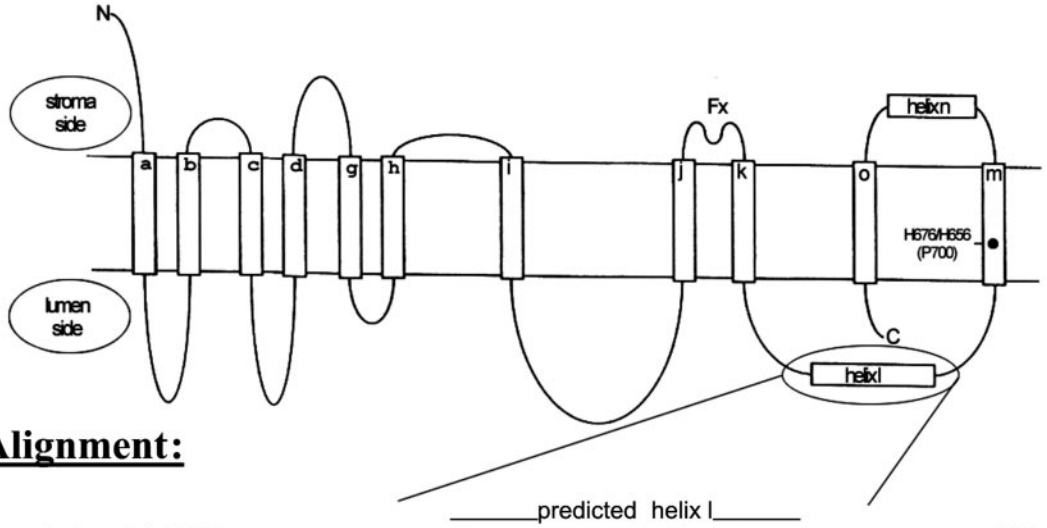
[1] The abbreviations used are: PSI, photosystem I; cyt, cytochrome; pc, plastocyanin; E, einstein.



Sequence Alignment:

predicted helix l

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Chlamydomonas reinhardtii 599 KHLTLWQGNV AQFDESSTYL MGWLRDYLWL NSSQLINGYN PFGMNSL 650
Synechococcus PCC7002     604 KHLGIWQGNV AQFNEKSTYL MGWFRDYLWA NSAQLINGYN PYGVNNL 655
Aanabaena variabilis      604 KHLGIWQGNV AQFNENSTYL MGWFRDYLWA NSAQLINGYN PYGVNNL 655
Spinacia oleracea         597 KHITLWQGNV SQFNESSTYL MGWLRDYLWL NSSQLINGYN PFGMNSL 648
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FIG. 1. **Schematic topographical model of helices in PsaB of *C. reinhardtii* and sequence alignment of loop *j* connecting helices *k* and *m* with corresponding regions in different organisms.** Helix *l* was predicted by using algorithms of Refs. 35 and 36. Highly conserved residues Glu[613], Asp[624], and Trp[627] (shown in *bold letters*) were changed.

crucial for the optimized binding equilibria of both donors. Furthermore, differences between both donors in the recognition and requirements for a correct docking and electron transfer can be identified. The data presented here will be discussed in light of the new structural information available for cyanobacterial PSI (4).

EXPERIMENTAL PROCEDURES

Strains and Media—C. reinhardtii wild type and mutant strains were grown as described previously (15). Tris acetate phosphate medium and high-salt medium were solidified with 2% Bacto agar (Difco) and supplemented with 150 μg/ml streptomycin (Sigma) when required.

Nucleic Acid Techniques—Procedures for the preparation of recombinant plasmids and DNA sequencing were performed as described previously (16). *Escherichia coli* DH5α was used as bacterial host. The site-directed change of PsaB Glu[613] to Asn and Lys was carried out in a single tube PCR as described by Picard *et al.* (17). We used degenerated mutagenic oligonucleotide N (5′-CAATCCGATAA(C/G)TCGTCTACT-3′) together with two oligonucleotides X (5′-GGTGCTCTAGATGCTCG-T-3′) and P (5′-ACCATTAATTAAAAGAGA-3′) complementary to the flanking regions about 150 bp upstream and downstream from the mutagenic site containing cleavage sites *Xba*I and *Pac*I, respectively. As template DNA, we used pKR162, a kind gift from K. Redding containing the *PsaB* coding sequence and 5′-untranslated region and also containing an *aadA* expression cassette conferring spectinomycin and streptomycin resistance to *C. reinhardtii* (18). The resulting DNA fragments were cloned into pBluescript SK, amplified in bacteria, and *Xba*I/*Pac*I-digested. After gel purification, the fragments were then cloned into pKR162, replacing the original sequence.

For the site-directed mutagenesis of PsaB Trp[627] to Phe and PsaB Asp[624] to Lys, we performed PCR using pKR162 as template, oligonucleotide X, and the degenerated mutagenic oligonucleotides F (5′-ACC-ATTAATTAATTGAGAAGTT(G/T)AAC(C/A)AT(C/A)GG(G/T)AGTCAC-GTAACCA-3′) or K (5′-ACCATTAATTAATTGAGAAGAGTTTAACCA-TAGGTA(G/C)TTACGTAACCA-3′), respectively, containing the *Pac*I cleavage site. The resulting DNA fragments were cloned into pKR162 as described above. For double mutants, PCR was performed with oligonucleotides F or K and X and with previously generated mutagenized pKR162 vectors as templates.

All mutations were verified by sequencing using the ABI310 capillary system.

Chloroplast Transformation and Analysis of Transformants—Chloroplast transformation in *C. reinhardtii* was carried out as described previously (19) using a helium-driven PDS-1000/He particle gun (Bio-Rad) with 1100 psi rupture discs (Bio-Rad). M10 tungsten particles (2.5 mg; Bio-Rad) were coated with 2 μg of the appropriate DNA as described and finally washed three times with 500 μl of absolute ethanol and resuspended in 25 μl of absolute ethanol by short sonification; 7 μl of the suspension were used per transformation. ΔPsaB cells lacking *psaB* were grown at 25 °C in liquid Tris acetate phosphate medium in the dark, and 4×10^7 cells were dispersed per Tris acetate phosphate plate containing 150 μg/ml spectinomycin. Once the plates were dry, the cells were bombarded with the DNA-coated particles. The bombarded cells were kept under low light (5 μE m^2 s) for 2 weeks. The appearing transformants were restreaked on fresh Tris acetate phosphate plates containing spectinomycin and used for further investigations.

Isolation of pc and cyt c_6—The isolation of pc and cyt c_6 followed previously published procedures (3, 20), with modifications as described in Ref. 21. The concentrations of pc and cyt c_6 were determined spectroscopically using an extinction coefficient of 4.9 mM^{-1} cm^{-1} at 597 nm for the oxidized form of pc (22) and 20 mM^{-1} cm^{-1} at 552 nm for the reduced form of cyt c_6 (2).

Isolation of Thylakoid Membranes and the PSI Complex—The isolation of thylakoid membranes purified by centrifugation through a sucrose step gradient and the isolation of PSI particles were as described previously (21, 23). Chlorophyll concentrations were determined according to Ref. 24.

SDS-PAGE and Western Analysis—SDS-PAGE (15.5% T, 2.66% C) was carried out according to Ref. 25. After the electrophoretic fractionation, the proteins were electroblotted onto nitrocellulose and incubated with antibodies as described previously (26). Immunodetection was carried out according to Ref. 21. To quantify the amount of PSI, wild type thylakoids were diluted with thylakoids from the Δ*psaB* strain (lacking PSI), resulting in fractions that contained 100%, 50%, 25%, and 0% PSI. Equal amounts of thylakoid proteins from these fractions and from mutant thylakoids were separated by SDS-PAGE and analyzed by immunoblotting using PsaF-specific antibodies to estimate the PSI content in the different strains. The blots were also probed with light harvesting complex II antibodies to verify equal loading.

Cross-linking Procedure—Cross-linking was performed as described in Ref. 21.

Flash Absorption Spectroscopy—Kinetics of flash-induced absorbance changes at 817 nm were measured essentially as described previously (13, 27). The measuring light was provided by a luminescence diode (Hitachi HE8404SG; 40 mW; full width at half-maximum, 30 nm) supplied with a stabilized battery-driven current source. The light was filtered through a 817-nm interference filter (full width at half-maxi-

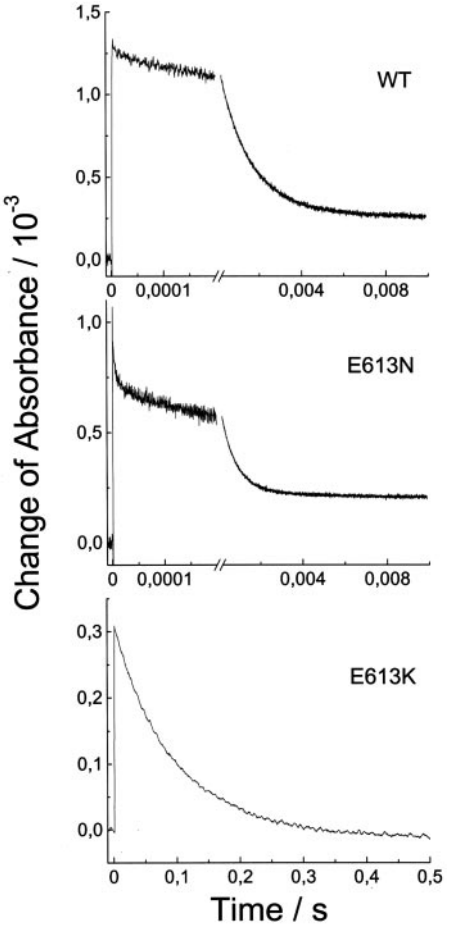

FIG. 2. **Complex formation between pc or cyt c_6 and PSI is enhanced in mutant PSI PsaB E613N compared with wild type but is completely abolished in mutant PSI PsaB E613K.** Absorbance changes at 817 nm induced by a laser flash in PSI particles were monitored in the presence of 20 μM cyt c_6. The relative amplitudes and half-times of the different kinetic components were A(1) = 0.14 and 0.32 with $t_{1/2}(1) = 3.5 \times 10^{-6}$ s, A(2) = 0.63 and 0.45 with $t_{1/2}(2) = 9.6 \times 10^{-4}$ s and 4.5×10^{-4} s, and A(3) = 0.23 with $t_{1/2}(3) = 4 \times 10^{-2}$ s and 4.9×10^{-2} s for wild type and mutant PSI E613N, respectively, and a monoexponential decay with A = 0.99, $t_{1/2} = 6.7 \times 10^{-2}$ s for E613K. The cuvette contained PSI particles at 120 μg chlorophyll/ml, 30 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.0, 0.2 mM methylviologen, 10 mM ascorbate, 0.05% β-dodecylmaltoside, and 0.3 mM $MgCl_2$. The absorbance changes are the result of 5–10 flashes. Breakpoints in the split time scales mark regions of different sampling rates.

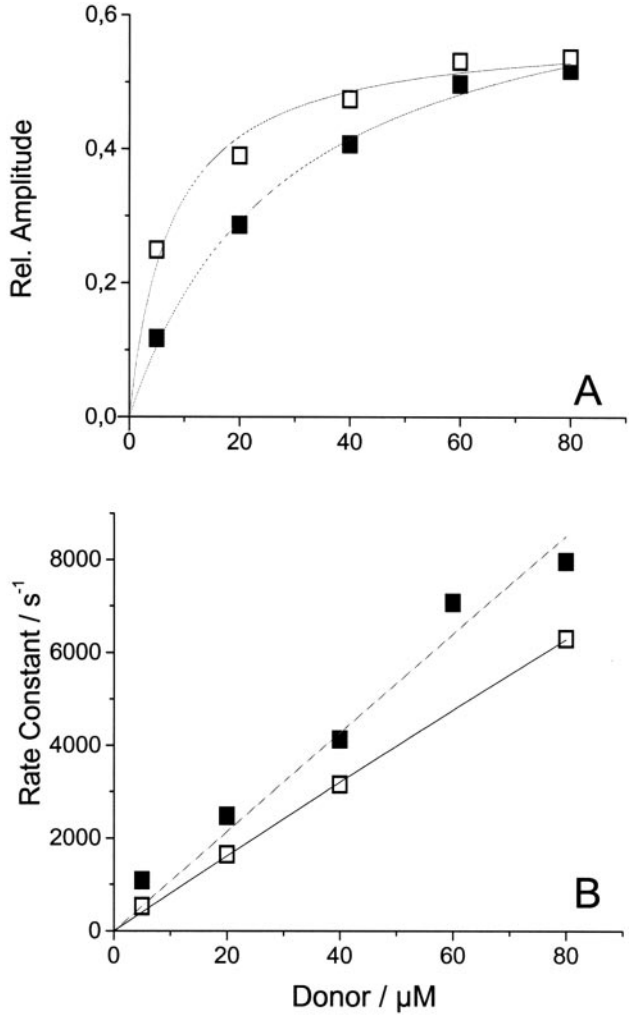

FIG. 3. **Determination of the kinetic constants for the electron transfer between mutant PSI PsaB E613N and the two electron donors.** *A,* amplitude of the fast kinetic component of $P700^+$ reduction as a function of the concentration of donor proteins. *B,* electron transfer rate constant as a function of the donor concentration. □, cyt c_6; ■, pc. Measurements were performed at 0.3 mM $MgCl_2$.

mum, 9 nm) and passed through a cuvette containing 200 μl or 50 μl of the sample with an optical pathlength of 1 cm or 3 mm, respectively.

RESULTS

PsaB Loop j Mutant Strains Are Sensitive to High Light—To investigate the role of loop *j* and especially that of helix *l* of the PsaB protein in binding and electron transfer of the soluble electron donors pc and cyt c_6, we performed a combinatorial site-directed mutagenesis approach. A plasmid containing the altered *psaB* and the *aadA* gene (encoding for aminoglycoside adenyl transferase) that can be expressed in the chloroplast to confer resistance to spectinomycin or streptomycin (18) was transformed into a PsaB-deficient mutant (14). In total, seven different mutant strains were obtained after selection on spectinomycin-containing medium (Table I). In spot test analyses, we found that all generated mutants could grow photoautotrophically at low light intensities (Table I). Mutant strains D624K, E613K/D624K, E613K/W627F, and D624K/W627F died under photoautotrophic or heterotrophic conditions at light intensities equivalent to or higher than 60 μE m^{-2} s^{-1} or 700 μE m^{-2} s^{-1}, respectively. In Western blot analyses of SDS-PAGE fractionated thylakoids using PSI-specific antibodies, such as anti-PsaD or anti-PsaF antibodies, the amount of PSI in these strains could be estimated to be <20% of the amount of PSI found in wild type thylakoids (Table I). For mutant strains E613N, E613K, and W627F, these estimations revealed that >50% PSI as compared with wild type accumulated in the mutant thylakoids. However, strains E613K and W627F died under photoautotrophic or heterotrophic conditions at light intensities equivalent to or higher than 700 μE m^{-2} s^{-1} (Table I). These growth phenotypes are comparable with the phenotype observed for the PsaF-deficient mutant. Mutant strain E613N showed a strong light sensitivity under photoautotrophic conditions and light intensities equivalent to or higher than 700 μE m^{-2} s^{-1} (Table I). The higher amounts of PSI in these three strains enabled us to isolate the altered PSI complexes and to perform detailed functional studies using flash-induced absorption spectroscopy.

Mutation E613N in PsaB Leads to Enhanced Binding of pc and cyt c_6 to PSI, whereas Mutation E613K Drastically Diminishes Binding—The electron transfer from pc or cyt c_6 to PSI isolated from wild type and mutants E613N and E613K was investigated using excitation by single turnover flashes. It should be noted that residue Glu^{613} is located in the inter-

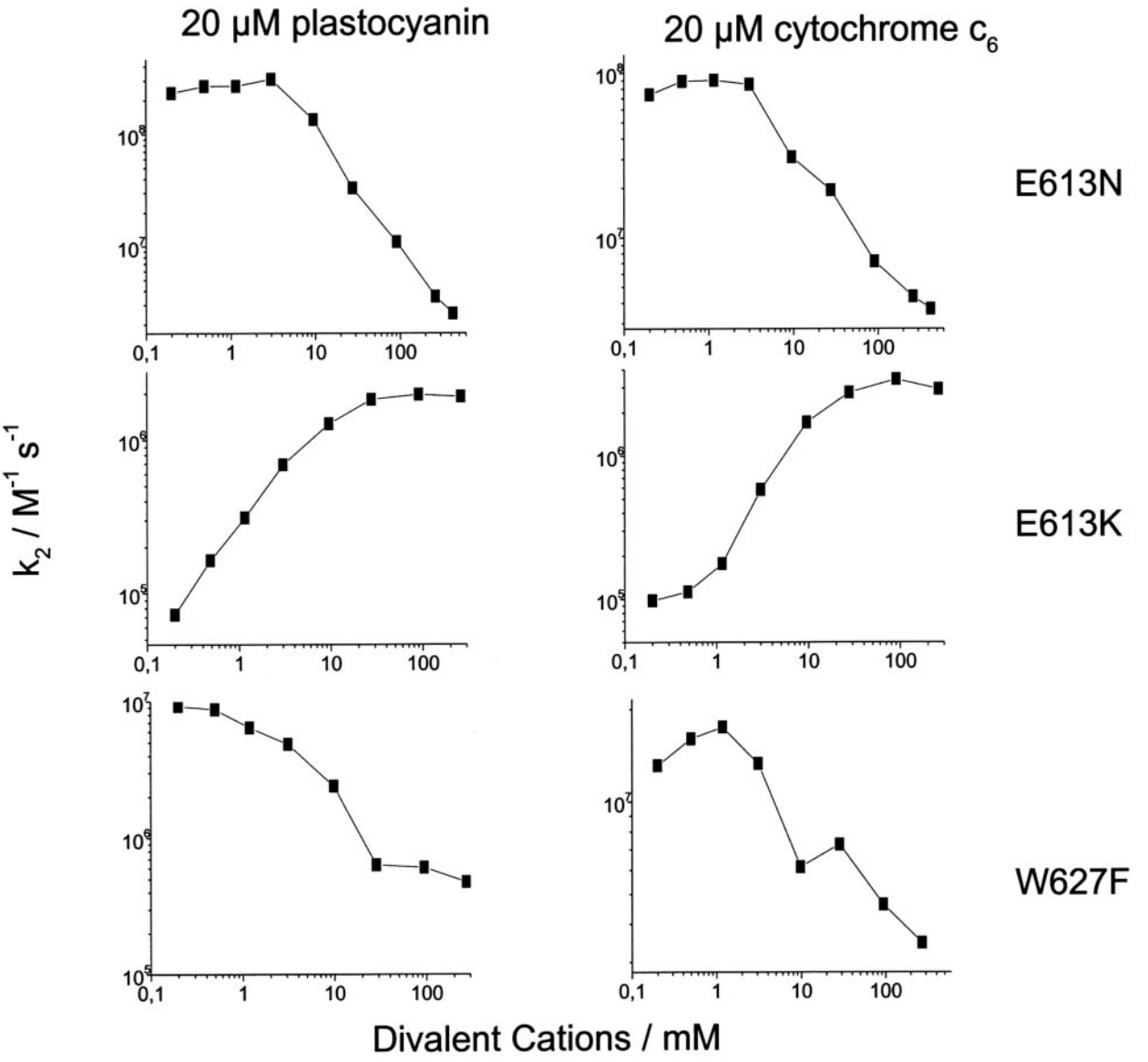

FIG. 4. **Salt dependences of the second-order rate constant of $P700^+$ reduction by 20 μM pc or cyt c_6.** For conditions, see Fig. 2. Small amounts of concentrated $MgCl_2$ solutions were added to increase ionic strength.

helical-loop region of loop *j*. Fig. 2 shows the absorbance transients at 817 nm induced by a laser flash for PSI particles in the presence of 20 μM cyt c_6. In the cases of wild type and mutant E613N, the time course of the $P700^+$ reduction can be deconvoluted into three kinetic components (for wild type, see also Fig. 6). The fast component with a constant half-life of 3–4 μs and a variable amplitude A(1) reflects a first-order electron transfer, the rate of which is independent of the concentration of the donor proteins. This phase can be explained by an electron transfer reaction within a preformed complex between the donor and PSI. The half-time of the fast phase, which was also identified in the kinetics of $P700^+$ reduction using pc as electron donor (data not shown), is found to be the same for wild type and mutant E613N PSI. The intermediate component with an amplitude A(2) shows a half-life that decreases with increasing concentration of reduced donor protein, as known for second-order reactions between soluble reactants (see Fig. 3). Amplitude A(1) increases with increasing concentration of reduced donor protein at the expense of A(2) (see Fig. 3). The third very slow component with an amplitude of about 25 and 40% of the total signal for PSI isolated from wild type and mutant E613N, respectively, has an electron transfer rate constant in the range of 7–9 $\times$ 10^5 M^{-1} s^{-1} for pc or cyt c_6, which is comparable with the values found for electron transfer between both donors and PSI from the PsaF-deficient mutant under similar conditions (21). In the case of mutant E613K, the time course of the $P700^+$ reduction can be deconvoluted into only one kinetic component, with a second-order rate constant of about 5 $\times$ 10^5 M^{-1} s^{-1}, which is again comparable with the values found for electron transfer between both donors and PSI from the PsaF-deficient mutant. Therefore, the very slow electron transfer between mutant PSI E613K and cyt c_6 or pc (Fig. 2, Table II) indicates that binding and electron transfer are independent of PsaF in this mutant.

For further analysis of PsaF-dependent functional electron transfer, we will consider mainly the two kinetic components A(1) and A(2). Drepper *et al.* (27) described a kinetic model for the binding and electron transfer between pc and PSI that also takes into account the redox equilibrium of the electron transfer. In this model, a simple dissociation equilibrium of the complex between the reduced donor protein ([*D*]) and PSI was used to describe the concentration dependence of the amplitude A(1). An estimate of the dissociation constant (K_D) can be determined using the following equation:

TABLE I
Growth properties of wild type, the PsaF-deficient mutant, and the ΔPsaB transformants with an altered PsaB protein on TAP[a] and HSM plates

Strains	10 μE m^{-2} s^{-1}		60 μE m^{-2} s^{-1}		700 μE m^{-2} s^{-1}		PSI content
	HSM	TAP	HSM	TAP	HSM	TAP	
WT	+	++	++	+++	++	++	100%
E613N	+	++	++	+++	−	+	>50%
E613K	+	++	+	++	−	−	>50%
W627F	+	++	+	++	−	−	>50%
E613K/W627F	+	+	−	+	−	−	>20%
D624K	+	+	−	+	−	−	<10%
E613K/D624K	+	+	−	+	−	−	<10%
D624K/W627F	+	+	−	+	−	−	<10%
3bF (Δ*psaF*)	+	+	+	+	−	−	>50%

[a] TAP, Tris acetate phosphate; HSM, high-salt medium; WT, wild type.

TABLE II
Properties of the electron transfer from pc and cyt c to PSI from wild type, the PsaF-deficient mutant and the ΔpsaB transformants with an altered PsaB protein

The second-order rate constants k_2 and the dissociation constants K_D determined for wild type and the PsaF-deficient strain 3bF are taken from Refs. 7 and 21.

	Plastocyanin			Cytochrome c_6		
	k_2[a]	K_D[b]	$t_{1/2(off)}$[c]	k_2	K_D	$t_{1/2(off)}$
	(10^7 M^{-1} s^{-1})	(μM)	(ms)	(10^7 M^{-1} s^{-1})	(μM)	(ms)
WT[d]	9 (0.3 mM)	83 (f = 0.69)	0.09	3.4 (0.3 mM)	83 (f = 0.66)	0.25
PsaB E613N	11 (0.3 mM)	29 (f = 0.71)	0.22	8.2 (0.3 mM)	7.7 (f = 0.58)	1.1
PsaB E613K	0.22 (0.1 M)	>1000	n.d.	0.34 (0.1 M)	>1000	n.d.
Δ*psaF*	0.13 (1 M)	>1000	n.d.	0.25 (1 M)	>1000	n.d.
PsaB W627F	0.74 (0.3 mM)	>1000	n.d.	1.6 (0.3 mM)	177 (f = 0.66)	0.24

[a] The second-order rate constant k_2 determined from linear regression of the observed rate constant of the donor-dependent kinetic phase as shown in Fig. 3*B* and Fig. 7*B* also gives an estimate of the on-rate for the formation of the complex. All kinetic constants refer to conditions close to the optimal concentrations of $MgCl_2$ as indicated in parentheses.
[b] The dissociation constant K_D of the active complex is estimated from the amplitude of the fast kinetic component as shown in Fig. 3*A* and Fig. 7*A*.
[c] The half-life of the active complex, $t_{1/2} = \ln(2)/k_{off}$, where k_{off} is the rate of dissociation of the donor from the photosystem estimated by using the approximate relation $k_{off} = K_D \times k_2$. For a detailed discussion of the limits of these estimates and a comparison of their results to a more refined kinetic analysis, see Ref. (27).
[d] WT, wild type; n.d., not determined.

$$A_1 = f \times \frac{[D]}{([D] + K_D)} \quad \text{(Eq. 1)}$$

where f represents an empirical factor ($f < 1$) that relates amplitude A(1) observed after the flash to the fraction of PSI in a complex with the reduced donor before the flash (27). Eq.1 indicates that the relative amplitude A(1) has a hyperbolic dependence on the concentration [D], *i.e.* shows a half-maximum saturation at a donor protein concentration equal to K_D and approaches a maximum value f at infinite concentration.

The amplitude of the fast phases of $P700^+$ reduction for kinetic experiments performed with PSI isolated from mutant E613N with various concentrations of pc and cyt c_6 is displayed in Fig. 3*A*. The data points can be analyzed by a hyperbolic curve according to Eq. 1. As a result, dissociation constants and the values of f are obtained for pc and cyt c_6 as summarized in Table II.

Because the concentration of pc and cyt c_6 exceeds that of P700 by >1 order of magnitude, the kinetic component A(2) follows an exponential time course. The plots of its rate constant, $k = \ln 2/t_{½}$, *versus* the donor concentration can be approximated by linear dependences throughout the concentration range used in the experiments (Fig. 3*B*). The second-order rate constant, $k_2 = \ln 2/(t_{½} \times [D])$, can be determined from the slope of the regression lines in Fig. 3*B*. The results for the reactions of cyt c_6 and pc with this mutant (E613N PSI) and with wild type PSI are summarized in Table II.

The dissociation constants of 29 and 7.7 μM as well as the second-order rate constants of 11 and 8.2×10^7 M^{-1} s^{-1} for pc and cyt c_6, respectively, are significantly smaller for mutant PSI E613N than for the wild type (Table II). The determination of k_2 and K_D values for binding of pc and cyt c_6 to PSI isolated from mutant E613N and the dissociation equilibrium of the complex, respectively, implies that the unbinding of the donors from altered PSI is about 3 times slower compared with wild type PSI. As mentioned above, mutation of residue Glu^{613} in PsaB to Lys has a dramatic effect on the binding of pc and cyt c_6 to the PSI complex. Their second-order rate constants for reduction of PSI are decreased by 2 orders of magnitude, which is comparable with the electron transfer reaction of both donors with the PsaF-deficient PSI. To further analyze the electron transfer properties of mutants E613N, E613K, and W627F, we investigated the salt dependence of the electron transfer between the mutant PSI and the two donors.

Salt Dependence of Electron Transfer from pc and cyt c_6 to PSI Isolated from Mutants E613N, E613K, and W627F—To analyze the role of electrostatic interactions in the reactions between the altered PSI and pc or cyt c_6, we measured the second-order rate constant of $P700^+$ reduction by pc and cyt c_6 as a function of the $MgCl_2$ concentration (Fig. 4). It was shown that the electron transfer rate constants from both donors to wild type PSI decreased at salt concentrations higher than 3 mM $MgCl_2$, whereas the electron transfer rates increased with

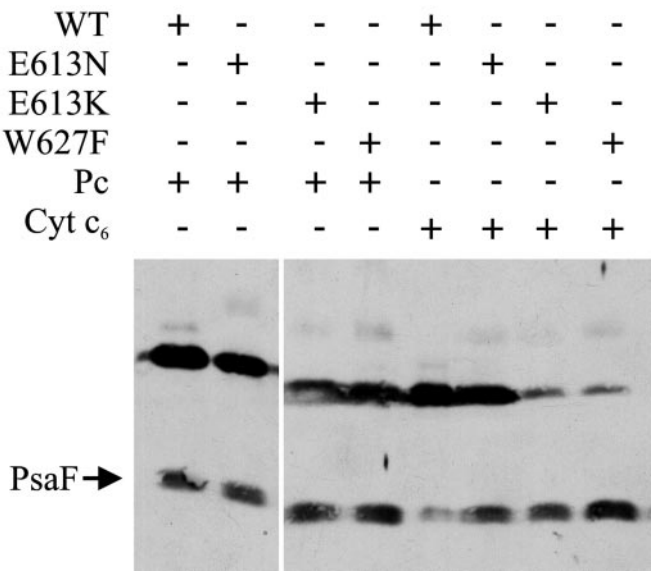

FIG. 5. **Cross-linking of pc or cyt c_6 to PsaF is diminished with PSI from mutant strains E613K and W627F.** Immunoblot analysis of cross-linked products between pc or cyt c_6 and PSI from wild type and the Δ*psaB* transformants E613N, E613K, and W627F is shown. 5 μg of chlorophyll are loaded on each lane. The blot was probed with anti-PsaF antibodies.

increasing salt concentration for the PsaF-deficient PSI (21). For mutants E613N and W627F, the electron transfer rates from pc or cyt c_6 to PSI show an optimum between 1 and 3 mM $MgCl_2$ and a decrease by >1 order of magnitude at higher concentrations. The optimal second-order rates are found for PSI E613N with pc and cyt c_6 at 30 and 9×10^7 M^{-1} s^{-1}, respectively, and for PSI W627F with pc and cyt c_6 at 0.9 and 1.75×10^7 M^{-1} s^{-1}, respectively. It is noteworthy that the optimum second-order rate constant for the altered PSI W627F in the interaction with pc is about 1 order of magnitude slower than that with wild type or mutant E613N PSI (Fig. 4, see also below). The electron transfer rates from pc or cyt c_6 to mutant PSI E613K increase with increasing salt concentrations, displaying an optimum between 100 and 300 mM $MgCl_2$. The optimal second-order rates are found at values of 1.9 and 3.4×10^6 M^{-1} s^{-1} for pc and cyt c_6, respectively. The shape of the salt dependence and the optimal electron transfer rates measured for interaction between the two donors and mutant PSI E613K are very similar to what was found with the PsaF-deficient PSI (21), thus suggesting that mutation PsaB E613K drastically disturbs the interaction between the donors and the positively charged N-terminal domain of PsaF. However, another possible explanation could be that the PsaF subunit is lost during the isolation of mutant E613K PSI, although it is present in thylakoids of the mutant. To analyze this question, equal amounts of protein from enriched PSI particles isolated from wild type or mutant E613K were fractionated by SDS-PAGE and analyzed by immunoblotting using anti-PsaF antibodies. These immunoblots show that PsaF is present in PSI from mutant E613K at almost equal amounts as compared with the amount of PsaF present in a wild type PSI preparation (data not shown). To check whether PsaF is reachable in the isolated altered PSI E613K particles for the soluble donors, we performed cross-linking experiments.

Cross-linking of pc or cyt c_6 to PsaF Is Diminished with PSI from Mutant Strains E613K and W627F—The interactions between PsaF subunit and pc or cyt c_6 were examined by cross-linking studies, using purified PSI particles from the E613N, E613K, and W627F transformants and from wild type (Fig. 5). The cross-linked products were fractionated by SDS-PAGE and identified by immunoblotting using PsaF antibodies (Fig. 5). The cross-linking products between pc or cyt c_6 and PsaF were found at about 29 kDa. It was shown in a previous study that the cross-linked products consist of either PsaF and pc or PsaF and cyt c_6 (21). The data confirm that the PsaF subunit is present in the E613K PSI particles. However, the cross-linking efficiency is clearly diminished for both donors. Cross-linking of pc or cyt c_6 to PsaF in PSI particles isolated from mutant W627F is also strongly reduced. This result indicates that efficient cross-linking depends not only on the electrostatic interaction between the donors and PsaF but also on a direct contact between the donors and the core of PSI.

Mutation W627F Abolishes Formation of an Electron Transfer Complex between pc and the Altered PSI—The cross-linking results and the salt dependence of the second-order rate constant indicate that mutation W627F has a strong impact on the interaction between the altered PSI and cyt c_6 or pc. To further characterize the electron transfer reactions between pc or cyt c_6 and the mutant W627F PSI, we measured reduction of $P700^+$ at different donor concentrations (see Fig. 7). Fig. 6 shows absorbance transients at 817 nm of PSI particles from wild type (*top panels*) and the W627F transformant (*bottom panels*) induced by a laser flash in the presence of 120 μM pc (*left panels*) or 120 μM cyt c_6 (*right panels*). In the case of wild type, the time course of the $P700^+$ reduction can be deconvoluted into three kinetic components, as described above. In contrast, no fast phase A(1) can be observed for the time course of $P700^+$ reduction in the case of mutant W627F when pc functions as electron donor. A fast phase with a half-time of 30 μs can be deconvoluted from the flash-induced absorbance transient of mutant W627F PSI in the presence of 120 μM cyt c_6. This half-time is about 10-fold slower than the half-lives found for the intermolecular electron transfer complex formed between wild type PSI and pc or cyt c_6. The amplitude of the 30-μs phase increases with increasing concentration of reduced donor protein at the expense of A(2) (see Fig. 7), indicating that it reflects a first-order electron transfer reaction.

If a *f* value of 0.66 is considered, as found for the reaction of cyt c_6 with wild type PSI, a dissociation constant of 177 μM can be estimated. However, we cannot exclude from our data that the maximum value *f* of the relative amplitude is higher in the mutant than in the wild type. Assuming a value *f* of 1.0 in the fit of the curve to the data in Fig. 7 would translate into a dissociation constant of 310 μM. The slower kinetic components of the time course of $P700^+$ reduction for pc or cyt c_6 show a rate constant that increases with increasing concentration of reduced donor protein (Fig. 7*B*). Evaluations of the second-order rate constants, which can be determined from the slopes of the curves in Fig. 7*B* at low concentrations ($k_2 = \ln 2/(t_{½} \times [D])$), result in values of 0.74 and 1.6×10^7 M^{-1} s^{-1} for pc and cyt c_6, respectively.

DISCUSSION

In previous studies, we could show that efficient electron transfer from both pc and cyt c_6 to PSI in eukaryotic organisms depends on PsaF (6, 7, 13, 21). In this study, we have taken advantage of a PsaB-deficient mutant (14) to modify lumenal loop *j* including helix *l* of PsaB using chloroplast transformation and site-directed mutagenesis. PSI particles from mutant strains containing a specific amino acid change in the PsaB protein, E613N, E613K, and W627F, were isolated together with PSI particles from wild type and used to characterize the electron transfer from purified pc and cyt c_6 to $P700^+$ *in vitro*. Our results indicate that helix *l* of PsaB is, in addition to the N-terminal domain of PsaF, a second structural element that is

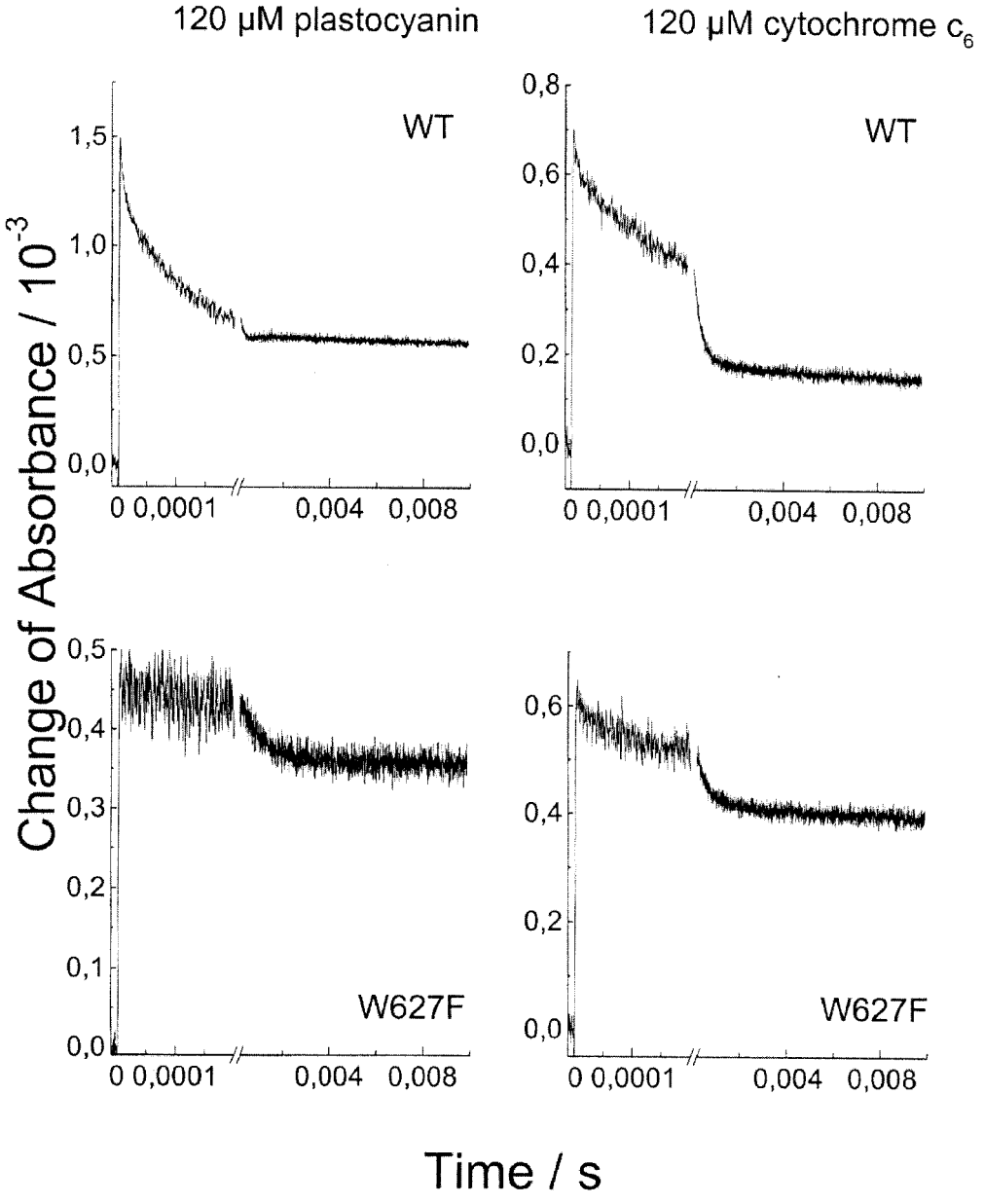

FIG. 6. **Mutation of Trp^{627} to Phe in PsaB abolishes complex formation of PSI with pc but not with cyt c_6.** Absorbance changes at 817 nm induced by a laser flash in PSI particles from wild type and mutant W627F in the presence of 120 μM pc or 120 μM cyt c_6; for conditions, see Fig. 2. The relative amplitudes and half-times of the different kinetic components were as follows: for wild type, A(1) = 0.25 and 0.24 with $t_{1/2}(1) = 3.5 \times 10^{-6}$ s, A(2) = 0.4 and 0.54 with $t_{1/2}(2) = 6.7 \times 10^{-5}$ s and 2×10^{-4} s, and A(3) = 0.35 and 0.22 with $t_{1/2}(3) = 1.5 \times 10^{-1}$ s and 3×10^{-2} s for pc and cyt c_6, respectively, and for mutant PSI W627F, a two exponential decay for pc with A(1) = 0.2 with $t_{1/2}(1) = 6 \times 10^{-4}$ s, A(2) = 0.73 with $t_{1/2}(2) = 3.4 \times 10^{-1}$ s, and a triphasic decay with A(1) = 0.09 with $t_{1/2}(1) = 3 \times 10^{-5}$ s, A(2) = 0.24 with $t_{1/2}(2) = 3.5 \times 10^{-4}$ s, and A(3) = 0.67 with $t_{1/2}(3) = 1.1 \times 10^{-1}$ s.

essential for recognition of pc and cyt c_6 to the core of PSI, leading to an intermolecular complex competent in fast electron transfer.

Mutation of Glu^{613} to Asn resulted in a clear acceleration of electron transfer rates as well as improved binding of both donors to PSI as compared with wild type. It is therefore likely that the negative charge provided by residue Glu^{613} is exposed on the surface of PSI. This view is supported by the data obtained from the three-dimensional structure of the cyanobacterial PSI at 2.5 Å resolution (4). In the structure of the cyanobacterial PSI, the side chain of Glu^{617} corresponding to Glu^{613} in *C. reinhardtii* points into the lumenal space (Fig. 8). Thus, the change of Glu to Asn could lead to a tighter binding of the donors to PSI in mutant E613N because this mutation decreases the electrostatic repulsion between the negatively charged donors and PSI. This in turn results in a slower unbinding of both donors from the reaction center (see Table II). Drepper *et al.* (27) proposed that the rate of unbinding of pc from PSI is limiting for the rate of electron transfer between PSI and the cyt b_6/f complex. Such a limitation could be a possible explanation of why mutant E613N dies in strong light (see Table I). Fig. 8 shows that the corresponding Glu^{613} of *C. reinhardtii* is in a close distance to the N terminus of the PsaF subunit of the cyanobacterial PSI. Because the N-terminal domain of PsaF in *C. reinhardtii* is even larger, mutation E613K may cause a distortion of the positively charged N-terminal domain of PsaF by charge repulsion from the positively charged Lys at position 613. Such a disorientation may have a strong effect on the binding and electron transfer of both donors to PSI because the electrostatic interaction with the

N-terminal domain of PsaF would be strongly impaired. This interpretation can explain why the salt dependence of electron transfer between both donors and the altered PSI E613K resembles the data obtained with PSI from the PsaF-deficient mutant. It may also explain why mutations E613N and E613K have opposite effects on binding and electron transfer between the altered PSI particles and the two donors. From these data, we can conclude that (i) residue Glu^{613} supports unbinding of the two donors from PSI because of electrostatic repulsion, and (ii) residue Glu^{613} orientates the positively charged N-terminal domain of PsaF in a way that allows efficient binding of donors pc and cyt c_6 to PSI.

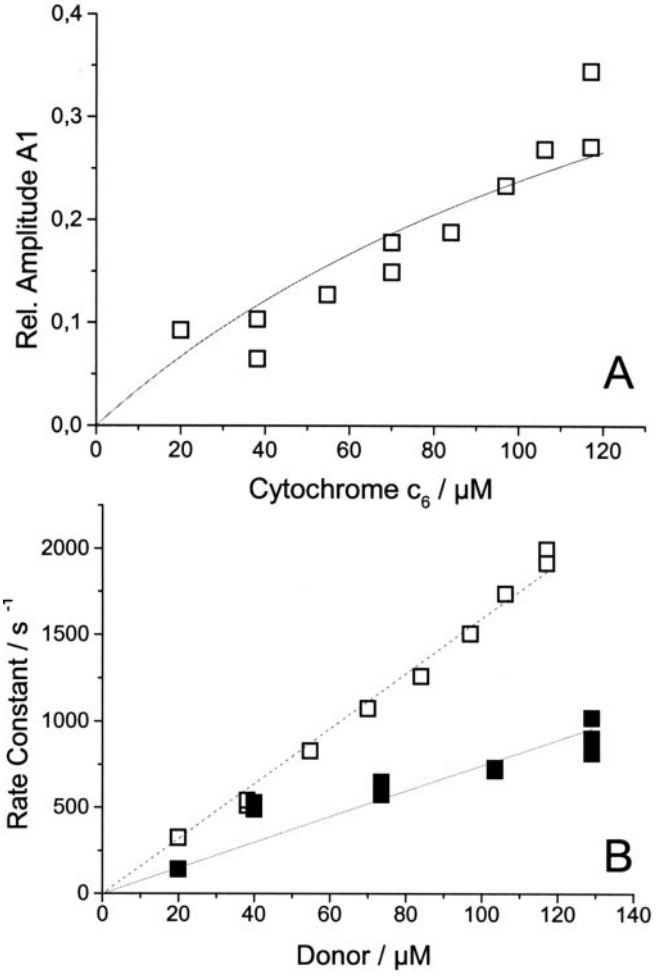

FIG. 7. **Determination of the kinetic constants for the electron transfer between mutant PSI PsaB W627F and the two electron donors.** *A,* amplitude of the fast kinetic component of $P700^+$ reduction as a function of the concentration of cyt c_6. *B,* electron transfer rate constant as a function of the donor concentration. □, cyt c_6; ■, pc. Measurements were performed at 0.3 mM $MgCl_2$.

For eukaryotic organisms, site-directed mutagenesis of pc and analysis of binding and electron transfer between the altered pc and PSI (6, 8, 28, 29) already suggested that besides the long-range electrostatic interaction between the positively charged PsaF and the negative patches of pc, a second recognition site is required that brings the flat hydrophobic surface of pc in close contact with the core of PSI to allow efficient electron transfer from the copper center to $P700^+$. The fact that mutation PsaB W627F abolishes the formation of an intermolecular electron transfer complex between the altered PSI and pc (see Fig. 6) indicates that Trp^{627} of helix *l* is part of the suggested recognition site required for binding of pc to the core of PSI. This interpretation is strongly supported by the high resolution structural data on PSI (4). Fig. 8 shows that Trp^{631} (corresponding to Trp^{627} in *C. reinhardtii*) from helix *l* of PsaB and Trp^{655} (corresponding to Trp^{651} in *C. reinhardtii*) from PsaA form a sandwich with their indole groups stacked at van der Waals distance (4), situated directly above P700. Thus, binding of pc to this structural element would possibly allow electron transfer from His^{87} of pc via the π-electron system of the aromatic Trp residues directly to $P700^+$. The mutation of Trp to Phe at position 627 decreases the hydrophobic surface that is exposed into the putative binding site from a value of about 255 to 210 ($Å^2$) (30). This change is apparently too large to allow a hydrophobic contact sufficient for the stable formation of an electron transfer complex between the northern face of pc and helix *l* of the mutated W627F PSI.

The electron transfer between cyt c_6 and the altered W627F PSI is not as much disturbed as that between the mutant PSI and pc (see Table II). How can this be explained? In cyanobacteria, site-directed mutagenesis of pc or cyt c_6 from *Synechocystis* sp. PCC 6803 and *Anabaena* PCC 7119 and analysis of binding and electron transfer between the altered donors and the cyanobacterial PSI revealed that a positively charged amino acid located at the northern face of either pc or cyt c_6 is crucial for the interaction with the reaction center (31, 32). Interestingly, an equivalent positively charged amino acid is present in cyt c_6 from *C. reinhardtii* (20) but absent in pc of *C. reinhardtii* and other eukaryotic pc structures. This implies that this positively charged amino acid is an important structural motif that promotes binding of cyt c_6 from *C. reinhardtii* to the core of PSI. The presence of this additional recognition site in cyt c_6, which is absent from pc, may therefore explain why binding of cyt c_6 to the mutant W627F PSI is less affected as compared with pc. However, the rate of electron transfer within the intermolecular complex between cyt c_6 and the mutated PSI is 10 times slower as compared with wild type. This implies that the orientation and/or conformation of the cyt c_6/W627F PSI complex is altered in comparison to wild type because the rate of electron transfer is very sensitive to

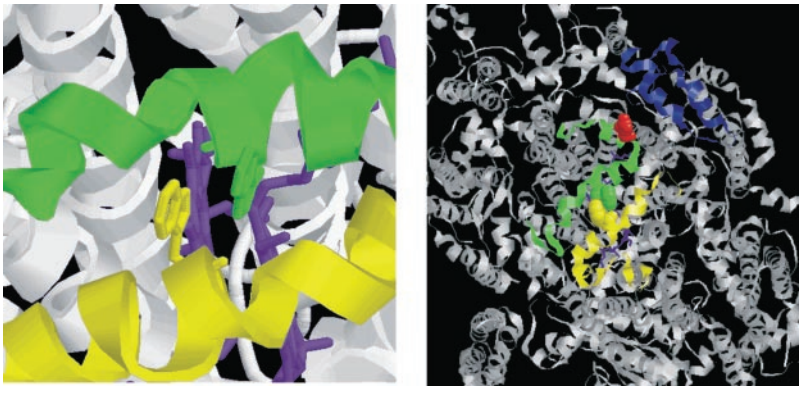

FIG. 8. **Crystal structure of *S. elongatus* PSI at a resolution of 2.5 Å with a focus on the lumenal side (4).** The N-terminal part of PsaF is shown in *blue*, loop *j* is shown in *green* (PsaB) and *yellow* (PsaA), the corresponding PsaB Glu^{613} of *Chlamydomonas* is shown in *red,* and P700 is shown in *purple*. The structure suggests (i) interaction of negatively charged PsaB Glu^{613} with the positively charged PsaF in *Chlamydomonas* and (ii) participation of PsaB Trp^{627} in binding of the electron donors.

changes in distance between electron transfer partners (33). Thus, W627 is also important for binding and electron transfer of cyt c_6 to PSI and required for the formation of the intermolecular electron transfer complex competent in the 3-μs electron transfer.

A functional characterization of a cyanobacterial PSI complex carrying an algal-type PsaF subunit has already suggested that cyt c_6 from *C. reinhardtii* is regarding its binding mechanism to PSI an evolutionary intermediate between cyt c_6 from *S. elongatus* and pc from *C. reinhardtii* (13). This view is substantiated with the data and interpretations presented above.

Interestingly, mutation E613K and mutation W627F cause a strong limitation of electron transfer between PSI and pc or cyt c_6, as already observed for the PsaF-deficient mutant. In comparison with the PsaF-deficient mutant, this restriction results in a light-sensitive phenotype, which supports the interpretation that a limitation of electron transfer at the oxidizing side of PSI is harmful to these mutant cells at higher light intensities and may lead to photooxidative stress (34).

In summary, we can conclude that lumenal loop *j* (especially helix *l*) is essential for efficient binding and fast electron transfer between PSI and pc or cyt c_6 in *C. reinhardtii*. It provides a negatively charged residue (Glu^{613}) of PsaB that (i) supports unbinding of the two donors from PSI and (ii) orientates the positively charged N-terminal domain of PsaF to enable efficient binding of both donors to PSI. Trp^{627} from PsaB, most likely together with Trp^{651} from PsaA, forms the specific hydrophobic recognition site of the core of PSI that is required for effective binding and electron transfer and stable complex formation between pc and PSI and is important for proper electron transfer from cyt c_6 to PSI.

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The hydrophobic recognition site formed by residues PsaA-W651 and PsaB-W627 of photosystem I in *Chlamydomonas reinhardtii* confers distinct selectivity for binding of plastocyanin and cytochrome c_6

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running title: Electron transfer between pc or cyt c_6 and PSI from mutants in PsaA-W651

Key words: photosystem I, plastocyanin, cytochrome c6, electron transfer, site directed mutation

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Abbreviations

WT wild-type; K_D dissociation constant; pc plastocyanin; cyt c_6 cytochrome c_6; E einstein

Abstract

Residue PsaA-W651 is located in loop j' on the lumenal side of PSI facing W627 of PsaB. In a former study it has been shown that PsaB-W627 is part of the hydrophobic recognition site that is essential for proper binding of the two electron donors plastocyanin and cytochrome c_6 to the donor side of PSI (F. Sommer, F. Drepper and M. Hippler, J. Biol. Chem. 277, 6573-81). To question the function of PsaA-W651 in binding and electron transfer of both donors to PSI we generated mutants PsaA-W651F and PsaA-W651S by site directed mutagenesis and biolistic transformation of *Chlamydomonas reinhardtii.* The electron transfer between the altered PSI and the donors was analysed by flash absorption spectroscopy. Mutation of PsaA-W651F completely abolished the formation of first order electron transfer complex between pc and the altered PSI and increased the dissociation constant for binding of cyt c_6 by more than a factor five. Mutation PsaA-W651S lowered the affinity for binding of cyt c_6 even further and resulted in a dissociation constant 10 fold larger as compared to wild type. In line with this cross-linking between PSI from PsaA-W651S and cyt c_6 was almost completely abolished. Interestingly, mutation PsaA-W651F had an opposite effect on binding of pc to the altered PSI and admitted the formation of a first-order electron transfer complex. These results indicate that the PsaA-W651 forms together with PsaB-W627 the hydrophobic area, that is crucial for high affinity binding of pc and cyt c_6 to PSI. Our results also indicate that the Trp dimer confers a differential selectivity in binding of both donors to PSI.

Introduction

Photosystem I (PSI) is an integral light driven plastocyanin:ferredoxin oxidoreductase that is embedded in the thylakoid membrane. It is a multiprotein complex that uses light energy to transport electrons from the lumenal soluble electron carrier plastocyanin (pc) across the membrane to the stromal soluble electron acceptor ferredoxin. In cyanobacteria and green algae cytochrome c_6 (cyt c_6) can substitute plastocyanin depending on the availability of copper in the medium (1-4). The crystal structure of PSI from *Synechococcus elongatus* has been refined to a resolution of 2.5 Å (5). PSI consists of about 11-14 subunits of which PsaA and PsaB build up the core of the complex each carrying a set of cofactors required for a functional electron transport chain through PSI. The 4Fe-4S cluster F_X is located on both, PsaA and PsaB, and the terminal 4Fe-4S clusters F_A and F_B are bound to PsaC on the stromal side where binding of ferredoxin takes place facilitated by PsaC, PsaD and PsaE (6).

On the lumenal side of PSI the primary electron donor P700, a chlorophyll dimer, is located near the lumenal surface. It is separated from the lumenal space by two α helices *l'* and *l* formed by loops *j'* and *j* in PsaA and PsaB, respectively, which are arranged in parallel to the membrane plane. Characteristic for algae and vascular plants is an extension of the lumenal N-terminal domain of PsaF that is absent from cyanobacteria and that was proposed to form an amphipatic helix with basic patches facing the binding site of the soluble electron donors (7). The electron transfer to eukaryotic PSI shows a complex kinetic behaviour and can be described by a multi step process involving donor binding, PSI-donor complex formation, electron transfer and unbinding of the donor (8). Docking of pc or cyt c_6 to the PSI is mainly promoted by two highly conserved structural interaction patterns which are 1. long range electrostatic attractions between basic patches of PsaF and acidic regions of pc and cyt c_6 (7,9-12) and 2. a hydrophobic region around the electron transfer site of the donors interacting with a hydrophobic region site on PSI including PsaB-W627 in *C. reinhardtii* (10,13).

The function of the positively charged residues in the eukaryotic N-terminus of PsaF in binding of both donors have been studied extensively by cross-linking, knock out and reverse genetics experiments (7,12,14-16). These studies showed that the basic patch present in the N-terminal domain of PsaF is crucial for proper binding, complex formation between donor and PSI and fast electron transfer. In contrast to eukaryotic organisms in the cyanobacterium *Synechocystis sp.* PCC6803 efficient binding and electron transfer between PSI and pc or cyt c_6 does not depend on the PsaF subunit, since the specific deletion of the *psaF* gene in cyanobacteria did not affect photoautotrophic growth (17) and the *in vivo* measured electron transfer rate between cyt c_{553} and PSI was the same as in WT (18).

The hydrophobic interaction site of the reaction core formed by PsaB has been studied by site directed mutagenesis. Sun *et al* introduced short stretches of mutations in the lumenal loop j of the PsaB protein from *Synechocystis* PCC 6803 and could isolate a double mutant (W622C/A623R) which was highly photosensitive and showed a severe defect in the interaction with pc or cyt c_6 (19). A more conservative mutation of W627 (corresponding to W622 in *Synechocystis* and W631 in *S. elongatus*) to F in the eukaryotic PSI of *C. reinhardtii* displayed also a strong effect on growth (13). Cells became strongly photosensitive and *in vitro* analysis of the electron transfer reactions revealed a differential effect on the binding constants of pc and cyt c_6. No complex formation was observed for the interaction of pc with the altered PSI while it was still present with cyt c_6 displaying a ten fold decreased electron transfer rate. Interestingly, as seen from the crystal structure W631 in loop j of PsaB (corresponding to W627 in Chlamydomonas) forms a sandwich complex with the corresponding W655 of PsaA (corresponding to W651 in Chlamydomonas). This stacked π electron system is located in close distance to P700.

In this study we questioned the role of PsaA-W651 in donor binding and electron transfer. Mutants PsaA-W651F and PsaA-W651S were generated by site directed mutagenesis and biolistic transformation of *Chlamydomonas reinhardtii.* Both mutations had large effects

on the *in vitro* measured electron transfer kinetics between pc or cyt c_6 and the altered PSI. The results are discussed in respect to the function of residue PsaB-W627F in binding and electron transfer between the donors and PSI.

Materials and Methods

Strains and media. C. reinhardtii wild-type and mutant strains were grown as descried (20). If necessary the media (Tris acetate phosphate medium (TAP) or high salt minimal medium (HSM)) were solidified with 1.5 % Kolbe agar (Roth) and supplemented with 150 µg/ml streptomycin (Sigma) when required.

Nucleic acid techniques. In vitro site directed mutagenesis was performed according to standard protocols (21) using *E. coli* strain DH5α as a bacterial host. For the single exchange of PsaA-W651 PCR was performed using as template plasmid pKR154 (22) containing the psaA gene and the aadA-cassette (23) and as mutation carrying primers 5`-GGTTACGTGACTTCTTATTTGCACAATCATCAC-3` and the antisense oligonucleotide for the exchange of PsaA-W651 to F or 5´-CGTGACTTCTTATCGGCACAATC-3´ and the antisense oligonucleotid for the exchange PsaA-W651S. After PCR the template was Dpn1 digested and the PCR product was transformed and amplified in *E. coli*. The mutations were verified by sequencing. Biolistic transformation of chloroplasts using the PDS-1000/He device (BioRad) and selection on spectinomycin was carried out as described (13). As recipient strain ΔPsaA cells lacking the *psaA* gene were chosen (22).

Isolation of proteins from C. reinhardtii Thylakoid membranes and PSI from wild-type and mutant strains were isolated as described (11), chlorophyll content was determined according to (24). Isolation of pc and cyt c_6 was done as published (11), the concentrations of pc and cyt c_6 were determined spectroscopically using an extinction coefficient of 4.9 mM^{-1} cm^{-1} at 597 nm for the oxidized form of pc and 20 mM^{-1} cm^{-1} at 552nm for the reduced form of cyt c_6.

SDS-PAGE and immuno analysis. SDS-PAGE (15.5 % T, 2.66 % C) was carried out as described (25). Western blotting and immuno detection followed the protocols according to

(11). For the quantification of PSI content in mutant strains compared to wild type different amounts of the thylakoid fraction isolated from wild type supplemented with thylakoids from the PSI lacking ΔPsaB strain to a final amount of 20 µg proteins were fractionated on a SDS-PAGE gel together with thylakoids isolated from mutant strains according to 20 µg proteins. Immuno analysis was carried out using anti-PsaF antibodies and anti LHCII antibodies to verify equal loading.

Cross-linking procedure. Cross-linking was performed as described (11).

Redox potentiometrie. Determination of the redox midpoint potential for P700 from WT and the different mutant strains was done essentially as described (8). A cuvette with an optical path length of 10 mm containing 3.5 ml of sample under argon was used. As redox mediators duroquinone and N,N,N´,N´-tetramethyl-p-phenylenediamine at 5µM, ferro- and ferricyanide at 10µM and pc at 2µM were added and the redox potential was adjusted by additions of ferricyanide or ascorbate. For measurement of the redox potential a platinum electrode and an Ag/AgCl reference electrode was used. Calibration of the reference electrode was done with quinhydrone at given pH. All redox potentials are displayed relative to the standard hydrogen electrode.

Flash absorption spectroscopy. Kinetics of flash induced absorbance changes at 817 nm were measured essentially as described (8). The measuring light was provided by a luminescence diode (Hitachi HE8404SG; 40 mW; full width at half maximum 30 nm) supplied by a stabilized battery-driven current source. The light was filtered through a 817 nm interference filter (full width at half maximum 9 nm) and passed through a cuvette containing 200 µl of the sample with an optical path length of 1 cm.

Analysis of more complex electron transfer kinetics was performed using global fit analysis. For the evaluation of the kinetic traces obtained at the presence of high concentrations of $MgCl_2$ (> 30 mM), where the donor dependent and independent reduction rate and amplitude were hard to distinguish, a global fit analysis was performed. Hereby a constant ratio of the amplitudes was assumed, that was derived from measurements at concentration below 30 mM $MgCl_2$. A global fit analysis was also performed to determine the amplitude A1 at low donor concentrations. In this case the lifetime for the fast kinetic phase was fitted to the same value over the range of donor concentrations. All fits were performed on two independent sets of measurement.

Results

Growth properties. To investigate the impact of PsaA-W651 on the binding and the electron transport from pc or cyt c_6 to PSI we performed site specific mutagenesis and altered this residue to F and S. A plasmid containing the altered PsaA together with the aadA cassette conferring resistance to spectomycin was introduced into *C. reinhadtii* strain ΔPsaA, lacking *psaA*, by biolistic transformation. Growth properties of the resulting mutant strains PsaA-W651F and PsaA-W651S were investigated. Hereby the mutant strains together with a WT strain were spotted on plates containing different media (TAP and HSM) that were exposed to different light intensities and atmospheric conditions (Table I). All strains were able to grow heterotrophically under normal atmospheric conditions and all light intensities tested whereas under photoautotrophic conditions the mutant strains became sensitive to light intensities higher than 700 μ E m^{-2} s^{-1}. Photoautotrophic growth could be restored under anaerobic conditions.

PSI content. To test whether the mutations affected the amount of PSI present in the mutant strains, thylakoids were isolated from both mutants and fractionated by SDS-PAGE. PSI was detected by Western-blotting using PsaF specific antibodies (Fig. 1) and the amount was estimated by comparison to the PsaF signal originated from a dilution series of wild type thylakoids. The PSI content in strain PsaA-W651S could be estimated to be above 70% whereas in strain PsaA-W651F the amount of PSI was close to 100% as compared to WT. Therefore the light sensitivity of both strains can not be explained by a decrease in PSI content.

Cross-linking experiments of wild type and mutant PSI with pc or cyt c_6. To assess the binding and accessibility of PsaF for the two electron donors in wild type and the mutant PSI

complexes we performed cross-linking experiments using the zero length linker EDC/NHS that leads to the formation of covalent bounds between the basic and acidic patches of PsaF and pc or cyt c_6, respectively. After cross-linking, the reaction mixture was fractionated by SDS-PAGE and analyzed by Western blotting using anti-PsaF antibodies. The hem group of cyt c_6 gives an antibody independent light reaction in the presence of ECL and is also detectable in Fig. 2. PsaF could be cross-linked to both donors in wild type and mutants PsaA-W651F. Whereas the cross-link product between PSI from PsaA-W651S and cyt c_6 could only be detected as a very light band it was clearly detectable between pc and PsaF from mutant PsaA-W651S. Thus the interaction between PsaF of PSI PsaA-W651S and cyt c_6 appears to be strongly disturbed.

Incubation of the different PSI particles with the cross-linking mixture in absence of donor gave rise to an additional band detectable with the anti-PsaF antibodies about 2 - 5 kDa above the PsaF band. In the presence of the electron donors this band disappeared completely for wild type PSI but was still detectable to some extend in the presence the altered PSI complexes (data not shown).

Flash-absorption spectroscopy. To gather more detailed information on the effects of the mutations on binding and electron transfer between the two donors pc or cyt c_6 and the altered PSI complexes we performed flash induced absorbance spectroscopy. Hereby we measured the kinetics of the absorption changes at 817 nm caused by P700 oxidation due to a single flash and the subsequent reduction of $P700^+$ by donor binding and electron transfer. In WT PSI the reduction of $P700^+$ by either of the two electron donors resulted in kinetic traces that can be deconvoluted into three distinct components (not shown). A fast first order phase with a donor independent half life $t_{1/2}(1)$ of 3-4 µs and an amplitude A1 that is dependent on the donor concentration. A slower second order component where the half life $t_{1/2}(2)$ (ms to µs range) and amplitude A2 is dependent on the donor concentration while the third minor

component is not related to the donor concentration and assumed to derive from very slow ascorbate reduction of pc and/or damaged PSI that is only accessible for the donors at very slow rates. For further discussion we will only consider the two former components.

Fig. 3 shows absorption transients at 817 nm for the altered PSI and the electron donors pc or cyt c_6, respectively. Reduction of PSI PsaA-W651F by pc follows a monoexponential time course (Fig. 3 A). A fast phase as measurable for wild type PSI in the presence of 160 µM pc is not detectable, which indicates that binding between the reduced PSI and pc prior to flash oxidation is strongly disturbed. In contrast the kinetic traces for reduction of PSI from PsaA-W651F by cyt c_6 (Fig. 3 B) as well as for reduction of PSI from PsaA-W651S (Fig. 3, C, D) by both donor molecules displayed biexponential decay with a faster and a slower phase.

In further investigations of the kinetic components we used donor concentrations up to 300 µM for cyt c_6 and 260 µM for pc. Because the donor concentration exceeds the concentration of PSI by more then one order of magnitude the amplitude A2 of the slower component follows an exponential time course. The reaction rate $k = \ln(2) / t_{1/2}(2)$ for the slower, second order phase exhibited a slight saturation profile at high concentrations for both donors. For the determination of the second order rate constant k_2 only the linear part of the reaction rate dependence was taken into account (Fig. 4). The rates were estimated to be $1.9*10^7$ $s^{-1}M^{-1}$ and $5.5*10^6$ $s^{-1}M^{-1}$ for the reduction of PSI from PsaA-W651F by pc and cyt c_6, respectively (Fig. 4 A). These rates are four-fold and six-fold slower for pc and cyt c_6, respectively, as compared to the rates obtained with wild type PSI. Estimation of the second order rate constant for the electron transfer reaction between PSI from PsaA-W651S and both donors resulted in values of $3.1*10^7$ $s^{-1}M^{-1}$ and $2.1*10^6$ $s^{-1}M^{-1}$ for pc and cyt c_6, respectively (Fig. 4 B), corresponding to a three- and 16-fold decrease as compared to rate measured for wild type PSI.

Whereas no fast component could be observed with the altered PSI from mutant PsaA-W651F at concentrations of pc up to 260 µM, the amplitude of the fast phase A1 increased with increasing concentrations of cyt c_6 at the expense of A2 (amplitude for the slower component) with a halftime $t_{1/2}(1)$ of approximately 30 µs that remained constant. These data are similar to the results with PSI from mutant PsaB-W627F, where the halftime $t_{1/2}(1)$ increased by about ten-fold as compared to WT when cyt c_6 was used as an electron donor. No first order component was detectable when pc was the electron donor (13). Interestingly both donors were able to form a transient complex with PSI PsaA-W651S.

More detailed analysis of the amplitude A1 representing the first order electron transfer reaction at different donor concentrations was carried out as described by (8). Hereby an equilibrium of the forward and back electron transfer reaction is taken into account. In this model, a simple dissociation equilibrium of the complex between the reduced donor protein [D] and PSI was used to describe the concentration dependence of the amplitude A(1). An estimate of the dissociation constant, K_D, can be determined using the following equation:

$$relA_1 = f \times \frac{[D]}{[D] \times K_D} \qquad \text{Equation 1}$$

where f represents an empirical factor (f < 1) which relates the amplitude A(1) observed after the flash to the fraction of PSI in a complex with the reduced donor prior to the flash (8). Eq.1 indicates that the relative amplitude A(1) has a hyperbolic dependency of the donor concentration [D], i.e. shows a half maximum saturation at a donor protein concentration equal to K_D and approaching a maximum value *f* at infinite concentration. For the electron transfer reaction between wild type PSI and pc or cyt c_6 K_D values of 83 µM and 81 µM and f values of 0.67 and 0.68 could be determined, respectively, ((12); this study). Measurements with PSI isolated from the PsaA mutant strains revealed that even at the highest donor concentrations the relative amplitude A1 exhibited only 10 - 15 %. Hereby the electron transfer reaction between PsaA-W651S and cyt c_6 revealed only lowest relative amplitude.

Fitting eq. 1 to the dependence of the relative amplitude from the donor concentration a f value of 0.38 (Fig. 5A solid line) was determined. For the estimation of the K_D values for the interaction between the altered PSI particles and the two donors we took this f value as a lower (Table II) and the f value obtained for the electron transfer between pc or cyt c_6 and wild type PSI as an upper limit. Thus K_D values can be estimated to be between 510-1087 μM and 1310–2560 μM for the electron transfer reaction between cyt c_6 and PSI from PsaA-W651F PsaA-W651S, respectively, and in a range of 254-554 μM for the interaction between pc and PSI from PsaA-W651S.

Salt dependence. Long range electrostatic interactions between the positively charged residues of PsaF and conserved acidic patches of pc or cyt c_6 play an important role in recognition and correct binding between the donor molecules and PSI enabling fast and efficient electron transfer. Therefore the salt dependence of the second order electron transfer rate constants for electron transfer between PSI and pc or cyt c_6 was investigated (Fig. 6). As described before the second order rate constant decreases at increasing ionic strength for wild type and both donors (11-13). The same behavior was found at increasing salt concentrations for the electron transfer reaction between PSI from the PsaA mutant strains and the two donors.

Redox potentiometrie. The change of residues close to the special chlorophyll pair P700 could alter the P700 redox midpoint potential. A change in the P700 redox midpoint potential would in turn change the free energy of the electron transfer reaction. To test whether this is the case we measured the P700 redox midpoint potentials of PSI from the altered PsaA strains as well as from the PsaB strain PsaB-W627F. As shown in Table II the redox midpoint potentials decreased only by 10 mV for the changes of W to F and remained constant for the change W651S as compared to WT. The standard deviations derived from the Nernst fits were approximately ± 1.5 mV. A change of 10 mV would slow down the half life of the first order

electron transfer reaction by about 0.5 µs using an approximation for electron transfer from (26) and a reorganisation energy λ of 545 mV as estimated by (27). Therefore the change in the redox midpoint potential can not account for the observed differences in the first order electron transfer rates obtained for the donors and PSI from PsaA or the PsaB mutant strains. Our data would rather indicate that an alteration of the hydrophobic binding site induces a change in distance between the redox centres of the donors and P700 which is supposed to be in the range of 0.5 – 1 Å according to the estimations mentioned above.

Discussion

In a previous study we could show that residue PsaB-W627F is important for proper binding and efficient electron transfer from both pc and cyt c_6 to PSI (13). In this study we have taken advantage of a PsaA-deficient mutant (22) to modify the PsaA-W651 using chloroplast transformation and site directed mutagenesis. PSI particles from the mutant strains containing a specific amino acid change in the PsaA protein, W651F and W651S were isolated together with PSI particles from wild type and used with purified pc and cyt c_6 to characterize the electron transfer between these donors and PSI *in vitro*. Our results indicate that PsaA-W651 together with PsaB-W627 forms the hydrophobic recognition site that is essential for binding of pc and cyt c_6 to the core of PSI. Our results also indicate that pc and cyt c_6 bind slightly different to the core of PSI, since both donors are affected differently in their binding affinities by mutations at the positions of the two tryptophan amino acid residues.

As shown by (13) the mutation of PsaB-W627 to F abolished the formation of a stable first order electron transfer complex between pc and the altered PSI but allowed the formation of an inter-molecular electron transfer complex between cyt c_6 and the PsaB-W627F PSI. The corresponding mutation of PsaA-W651 to F had a similar impact. However the dissociation constant for binding of cyt c_6 to PSI from PsaA-W651F was 3- to 5-fold larger than for the binding to PsaB-W627F PSI (Table II). In the same line, the rate constant for electron transfer between cyt c_6 and PSI from PsaA-W651F was about 3 times slower than for the electron transfer with PSI from PsaB-W627F. Mutation of PsaA-W651 to S had an even larger impact on the dissociation and rate constants. The K_D value increased and the rate constant decreased another two-fold when the values obtained for the interaction and electron transfer between cyt c_6 and PSI from PsaA-W651S and PsaA-W651F are compared (Table II). In contrast binding and electron transfer of pc to PSI from PsaA-W651S improved as compared to PSI from PsaA-W651F. It is of note that rate constants for the electron transfer between pc and PSI from PsaA-W651F and PsaA-W651S increase 3- and 5-fold, respectively, as compared to

the rate constant measured for PSI from PsaB-W627F. This indicates that alterations of PsaB-W627 or PsaA-W651 have different impacts on binding and electron transfer between the PsaA or PsaB mutant PSI and pc or cyt c_6. From these results we can deduce that pc binds more tightly to the PsaB-W627 whereas cyt c_6 interacts more directly with PsaA-W651. This conclusion is also supported by the cross-linking experiments (Fig. 2), where the mutation PsaA-W651S almost completely abolished the formation of a cross-link product between PsaF and cyt c_6. In this case the salt dependence of the second order rate constant k_2 suggested that the negatively charged cyt c_6 interacts with PsaF, which supports our argumentation that residue PsaA-W651 is crucial for a productive formation of an electron transfer complex between PSI and cyt c_6.

Interestingly the change of residue PsaA-W651 to the hydrophilic S restored slightly the formation of the electron transfer complex between pc and the altered PSI as compared to the interaction of pc with PSI from PsaA-W651F. It is tempting to speculate that a hydrophilic interaction between the S residue at position W651S and an appropriate residue on the surface of pc eventually H87 on the “northern” hydrophobic interaction site of pc results in an equivalent gain of binding energy that compensates the loss of hydrophobic interaction surface.

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Table I

Growth inhibition of mutants under high light is restored under anaerobic conditions.

Cells from liquid cultures were spotted on plates containing TAP or HSM medium for heterotrophic or photoautotrophic growth respectively and placed under different atmospheric and light conditions.

Strain	TAP 60µE/m^{2}s	HSM 60µE/m^{2}s	TAP 700µE/m^{2}s	HSM 700µE/m^{2}s	TAP 700µE/m^{2}s	HSM 700µE/m^{2}s
	AEROB				ANAEROB	
WT	++	++	++	++	++	++
PsaA-W651F	++	++	+	-	++	++
PsaA-W651S	++	++	+	-	++	++

Table II:

P700 midpoint potentials and electron transfer properties from pc or cyt c_6 to PSI isolated from WT and the different strains mutated either on PsaA-W651 or PsaB-W627.

	Plastocyanin		Cytochrome c_6		Midpoint-potential
	k_2 [$s^{-1} * 10^6$]	K_D [µM] (*f*)	k_2 [$s^{-1} * 10^6$]	K_D [µM] (*f*)	E_m [mV] [c]
WT[a]	90	83 (f=0.67)	34	81 (f=0.68)	473.1
PsaA-W651F	19	-	5.5	510 - 1087 (f=0.38-0.68)	463.9
PsaA-W651S	31	254 - 554 (f=0.38-0.67)	2.1	1310 - 2560 (f=0.38-0.68)	477
PsaB-W627F[b]	6.4	-	16	177 (f=0.66)	463.9

[a] Values for the kinetic constants were taken from (12)

[b] Values for the kinetic constants were taken from (13)

[c] Standard deviation from the Nernst fits were less then 1.5 mV.

Figure 1: PSI content in mutants PsaA-W651F/S is only slightly reduced. Thylakoids (20µg proteins) isolated from WT and mutants PsaA-W651F and PsaA-W651S as well as from a PSI-deficient mutant (ΔPsaB) were separated by SDS-PAGE and immuoblotted. The blots were probed with anti-PsaF antibodies. To estimate the relative amount of PSI, thylakoids from WT were diluted with thylakoids from ΔPsaB (PSI-lacking) by keeping the total protein concentration constant.

Figure 2: Cross-link of mutant photosystem PsaA-W651S with the electron donor cyt c6 is strongly impaired. The cross-linking reaction mixture (x-link) of pc or cyt c_6 and PSI (according to 2µg chl) from WT and mutants PsaA-W651F and PsaA-W651S was separated by SDS-PAGE and immuno-probed with anti-PsaF antibodies, the hem group of cyt c_6 is also detectable under these conditions.

Figure 3: Kinetic traces for reduction of PSI PsaA-W651F (A and B) and PsaA-W651S (C and D) in the presence of 160 µM pc or 150 µM cyt c_6, respectively. Only PSI PsaA-W651F/pc (A) shows a monoexponential decay while the other traces B, C and D exhibit a first order phase with a half-life of about 15-30 µs.

Figure 4: Determination of the second order rate constants for electron transfer between the two electron donors pc (■ ; □) or cyt c_6(● ; ○) and PSI from PsaA-W651S (A) or PsaA-W651F (B). The measurements were performed at optimal $MgCl_2$ concentration. For estimation of k_2 only measurements in the linear part i.e. donor concentrations up to 200 µM were taken into account.

Figure 5: Relative amplitude of the fast kinetic phase of $P700^+$ reduction as a function of the donor concentration. Determination of the dissociation constants for the electron transfer between the two donors and the altered PsaA. In A the solid line represents a fit with an f value of 0.38 and the dotted line a fit with an f value of 0.68. For B (PsaA-W651S/pc) and C (PsaA-W651F/cyt c_6) fits with f=0.68 and 0.67 respectively are shown. Results are summarized in table 2.

Figure 6: Dependence of the second order rate constant k_2 from the ionic strength. Measurements were performed in the presence of 20 µM of the respective donor, ionic strength was adjusted by adding small amounts of a concentrated $MgCl_2$ solution.

Figure 1

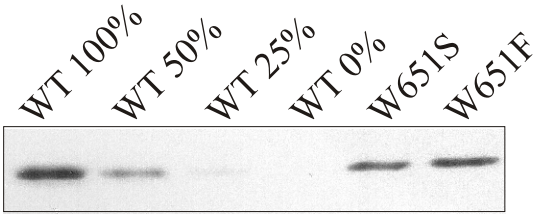


Figure 2

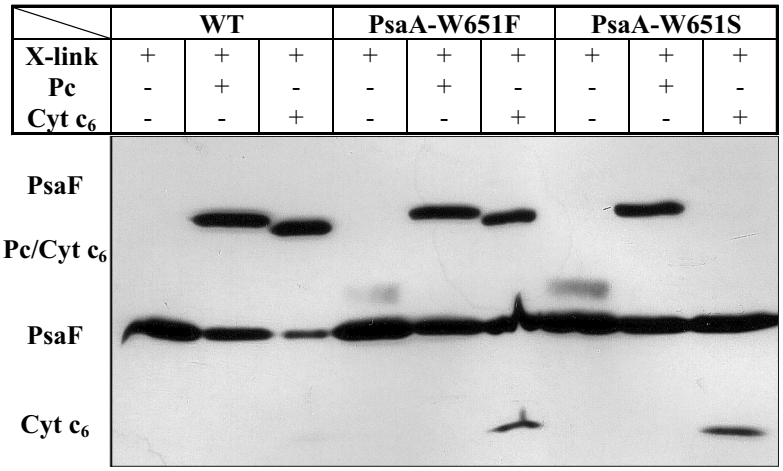


Figure 3

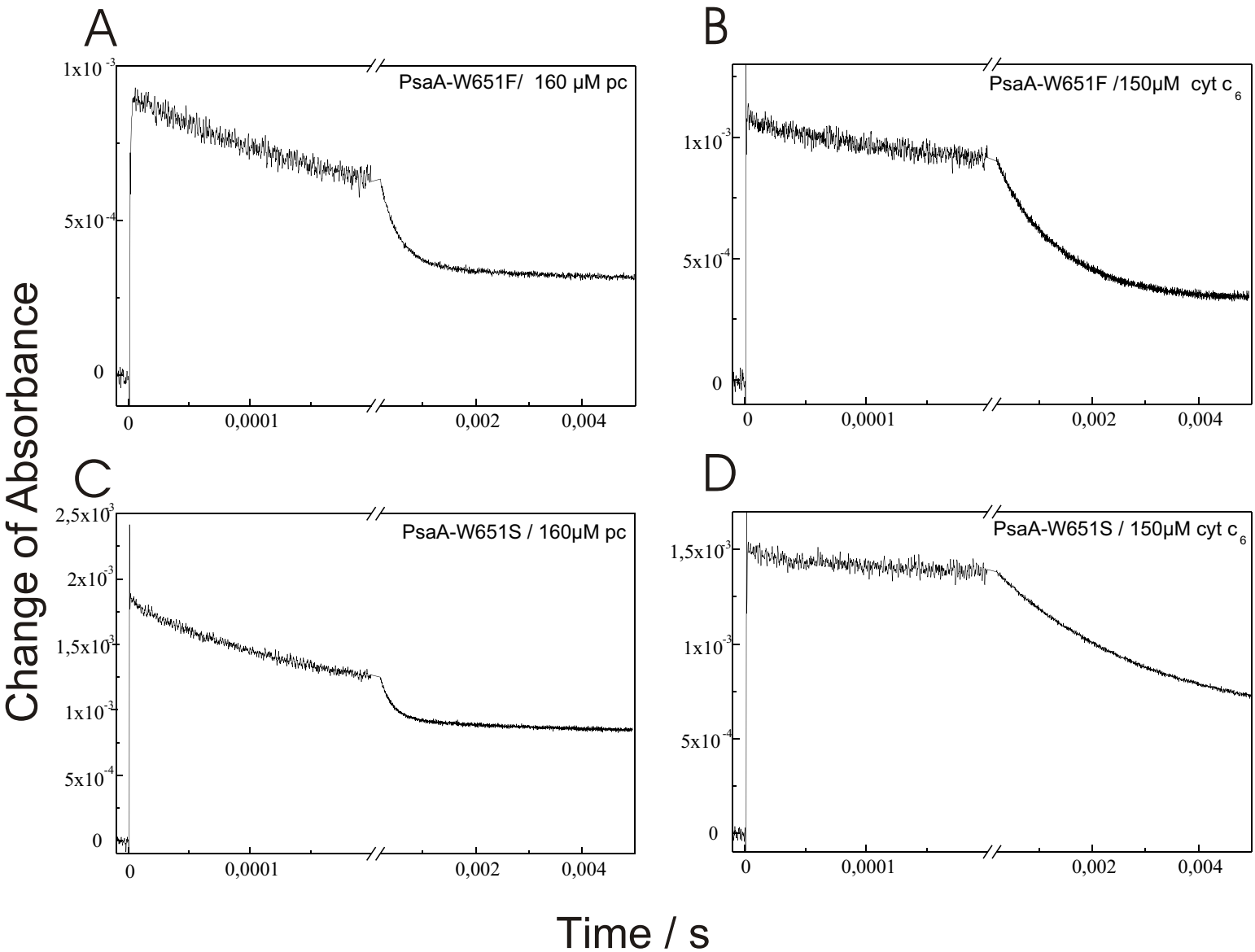

A
PsaA-W651F/ 160 µM pc
B
PsaA-W651F /150µM cyt c6
C
PsaA-W651S / 160µM pc
D
PsaA-W651S / 150µM cyt c6
Change of Absorbance
Time / s

Figure 4

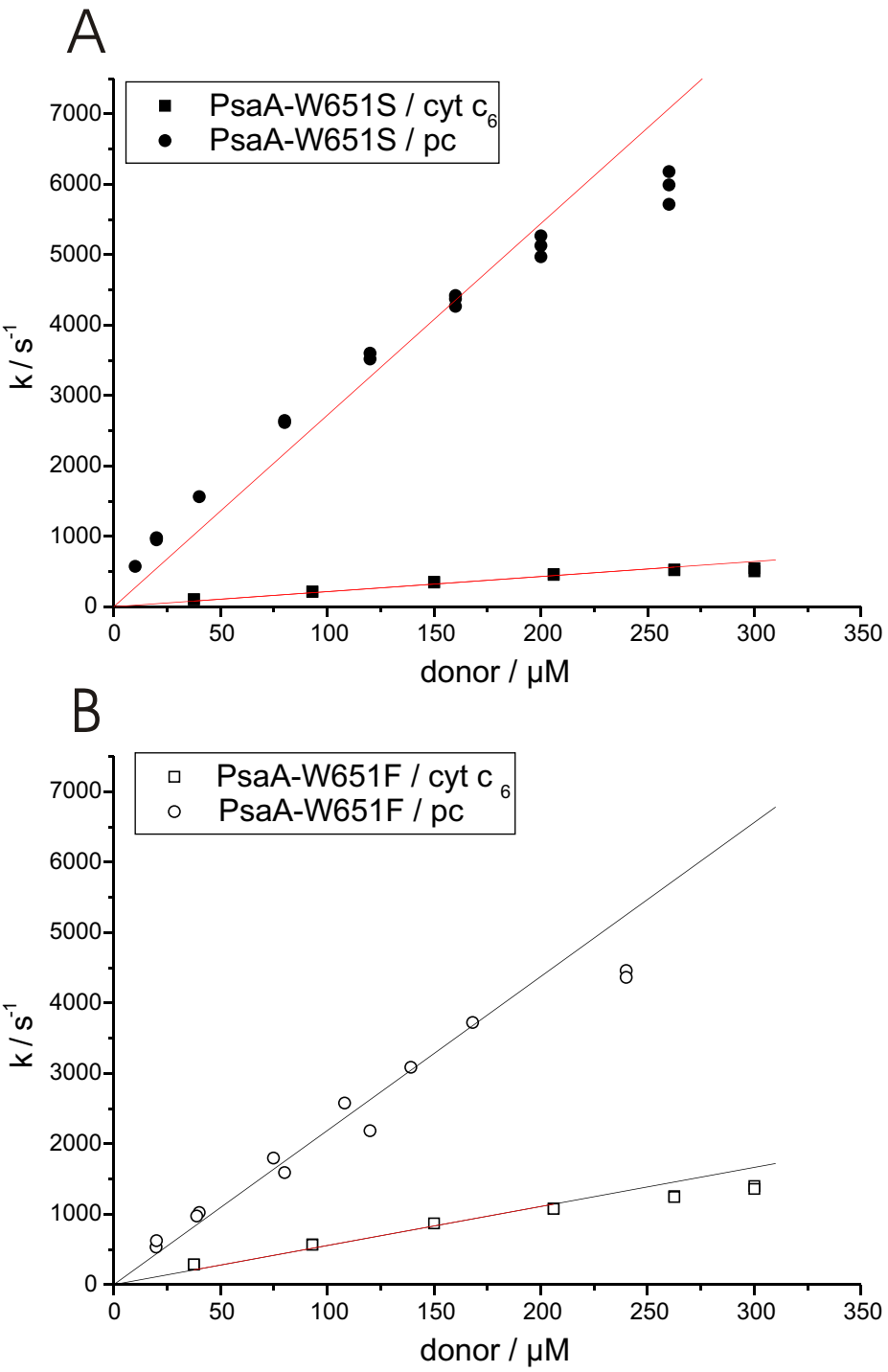

A
PsaA-W651S / cyt c6
PsaA-W651S / pc
k / s-1
donor / µM
B
PsaA-W651F / cyt c 6
PsaA-W651F / pc
k / s-1
donor / µM

Figure 5

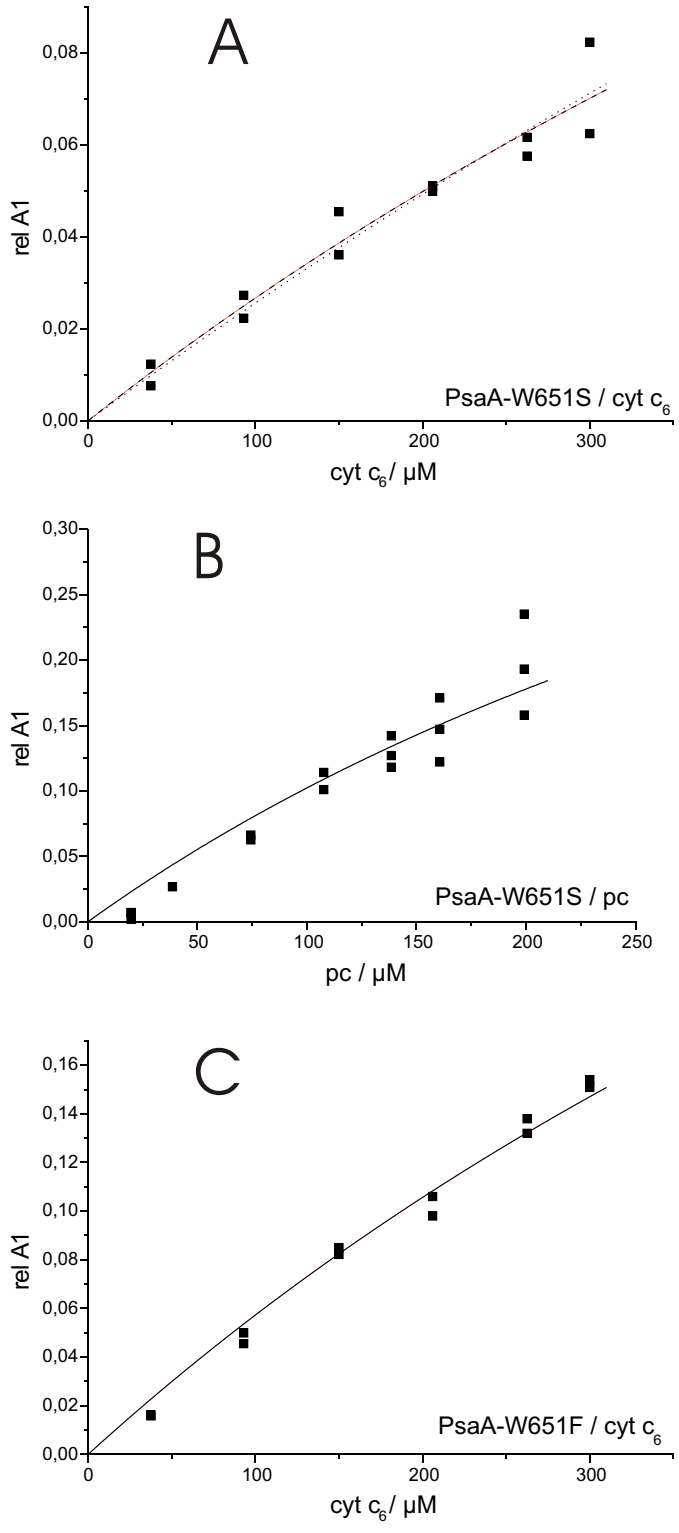
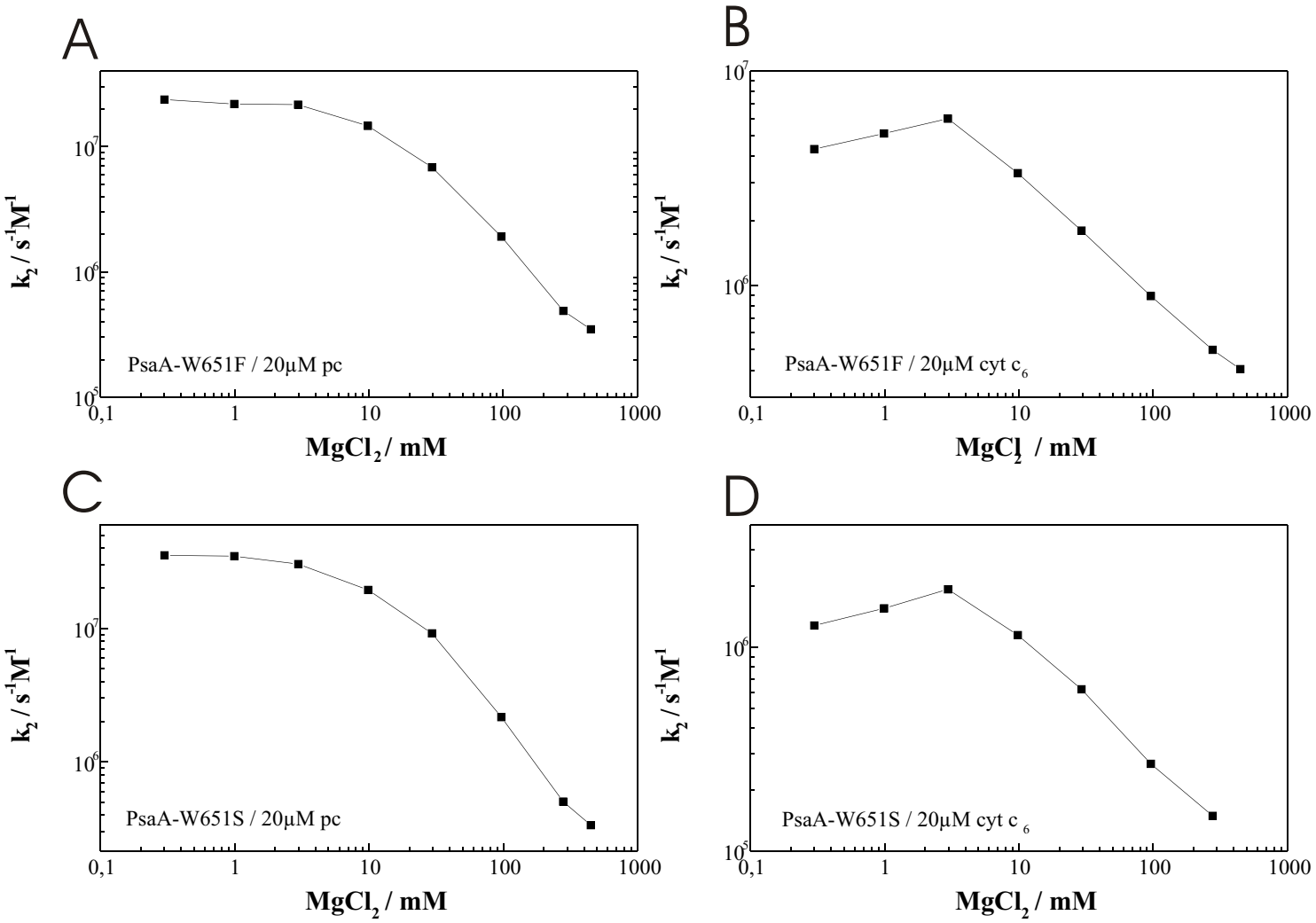

A
rel A1
0,00
0,02
0,04
0,06
0,08
0
100
200
300
cyt c6/ µM
PsaA-W651S / cyt c6
B
rel A1
0,00
0,05
0,10
0,15
0,20
0,25
0,30
0
50
100
150
200
250
pc / µM
PsaA-W651S / pc
C
rel A1
0,00
0,02
0,04
0,06
0,08
0,10
0,12
0,14
0,16
0
100
200
300
cyt c6/ µM
PsaA-W651F / cyt c6

Figure 6


A
B
C
D
PsaA-W651F / 20µM pc
PsaA-W651F / 20µM cyt c6
PsaA-W651S / 20µM pc
PsaA-W651S / 20µM cyt c6
k2 / s-1M-1
MgCl2 / mM
0,1
1
10
100
1000
10^5
10^6
10^7

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Comparative analysis of photosensitivity in photosystem I donor and acceptor side mutants of *Chlamydomonas reinhardtii*

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ABSTRACT

Electron input from plastocyanin into photosystem I (PSI) is slowed down in the *Chlamydomonas reinhardtii* mutants affected at the donor side (PsaF or PsaB, lumenal loop *j*) of PSI. In contrast, electron exit from PSI to ferredoxin is diminished in the PSI acceptor side PsaC mutants K35E and FB_1. Although, the electron transfer reactions are diminished to a similar extent in both type of mutants, the PsaC mutants K35E and FB_1 are more light-sensitive than the PsaF-deficient strain 3bF or the PsaB mutants E613N and W627F. To assess the differential photosensitivity of donor and acceptor side mutants fluorescence transients, gross oxygen evolution and uptake, PSII photo-inhibition and rate of recovery were measured as well as $NADP^+$ photoreduction. The $NADP^+$ photoreduction measurements indicated that the donor side is limiting the reduction rate. In contrast, measurements of gross oxygen evolution and uptake showed that the reducing side limits linear electron transfer. However, under high light, donor and acceptor side mutations lead to PSII photo-inhibition and to a diminished rate of PSII recovery, cause lipid peroxidation and result in a decrease in the levels of PSI and PSII. The wild type is not affected under the same conditions. These responses are most pronounced in the PsaC-K35E and PsaB-W627F mutants, and they correlate with the light sensitivity of these strains. The correlation between limitation of electron transfer through PSI and the formation of reactive oxygen species as a cause for the light-sensitivity is discussed.

Key-words: *Chlamydomonas reinhardtii*; photo-inhibition; photosystem I; site-directed mutagenesis.

Abbreviations: Cyt, cytochrome; Fd, ferredoxin; MDA, malondialdehyde; PSI, photosystem I; Pc, plastocyanin; TBARS, thiobarbituric acid-reactive substances.

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INTRODUCTION

Photosynthetic activity is dependent on light. However, its efficiency can decrease under excessive illumination. This process is called photo-inhibition. The primary target of photo-inhibition is often considered to be photosystem II (PSII), as photosystem I (PSI) is stable under strong illumination (Powles 1984). The process leads to the specific inactivation of the D1 polypeptide of PSII and is a direct consequence of the chemistry taking place in the complex which involves highly oxidizing radicals and toxic oxygen species. The damaged D1 protein is degraded and replaced by newly synthesized D1 polypeptide, leading to the re-assembly of a functional PSII complex (Barber & Andersson 1992). In addition to the efficient repair of PSII via the turnover of D1, other mechanisms such as non-photochemical thermal dissipation and dynamic regulation of antenna size, protect the photosynthetic apparatus from excessive illumination (Horton *et al*. 1994; Niyogi 1999).

Over-reduction of the electron carriers can directly reduce O_2 through PSI (Mehler 1951) and can also generate reactive oxygen species such as the superoxide anion radical ($O_2^{-\bullet}$). Accumulation of excitation energy can cause the formation of singlet oxygen ($^1O_2$) through energy transfer from excited triplet chlorophyll to ground-state O_2 (Asada 1994, 1996). The reduction of O_2 to O_2^- has been observed with washed thylakoids depleted of ferredoxin (Fd). The terminal iron-sulphur clusters of PSI are most likely the electron donors to O_2 (Takahashi & Asada 1982; Asada 1994). PSI is also a target of photo-inhibition (Inoue, Sakurai & Hiyama 1986, Inoue *et al*. 1989), in particular under weak illumination at chilling temperature (Terashima, Funayama & Sonoike 1994; Sonoike *et al*. 1995). Oxygen is required for light-induced inactivation of PSI, and damage is probably mediated by active oxygen species (Inoue *et al*. 1989). Oxygen scavenging enzymes such as Cu, Zn-superoxide dismutase appear to be localized in close vicinity of PSI (Ogawa *et al*. 1995) and play an important role in the protection of PSI against photo-oxidation. The scavenging of reactive oxygen species in intact chloroplasts involves a complex network of redox reactions which results in the

photoreduction of O_2 to H_2O, a process called the water–water cycle (Asada 1999).

If the scavenging system is not functional or if the production of superoxide exceeds the scavenging capacity, O_2^- and H_2O_2 can diffuse to stromal protein targets and inactivate them. The released Fe and Cu from inactivated metalloproteins can catalyse the production of highly reactive •OH radicals. The inactivation of PSI during photoinhibition has been suggested to occur in three steps: (1) inactivation of the acceptor side; (2) destruction of the reaction centre; and (3) specific degradation of the PsaB subunit (Sonoike 1996a, b). It has been shown that H_2O_2 can react with the photoreduced [4Fe-4S] clusters of PSI to generate .OH radicals (Jakob & Heber 1996). As the acceptor side of PSI is inactivated first and the [4Fe-4S] clusters are extremely sensitive to oxidation, it seems likely that reactive oxygen species can destroy the terminal electron acceptors of PSI (Tjus, Moller & Scheller 1998). Interestingly, the selective excitation of PSI in isolated thylakoids in the presence of reductants appears not only to damage PSI but also PSII, indicating that active oxygen species produced by PSI, such as superoxide and hydrogen peroxide, may diffuse to PSII and cause damage (Tjus *et al*. 2001). Furthermore, when the acceptor side is fully reduced, recombination between the radical pairs $P700^+/A_0^-$ or $P700^+/A_1^-$ can generate the P700 triplet state which leads to the formation of highly toxic singlet oxygen. Taken together, it appears that PSI is able to generate reactive oxygen species that can damage the complex and that scavenging mechanisms exist to protect PSI.

Recently, we have characterized several *Chlamydomonas reinhardtii* mutant strains in which mutations in the PsaC subunit alter the electron flow from PSI to Fd (Fischer *et al*. 1998, Fischer, Sétif & Rochaix 1999). In particular, mutations changing K35 had a strong effect on the rate of Fd reduction and on the affinity of Fd for the complex. These strains are photosensitive as they are unable to grow on an acetate-containing medium under high light intensities (Fischer *et al*. 1998). The FB_1 triple mutant is one of the most affected strains *in vivo* and is unable to grow when illuminated with more than 80 μE m^{-2} s^{-1}. The affinity of Fd for the PSI complex is dramatically increased and we proposed that the Fd K_{off} rate might be limiting in this mutant, leading to the observed phenotype (Fischer *et al*. 1999).

At the donor side of PSI, PsaF and the lumenal interhelical loops of PsaA and PsaB are important for docking of plastocyanin (Pc) or cytochrome c_6 (Cyt c_6) (Farah *et al*. 1995; Hippler *et al*. 1997; Hippler *et al*. 1998; Sommer, Drepper & Hippler 2002). The PsaF-deficient mutant 3bF of *C. reinhardtii* is drastically impaired in electron transfer from both Pc and Cyt c_6 to PSI *in vivo* and *in vitro* (Farah *et al*. 1995; Hippler *et al*. 1997). The positively charged N-terminal domain in the algal and plant-type PsaF is responsible for binding of Cyt c_6 or Pc to PSI and crucial for fast electron transfer (Hippler *et al*. 1996; Hippler *et al*. 1998). PsaB mutants W627F (corresponding to W631 in *Synechococcus*) and E613N were generated by site-directed mutagenesis and biolistic transformation of *C. reinhardtii* (Sommer *et al*. 2002). The structural data on cyanobacterial PSI (Jordan *et al*. 2001) together with the data obtained for analysis of mutant W627F strongly suggest that W627 of helix *l* is part of the suggested hydrophobic recognition site required for binding of Pc to the core of PSI. A detailed analysis of the electron transfer between PSI from the mutant PsaB-E613N and the two algal donors indicated that this mutation leads to an increased rate of electron transfer which is concomitant with a slower release of the two donors from PSI (Sommer *et al*. 2002). Interestingly, both PsaB mutant strains become light sensitive at light intensities higher than 400 μE m^{-2} s^{-1}.

The same holds for the PsaF-deficient strain which becomes photosensitive under photo-autotrophic growth conditions at light intensities higher than 400 μE m^{-2} s^{-1} (Hippler *et al*. 2000). A light-resistant suppressor strain could be isolated that lacks functional light-harvesting complex proteins suggesting that, under high light intensities, electron transfer and light harvesting are not balanced in the absence of PsaF. This imbalance could cause photo-oxidative stress and may explain the photosensitivity of the PsaF-deficient strain in high light.

In this study we show that changes of PsaF at the donor side of PSI limit electron transfer from Pc to $NADP^+$ *in vitro* as revealed by $NADP^+$ photoreduction measurements. However, it appears that *in vivo,* overall electron transfer is limited by the impairment of the acceptor side of PSI as shown by mass spectrometric measurements of gross oxygen evolution and uptake of the PsaC and PsaF mutants. Although acceptor side mutants are more light sensitive than donor side mutants, both types of mutations result in photo-inhibitory effects on PSII, affect the recovery rate of PSII, lead to lipid peroxidation and in a decrease of PSI and PSII proteins under high light.

MATERIALS AND METHODS

Strains and media

Chlamydomonas reinhardtii wild-type and mutant strains were grown as described (Harris 1989). Strain $6a^+$ is a cell wall-containing, PsaF-deficient progeny from a backcross between the cell wall-less 3bF mutant and a wild-type strain.

In vivo growth tests

The growth properties of the different strains were determined by spotting 20 μL of an exponentially growing culture in low light (< 6 μE m^{-2} s^{-1}) on acetate containing (TAP) plates or on minimal plates (HSM). The plates were subsequently exposed to different light and atmospheric conditions. For anaerobic growth on plates, the 'GENbag anaer' system (BioMérieux, Marcy l'Etoile, France) was used.

$NADP^+$ photoreduction

Rates of $NADP^+$ photoreduction were measured by monitoring the absorption change of NADPH at 340 nm under

non-saturating light. The reaction was performed in 2 mL (20 mM Tricine, pH 7.6; 10 mM $MgCl_2$; 0.03% β-dodecyl maltoside) in the presence of 3 μM Fd, 4 μM Pc, 0.5 μM Fd:NADP reductase, 2 mM sodium ascorbate, 0.06 mM DCPIP (2, 6-dichlorphenol-indophenol) and 5 μg mL^{-1} chlorophyll (Chl) *a* of isolated PSI complex. Recombinant Fd from *C. reinhardtii* was kindly provided by Dr J.-P. Jacquot, Pc was purified from *C. reinhardtii* as described (Hippler *et al*. 1997) and spinach ferredoxin:NADP reductase was purchased from Sigma-Aldrich (Munich, Germany).

Fluorescence and photo-inhibition measurements

The different strains were grown on TAP plates in dim light for 3–4 d to a similar density. Cells were photo-inhibited for 1 h at 24 °C with a light intensity of approximately 2500 μE m^{-2} s^{-1} photosynthetic active radiation [PAR; measured with a Hansatech (Kings Lynn, UK) Quantitherm Lightmeter] provided by four strong light sources. Infrared radiation was cut off by a heat protection filter and the temperature only slightly increased by 1–2 °C during this period. After strong light treatment cells were allowed to recover in dim light at 20 μE m^{-2} s^{-1} PAR. The values of F_v/F_m and the fluorescence induction transients were recorded with a FluorCam 700MF device (Photon System Instruments, Brno, Czech Republic). Fluorescence induction transients were induced with an actinic light intensity of 90 μE m^{-2} s^{-1}.

Gross oxygen evolution and oxygen uptake

The measuring system was described earlier (Sültemeyer, Biehler & Fock 1993). Actinic light was obtained by a dia projector (Braun, Nürnberg, Germany) with heat filter. Light intensity was dimmed by neutral glass filters. The temperature was 25 °C. The rates of oxygen evolution and uptake were calculated according to Sültemeyer *et al*. (1993).

Isolation of PSI complexes and flash-induced spectroscopy

The isolation of PSI particles was as described (Hippler *et al*. 1997). Chl concentrations were determined according to Porra, Thompson & Kriedemann (1989). Electron transfer from Pc to PSI was monitored by flash-induced spectroscopy as described (Hippler *et al*. 1997).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblot analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (15.5% T, 2.66% C) was carried out according to Laemmli (1970). After the electrophoretic fractionation the proteins were electroblotted onto nitrocellulose and incubated with antibodies as described (Hippler *et al*. 1997). Immuno-detection was carried out according to Hippler *et al*. (1997).

Assay of malondialdehyde concentration

To measure the amounts of TBARS we followed the procedure as described by Slater (1984). Five hundred microlitres of cells (15 μg Chl mL^{-1}) were mixed with 1.8 mL of a degassed solution of 0.4% thiobarbituric acid in 5% trichloroacetic acid and heated in a boiling water bath for 15 min. After cooling, the reaction mixture was centrifuged and the absorbance of the supernatant at 532 nm, corrected for non-specific absorption at 600 nm, was determined. The concentration of TBARS was expressed through the concentration of malondialdehyde (MDA) using the extinction coefficient of the MDA–thiobarbituric acid complex of 155 mM cm^{-1}.

RESULTS

Growth phenotypes of the 3bF, 6a$^+$, PsaB-E613N, PsaB-W627F, PsaC-K35E and PsaC-FB1 mutant strains

The growth of wild type, 3bF, 6a$^+$, PsaB-E613N, PsaB-W627F, PsaC-K35E and PsaC-FB_1 were compared under different light regimes and atmospheric conditions on both minimal (HSM) and acetate-containing (TAP) media which are restrictive and permissive for the growth of mutant strains lacking photosynthetic activity, respectively (Fig. 1). The wild-type strain was able to grow under all conditions tested. The 3bF and 6a$^+$ mutant strains which both lack the PsaF subunit are relatively light-tolerant and their growth

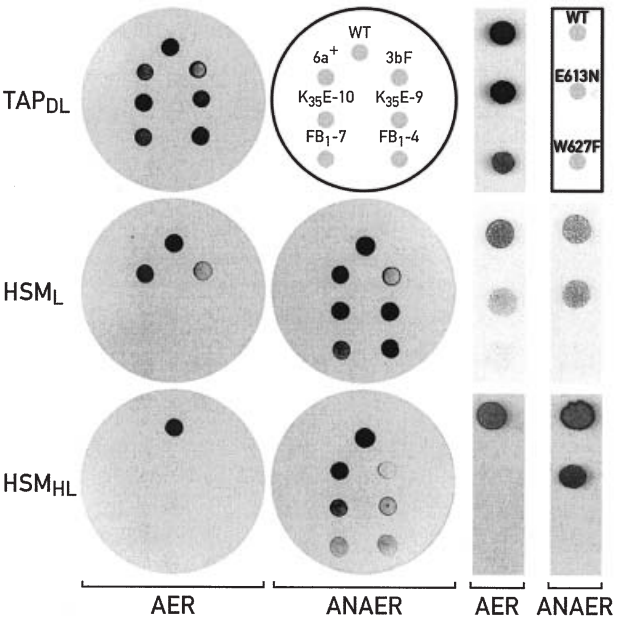

Figure 1. Growth properties of wild-type and mutant strains on plates. The difference observed for the two PsaF-deficient strains 6a$^+$ and 3bF is most likely due to the deficient cell wall in the 3bF strain. TAP, acetate containing medium; HSM, minimal medium; DL, dim light (< 6 μE m^{-2} s^{-1}); L, medium light (60 μE m^{-2} s^{-1}); HL, high light (800 μE m^{-2} s^{-1}); AER, aerobic conditions; ANAER, anaerobic conditions.

was only inhibited under high light on HSM. Although both of these mutants contain the same mutation, 3bF appears to be more light sensitive than 6a$^+$. This is possibly due to the fact that 3bF is cell wall-deficient and thus less robust than 6a$^+$. The PsaB-E613N mutant is only sensitive to high light when grown on minimal medium and is similar in this respect to 3bF (Fig. 1). In contrast, the PsaC mutant strains are photo-inhibited at light intensities of 60 and 800 μE m^{-2} s^{-1} on HSM and TAP medium, respectively (Fischer *et al*. 1998, 1999). Interestingly, growth of all the mutants, except mutant PsaB-W627F, is restored, at least to some extent, under anaerobic conditions, thus clearly indicating that inhibition of growth is mainly due to photo-oxidative damage. This is particularly striking for the PsaC K35E and FB_1 mutants that are unable to grow photo-autotrophically under aerobic conditions, but grow, at least to some extent, under high light and anaerobic conditions (Fig. 1).

Deletion and site-directed mutations affecting the donor side of PSI are limiting for overall electron transfer *in vitro*

In order to determine the effects of the mutations affecting either the donor or the acceptor side of PSI on the overall electron transfer through PSI, we measured NADP$^+$ photoreduction by reconstituting the electron transfer chain from Pc to NADP$^+$. The activity was measured by following the absorption change at 340 nm with 4 μM Pc isolated from *C. reinhardtii* as electron donor to PSI and 3 μM recombinant Fd as electron acceptor. The PSI complex of the 3bF mutant is clearly the most affected with a 50- to 80-fold reduction of activity in comparison with the wild-type complex (Table 1). Interestingly, this decrease is close to that observed for P700$^+$ reduction by Pc by flash absorption spectroscopy (Hippler *et al*. 1997). The same correlation is found for the altered PSI complexes K23Q and K16Q, in which Lys residues within the N-terminal part of PsaF are changed to Gln (Table 1). For the PsaB-W627F PSI a four-fold reduction in the NADP$^+$ reduction rate is observed, whereas the rate for PsaB-E613N PSI is comparable with WT PSI. In contrast, in the PsaC-K35E and PsaC-FB_1 mutants, the rate of NADP$^+$ reduction is only diminished by a factor of 1.9 and 3.3, respectively (Table 1). These data indicate that P700$^+$ reduction is limiting for overall electron transfer across PSI. Mutations affecting the acceptor side of PSI have only a limited effect on NADP$^+$ photoreduction.

Fluorescence transients of different PSI mutants affected at the donor or acceptor side

To assess the photosynthetic electron transfer properties in the different mutants *in vivo* we measured fluorescence transients (Fig. 2). The wild-type fluorescence transient of dark-adapted cells reaches a maximum after about 0.5 s and declines to a steady-state fluorescence level. This decline is caused mainly by the oxidation of Q_A by the PQ-pool which is determined by the subsequent photosynthetic electron transfer reactions. Other parameters responsible for a decrease of PSII fluorescence such as non-photochemical quenching (NPQ) or state transitions become prominent only at later times (reviewed in Krause & Weis 1991). In *Chlamydomonas* maximal NPQ is reached after several min (Niyogi, Bjorkman & Grossman 1997). We measured the level of NPQ after 5 s for wild type and 3bF and found NPQ values below 0.15, thus indicating that NPQ can be neglected as a cause of fluorescence quenching within the first 5 s. In the case of a PSI-deficient mutant (ΔPsaB) the fluorescence trace reaches a plateau before 0.5 s and remains constant thereafter. The fluorescence transients of the donor or acceptor side mutants decline to levels between those observed for the wild-type and the PSI-deficient mutant. Although the P700$^+$ reduction appears to be limiting for overall electron transfer across PSI as measured by the

Table 1. Electron transfer properties of wild-type and mutant PSI complexes

PSI complex (mutation)	P700$^+$ reduction rate by Pc (M^{-1} s^{-1}) (Limitation factor)	Fd reduction rate (M^{-1} s^{-1}) (Limitation factor)	NADP$^+$ reduction rate (μmol mg^{-1} chl h^{-1}) (Limitation factor)
WT	$6.5–9.2 \times 10^7$ (1)	$2.0–2.5 \times 10^8$ (1)	240 (1)
PsaC-K35E (acceptor side)	n.d.	2.8×10^6 (70–90)	125 (1.9)
PsaC-FB_1 (acceptor side)	n.d.	Not measurable	72 (3.3)
WT (cw15)	$6.5–9.2 \times 10^7$ (1)	n.d	252 (1)
PsaF-3bF (donor side)	1.32×10^6 (50–80)	n.d.	3 (80)
PsaF-$K23Q_{17}$ (donor side)	0.7×10^7 (9–13)	n.d	18 (13)
PsaF-$K23Q_{30}$ (donor side)	0.7×10^7 (9–13)	n.d	18 (13)
PsaF-K16Q (donor side)	1.6×10^7 (4–6)	n.d	144 (1.7)
PsaF-K30Q (donor side)	3.4×10^7 (2–3)	n.d	243 (1)
PsaB-E613N (donor side)	11×10^7(0.6–0.8)	n.d.	243 (1)
PsaB-W627F (donor side)	7.4×10^6(9–12)	n.d.	60 (4)

The second-order rate constants for P700$^+$ reduction by Pc and Fd reduction are taken from Hippler *et al*. (1998), Fischer *et al*. (1998, 1999) and Sommer *et al*. (2002), respectively. The limitation factor indicates the ratio between the wild-type and mutant values and is shown in parenthesis. All the PsaF mutants listed are in the cw15 nuclear background. PsaF-$K23Q_{17}$ and PsaF-$K23Q_{30}$ refer to two independently generated mutants. n.d., not determined.

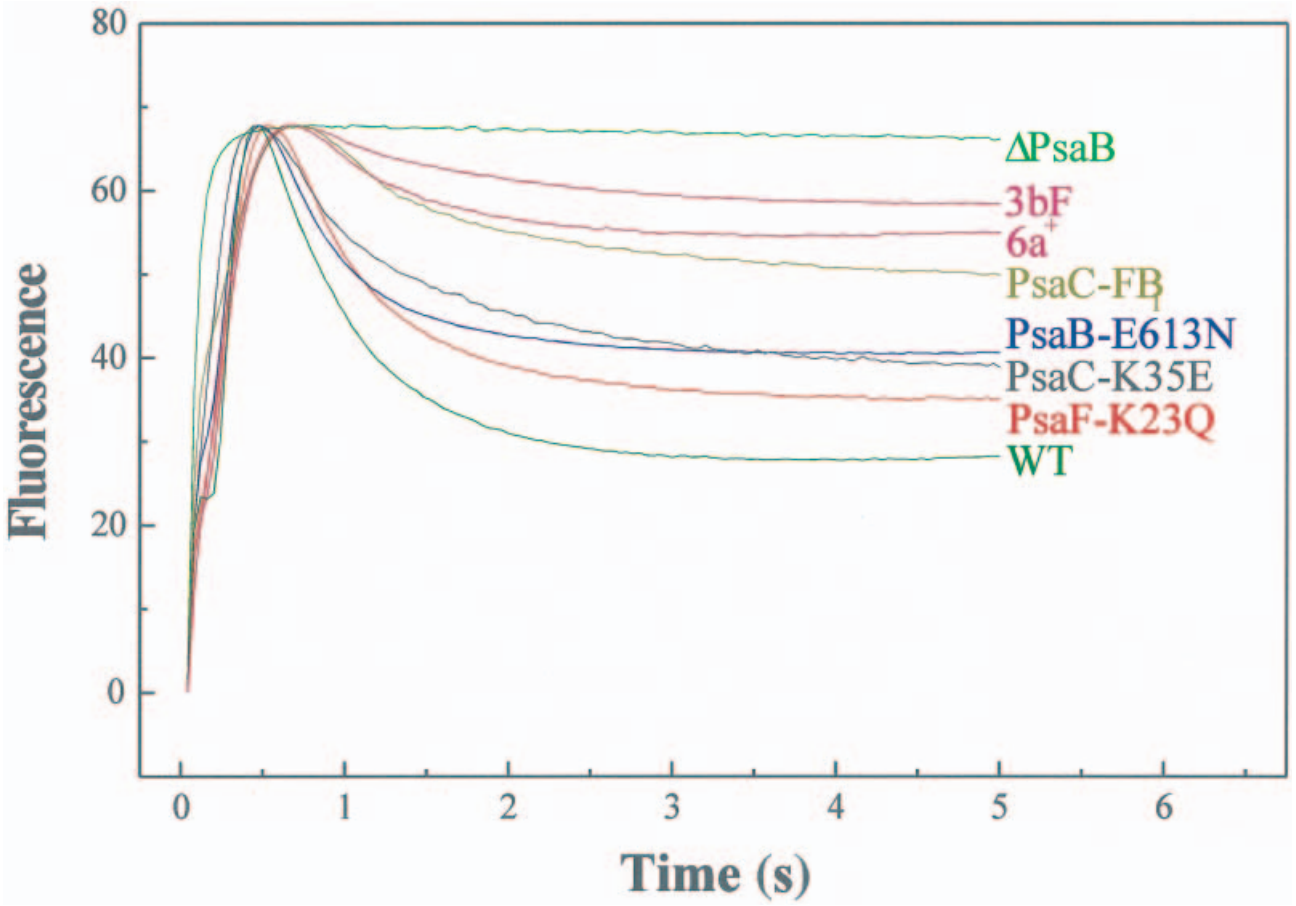

Figure 2. Fluorescence transients of different PSI mutants, affected either at the donor (3bF, $6a^+$, PsaB-E613N, PsaF-K23Q_{17}) or acceptor side (PsaC-K35E, PsaC-FB_1), WT and the PSI-deficient strain ÄPsaB show different levels of steady state fluorescence. Measurements from colonies on TAP plates were taken with a fluorcam system. Data were normalized to equal origin and amplitude.

$NADP^+$ reduction rate, the fluorescence transients show that the steady-state fluorescence level of mutant PsaC-FB_1 is much higher than that of the PsaF mutant K23Q. The fluorescence transient of the PsaC-K35E mutant, in contrast, declines approximately to a level that is comparable with that of PsaF-K23Q or PsaB-E613N. Although the NADP photoreduction rates are only 3.3- and 1.9-fold slower for PsaC mutants FB1 and K35E, respectively, compared to wild type, the steady-state levels of fluorescence are comprised between those of the donor side mutants PsaF-K23Q and 3bF, whose NADP photoreduction rates are slower by factors of 13 and 80, respectively (Table 1). These results indicate that the $NADP^+$ photoreduction measurements are not well suited to predict *in vivo* photosynthetic electron transfer properties (see also below).

Site-directed mutations affecting the acceptor side of PSI are limiting for overall electron transfer *in vivo*

To investigate photosynthetic electron transfer *in vivo* in mutants affected at the donor and acceptor side of PSI by another method, we determined gross oxygen evolution and uptake at increasing light intensities using mass spectrometry. These measurements were performed at high CO_2 concentrations, which completely suppress photorespiration, so that this process will not contribute to the oxygen uptake in the light (Peltier & Thibault 1985; Sültemeyer, Klug & Fock 1987). Under these conditions gross oxygen evolution and uptake follow saturation curves for the PsaF-deficient mutant and the same mutant rescued with the wild-type PsaF gene (Fig. 3b & a, respectively). These results differ from those reported previously at low CO_2 concentrations in which gross oxygen evolution was found to decrease at light intensities above 1800 μE m^{-2} s^{-1}, whereas oxygen uptake displayed a saturation curve (Hippler *et al.* 2000). Taken together, these data show that an increase of the electron sink rescues photosynthetic oxygen evolution in high light in the PsaF-deficient strain. It is expected that when the electron sink is saturated, back reaction from the terminal electron acceptors of PSI to $P700^+$ is favoured in this strain. The back reaction between the radical pairs $P700^+/A_0^-$ or $P700^+/A_1^-$ can cause chlorophyll triplet formation and thereby promote the formation of singlet oxygen. However, in the case of the PsaC mutants K35E and FB_1, gross

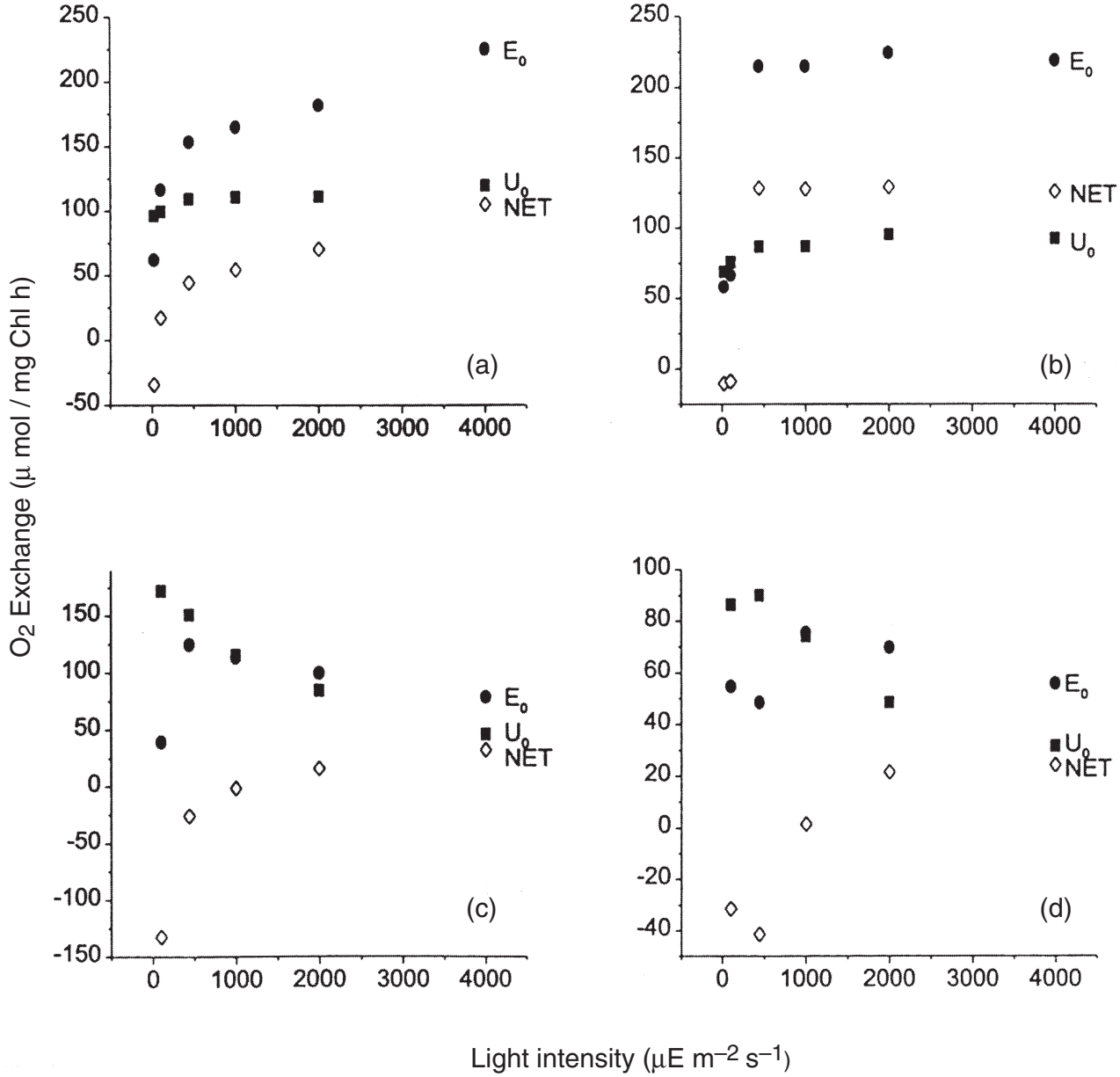

Figure 3. Gross oxygen evolution and uptake are diminished in the PsaC mutants K35E and FB_1 compared to the PsaF-deficient and PsaF-rescued strains at high light intensities. The measurements with the PsaC mutants were repeated twice and with the PsaF-deficient and PsaF-rescued strains three times. The results of one representative experiment are shown. The concentration of C_i (inorganic carbon, $HCO^-_3 + CO_2$) was 2 mM. Oxygen uptake and oxygen evolution in the light were distinguished using $^{18}O_2$ as a tracer and mass spectrometry. Rates of oxygen evolution (E_0, closed circles) and uptake (U_0, closed squares) were estimated at the end of an illumination period of 5 min. NET refers to net oxygen evolution ($E_0 - U_0$, open diamonds). The cells were grown in minimal medium at very low light and were used for measurements when they had reached a cell density of about 2×10^6 cells mL^{-1}. The panels show the light saturation curves of oxygen evolution, uptake and net oxygen evolution, respectively, of the following strains: (a), PsaF-rescued strain RPsaF; (b), PsaF-deficient 3bF; (c), PsaC-K35E and; (d), PsaC-FB_1

oxygen evolution and uptake decrease with increasing light intensity even at high CO_2 concentrations (compare the values at 1000 and 4000 μE m^{-2} s^{-1} in Fig. 3c & d). At 1000 μE m^{-2} s^{-1} gross oxygen evolution and oxygen uptake are nearly equal in the two PsaC mutants, whereas this point is already reached at a light intensity of about 100 μE m^{-2} s^{-1} in the PsaF-deficient and PsaF-rescued strains. Thus the compensation point for oxygen evolution is shifted to a 10-fold higher light intensity in the two PsaC mutants compared to the PsaF-deficient and -rescued

strains. The highest gross oxygen evolution rates found for K35E and FB_1 with about 120 and 80 μmol O_2 mg^{-1} Chl h^{-1}, respectively, are about 1.7- and 2.5-fold lower than the highest rates obtained with the PsaF-deficient and -rescued strain (about 200 μmol O_2 mg^{-1} Chl h^{-1}). This indicates that photosynthetic oxygen evolution *in vivo* is limited more severely by the mutations at the acceptor side of PSI under these conditions. It also shows that the down-regulation of electron transfer in the acceptor side mutants under high light can not be rescued by an increase of the electron sink, in agreement with our proposal that reduction of Fd by PSI limits electron flow to CO_2.

Mutants affected at the electron donor or acceptor side of PSI are subjected to pronounced photo-inhibitory damage to PSII

To test the impact of mutations at the donor or acceptor side of PSI on photo-inhibitory damage to PSII and on the recovery rate of PSII, mutant and wild-type strains were subjected to photo-inhibitory light (2500 μE m^{-2} s^{-1}) for 1 h and subsequently shifted to low light for recovery. PSII activity was measured by fluorescence induction and it is represented by the F_v/F_m ratios [$F_v = F_m - F_0$, where F_v is the variable fluorescence, and F_v and F_m are the fluorescence emitted when all PSII reaction centres are closed and open, respectively; (reviewed in Krause & Weis 1991)]. Figure 4 shows the values of F_v/F_m, averaged over four experiments with wild-type and PSI donor and acceptor side mutants. The wild-type strain displayed the weakest photo-inhibition and the fastest recovery rate and is followed by strains PsaB-E613N and PsaC-K35E. An even stronger decrease and a slower recovery rate of the F_v/F_m ratio indicating a more pronounced photo-inhibition, is observed with strains 3bF, $6a^+$, PsaC-FB_1 and PsaB-W627F. It is noteworthy that the photo-inhibitory damage and the rate of recovery of PSII correlate with the steady-state fluorescence levels of the fluorescence transients of the strains examined (Fig. 2). It thus appears that an increase of the level of reduced Q_A, due to mutations either at the

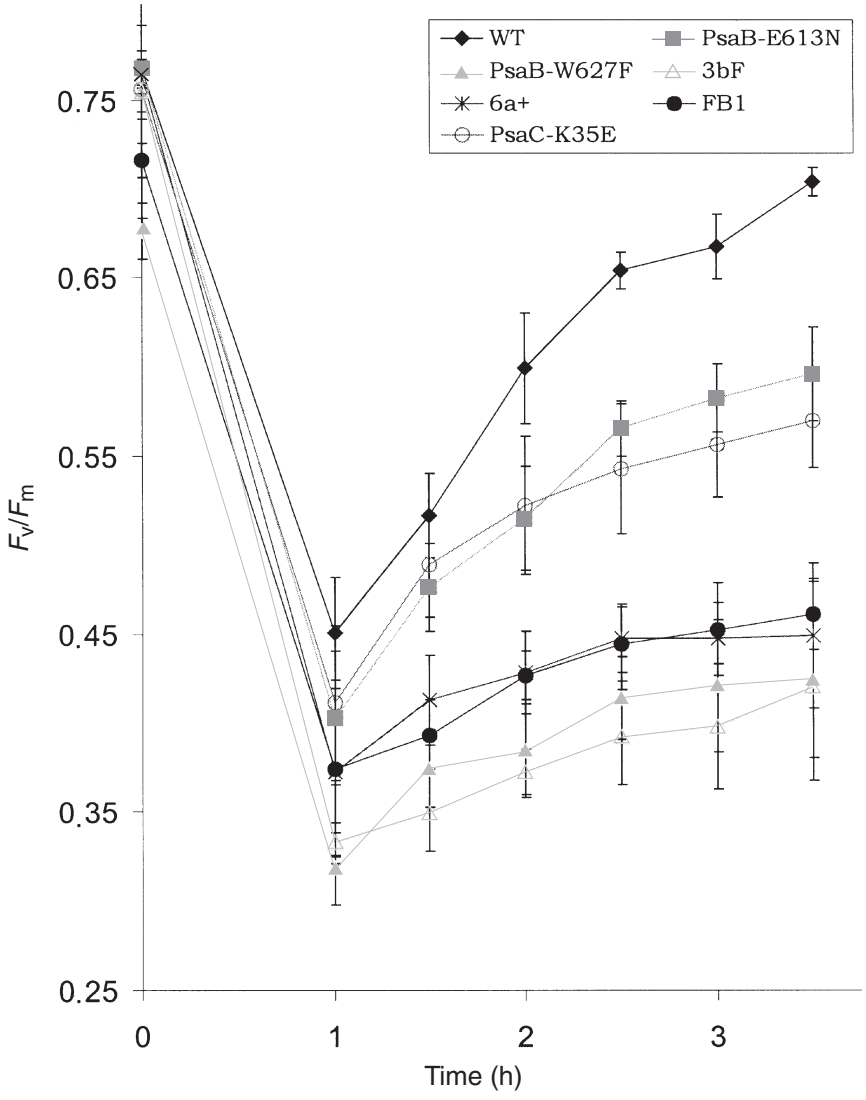

Figure 4. PSII photo-inhibition and recovery rate after high light treatment. PSII activity was monitored by measuring F_v/F_m values for wild type, PSI donor side (3bF, $6a^+$, PsaB-E613N, PsaB-W627F) and PSI acceptor side mutants (FB1, PsaC-K35E). The cells were treated with high light at 2500 μE m^{-2} s^{-1} for 1 h (0 h–1 h) and then allowed to recover in dim light at 20μE m^{-2} s^{-1}. Error bars are shown for four experiments.

oxidizing or reducing side of PSI, has a pronounced photoinhibitory effect and leads to a slower recovery rate of PSII.

Lipid peroxidation is enhanced after incubation of the PsaC-K35E and donor side mutants in the light

To quantify photo-oxidative damage we measured the amount of lipid peroxidation in whole cells after incubation in high light. Lipid peroxidation has been shown to occur in plants after exposure to photo-oxidative light stress (Vavilin & Ducruet 1998; Vavilin *et al*. 1998). Using the thiobabituric acid reaction, that is most generally used for studying lipid peroxidation in biological systems (Slater 1984), we found that lipid peroxidation is enhanced in the donor side mutants PsaB-W627F and PsaB-E613N (Sommer *et al*. 2002) and the PsaC-K35E strain in comparison with wild type (Fig. 5) after 30 h of light treatment (400 μE m^{-2} s^{-1}). Under our experimental conditions the PsaC-K35E strain started to display a chlorotic phenotype after 30 h. At this time point the amount of lipid peroxidation in the PsaC-K35E mutant is higher than in the PsaB-E613N and PsaB-W627F mutants (Fig. 5). In mutant PsaB-W627F, the binding and electron transfer of Pc to the core of the reaction centre is strongly perturbed and no first-order electron transfer kinetics are detectable (Sommer *et al*. 2002). However, the intramolecular electron transfer rate is probably altered, as determined for the first-order electron transfer rate between Cyt c_6 and PSI from this mutant. It is possible that the increase of lipid peroxidation and therefore the increase of reactive oxygen species is related to these properties. The light-sensitivity of mutant W627F cannot be suppressed by anaerobic conditions, indicating that in addition to oxidative damage an oxygen-independent mechanism leads to a destabilization of the photosystem I complex. This sensitivity could be related to the altered electron transfer within PSI in this mutant.

Donor and acceptor side mutations cause degradation of PSI and PSII under high light

To examine the effect of high light on the photosynthetic complexes of the mutants PsaC-K35E, of the donor side mutants and of the wild type, the strains were grown to a density of about 2–3 × 10^6 cells mL^{-1} at 60 μE m^{-2} s^{-1}, diluted to a concentration of 15 μg Chl mL^{-1} and incubated at 400 μE m^{-2} s^{-1}. Cells were then harvested after 10, 20 and 30 h of growth, equal volumes were fractionated on SDS-PAGE and analysed by immunoblotting, using anti-PsaA, anti-PsaD, anti-D1, anti-Cytf and anti-CF1 antisera (Fig. 6). In the wild-type strain, the amount of all proteins investigated increased during the time-course experiment, indicating that the strain is growing under these conditions. In contrast, PSI protein levels decreased in the donor side mutants in the light. This decrease was strongest in the PsaB-W627F strain, followed by K35E, in which PsaA decreased after 20 h and disappeared completely after 30 h in the light. A decrease of PsaA was also seen in E613N. Additionally, D1 protein levels started to decrease after 10 h in 3bF and 30 h in W627F, whereas the levels of CF1 remained stable. In mutant PsaB-E613N, D1 levels stayed constant. The Cytf protein levels decreased only slightly in the donor side mutants. The level of D1 protein also decreased in the K35E mutant after 20 and 30 h in the light. The levels of most proteins investigated decreased strongly after 30 h in the light, a time period at which a boost of lipid peroxidation occurs (Fig. 5). The disappearance of the PsaA signal in PsaC-K35E after 30 h in the light demonstrates that PSI itself becomes a target of the photo-oxidative stress. It thus appears that limiting electron exit from PSI is extremely deleterious. The fact that D1 protein levels diminish in the donor side mutants 3bF and PsaB-W627F and the acceptor side mutant PsaC-K35E shows that limitation of electron transfer through PSI causes degradation of PSII. This might occur through the production of reactive oxygen species or through accumulation of Q_A^-.

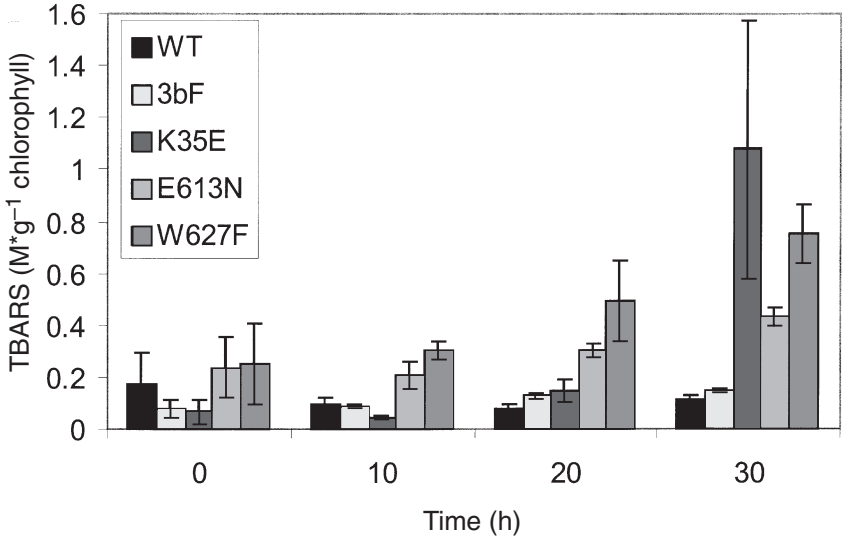

Figure 5. Lipid peroxidation is enhanced after incubation of the 3bF, PsaC-K35E, PsaB-E613N and PsaB-W627F mutants in the light. MDA accumulation was measured from whole cells (15 μg Chl mL^{-1}) before and after light treatment (grown in TAP, 0–30 h at 400μE m^{-2} s^{-1} and 25 °C). The data shown correspond to three independent experiments for each of which three samples per strain were analysed. Equal sample volumes were taken for the measurements.

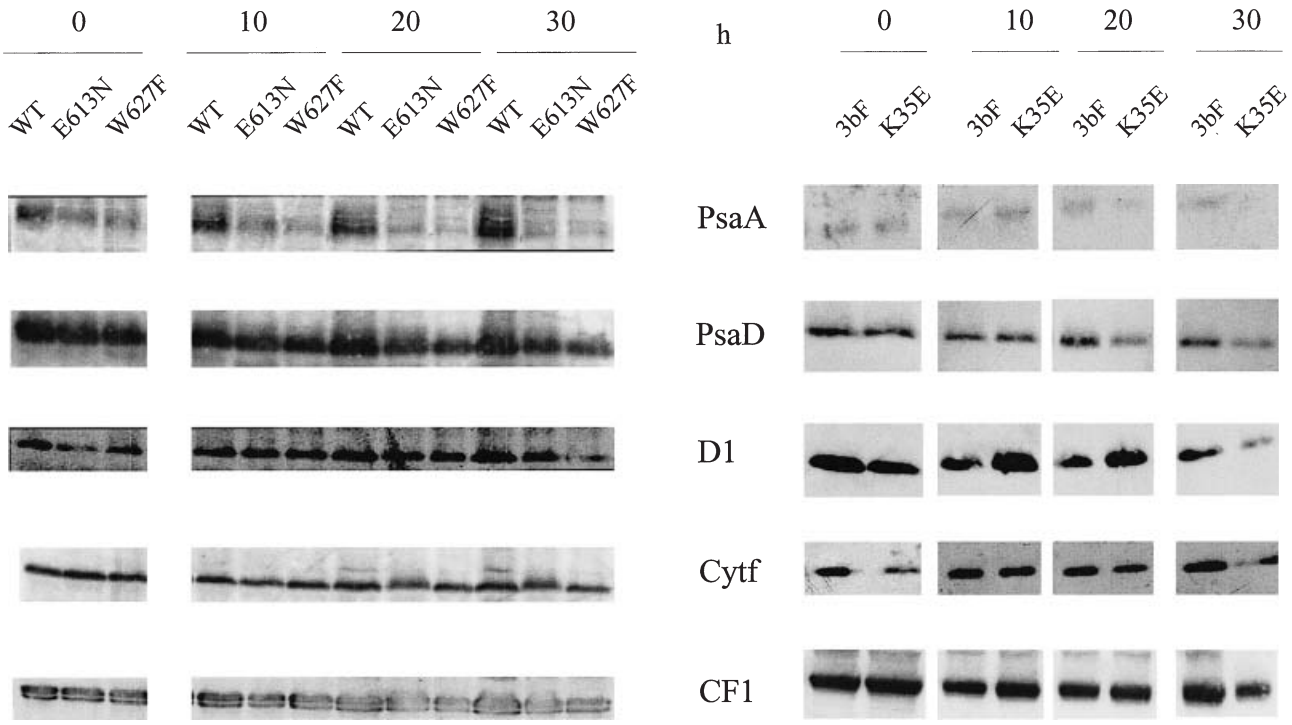

Figure 6. Donor and acceptor side mutations cause degradation of PSI and PSII under high light. PsaC-K35E, 3bF, PsaB-W627F, PsaB-E613N and wild-type cells were grown in TAP to a density of about 2–3 × 10^6 cells mL^{-1} at 60μE m^{-2} s^{-1}, diluted to a concentration of 15 μg Chl mL^{-1} (0 h) and incubated at 400μE m^{-2} s^{-1}. After 10, 20 and 30 h of growth 1.5 mL cells were harvested and re-suspended in 100 μL TAP. Equal volumes (15 μL) were fractionated on SDS-PAGE and analysed by immunoblotting, using anti-PsaD, anti-PsaA, anti-D1, anti-Cytf, and anti-CF1 antibodies.

From our data it is clear that the decrease of the photosystems in the time-course experiment (Fig. 5) correlates with the light sensitivity of the strains (Fig. 1). It is also apparent that after 30 h the amount of lipid peroxidation for mutant strains PsaC-K35E and PsaB-W627F, and to a lesser extent for mutant PsaB-E613N, correlates with their light sensitivity (Fig. 5). In contrast, in mutant strain 3bF the amount of lipid peroxidation is modest and does not correlate with its light sensitivity. However in all cases, except for PsaB-W627F, the light sensitivity of these strains is suppressed by anaerobic conditions. This strongly suggests that photo-oxidative stress is a cause of the light-sensitivity of the mutants.

DISCUSSION

In this study we performed a comparative analysis of PSI donor and acceptor side mutants to analyse the relation between limitation in electron transfer through PSI and the corresponding light sensitivity. The link between oxidative damage and photosensitivity is revealed by the fact that: (1) photosensitivity is suppressed under anaerobic conditions in most cases; and (2) there is a burst of lipid peroxidation in high light in some of the mutant strains. Our data clearly show that limitation in electron transfer either at the donor or acceptor side of PSI leads to photo-inhibition of PSII and affects the rate of recovery of PSII. Our data also demonstrate that acceptor side mutants limit linear electron transfer *in vivo* whereas the donor side mutants limit electron transfer from Pc to FNR *in vitro*.

PSI donor and acceptor mutations have a different impact on photosynthetic electron transfer

Mutations at the donor side of PSI affect the electron transfer from PSII to PSI *in vivo*. They lead to a higher level of reduced Q_A as seen from the fluorescence transients shown in Fig. 2 and from earlier observations (Farah *et al*. 1995; Hippler *et al*. 2000). In high light and at ambient CO_2, a decrease of gross oxygen evolution was observed in the PsaF-deficient mutant (Hippler *et al*. 2000). Here we have shown that this decrease in the rate of oxygen evolution at high light intensities no longer occurs in the presence of high CO_2 (Fig. 3). In this respect the impact of the donor side mutations on linear electron transfer needs to be considered. The rate-limiting step of linear electron transfer is the concerted reduction of Cytf and $Cytb_H$ by plastoquinol oxidation with a half-life of 10–15 ms (Stiehl & Witt 1969). Measurements with intact *Chlorella* cells under high light conditions, when the plastoquinone pool is fully reduced, show that this step can be accelerated to 1.2 ms as (Joliot & Joliot 1986). The *in vivo* electron transfer between the $Cytb_6$/f complex and $P700^+$ is 1.2 ms in the PsaF-deficient

strain 3bF, 20 times slower in comparison with wild type (Farah *et al*. 1995). Thus it is possible that under high light conditions electron transfer between the $Cytb_6/f$ complex and $P700^+$ proceeds at a similar rate as the concerted reduction of Cytf and $Cytb_H$ by plastoquinol oxidation in a PsaF-deficient strain. Additionally we have to consider that the level of PSI in the donor side mutant 3bF is slightly lower in comparison with the wild type, which can also contribute to a delayed oxidation of Pc (Farah *et al*. 1995; Hippler *et al*. 1997). Hence, the plastoquinone pool would remain reduced and this would explain the high steady-state fluorescence levels detected in the fluorescence transients. However, the argument that linear photosynthetic electron transfer could be limited by oxidation of Pc and not by the concerted reduction of Cytf and $Cytb_H$ by plastoquinol oxidation in the donor side mutants needs further experimental evidence and should be understood as a working hypothesis. How is the situation for the acceptor side mutants? If one considers the second-order rate constant for Fd reduction by PSI from the *psaC* mutant K35E (2.8×10^6 M^{-1} s^{-1}), one can estimate that a Fd concentration of approximately 250 μM would be needed for Fd reduction to occur with a half-life of 1 ms. At this concentration linear electron transfer would not be limited by Fd reduction in this mutant strain. Gross oxygen evolution rates in K35E and FB_1 are 1.7- and 2.5-fold lower, respectively, in comparison with the values found for the rescued 3bF strain. This is close to the differences of 1.9 and 3.3 when the $NADP^+$ photoreduction rates with PSI from K35E and FB_1, respectively, are compared with those obtained with wild-type PSI. We measured flash-induced electron transfer between Pc and PSI isolated from the PsaC-K35E mutant and found electron transfer rates that are comparable with those obtained with wild-type PSI. Thus, the lower rate of gross oxygen evolution in the two PsaC mutants is most likely due to the limitation in electron transfer from PSI to Fd. It implies that in the K35E strain the Fd concentration in the stroma is not sufficient to compensate for the lower reduction rate caused by the PsaC mutation. The ratios of Fd to NADP–ferredoxin–oxidoreductase to Pc have been estimated to be 5 : 3 : 4, respectively, per Cytf or P700 in spinach chloroplasts (Böhme 1978). Since the volume of the lumenal space is much smaller than that of the stroma, the concentration of Fd in the stroma should be considerably lower than that of Pc in the lumen, which is considered to be about 400 μM (Whitmarsh 1986), and thus below 250 μM. In the case of the FB_1 mutant, it is likely that the slower release of reduced Fd from PSI compared to wild type limits linear electron transfer. Interestingly, the fluorescence transients of PsaC-K35E show lower steady-state fluorescence levels in comparison with the PsaF-deficient mutants (Fig. 2), indicating that the level of reduced Q_A is considerably lower in the acceptor side mutant. This would indicate that in contrast to the conclusions reached from the oxygen evolution measurements, linear electron transfer is more limited in the PsaF-deficient mutants than in the PsaC-K35E mutant. How can this discrepancy be explained?

Electron trapping within the terminal electron acceptors of PSI is deleterious

Whereas gross oxygen evolution rates in K35E and FB_1 are about 1.7- and 2.5-fold lower, respectively, in comparison with the values found for the PsaF-deficient and -rescued strains, the highest net oxygen evolution rates at 4000 μE m^{-2} s^{-1} are four- and five-fold lower, respectively (Fig. 3). This difference becomes even larger when the net rates of oxygen evolution are compared at lower light intensities. The ratio between gross oxygen uptake and evolution is higher for the PsaC mutants, especially at lower light intensities, in comparison with that of the PsaF-deficient and -rescued strains. Thus, more electrons originating from photosynthetic water splitting are used for reducing oxygen in the PsaC mutants. We suggest that electron escape to oxygen explains the lower steady-state fluorescence transient levels of the acceptor side mutants in comparison with PsaF-deficient strains although these mutations limit linear electron transfer to Fd. Thereby direct reduction of O_2 through PSI (Mehler 1951) will generate reactive oxygen species such as the superoxide anion radical ($O_2^{-\bullet}$). As electron transfer between the acceptor side of PSI and Fd is considerably diminished in the two PsaC mutants, it is possible that under increasing light intensities that induce high electron flow into PSI, additional charge separations could fully reduce the iron–sulphur clusters, thus leading to recombination between the radical pairs $P700^+/A_0^-$ or $P700^+/A_1^-$ and to the generation of P700 triplet allowing singlet oxygen formation. It has been shown previously that under conditions in which the iron–sulphur clusters are completely reduced, the recombination between the radical pair $P700^+/A_1^-$ result in P700 triplet state (^{3}P700) formation with a yield of more than 85% at room temperature (Sétif, Bottin & Mathis 1985; Brettel & Sétif 1987). Under reducing conditions, back reaction and thereby formation of singlet oxygen is even more likely in the donor side mutants in which forward electron transfer from Pc to $P700^+$ is slowed down in comparison with wild type. The role of oxygen in the photoinhibitory process is clearly revealed by the restored growth capacity of the PsaC as well as the donor side mutants 3bF, $6a^+$ and PsaB-E613N mutants under anaerobic conditions.

Interestingly, data from Figs. 2 and 4 indicate a correlation between the Q_A redox status as measured by fluorescence transients in mutants affected at the donor or acceptor side of PSI, and the level of PSII photo-inhibition as well as the rate of PSII recovery. Thus mutant PsaC-K35E is less photo-inhibited in comparison with the PsaF-deficient strains. The extent of PsaC-K35E photo-inhibition is comparable with that of the donor side mutant PsaB-E613N. However the acceptor side mutant is significantly more light sensitive than the two donor side mutants. This supports the hypothesis that an increase in the excitation pressure on PSII, which causes damage to the system, is not as dangerous as blocking electrons at the acceptor side of PSI, which would result in electron escape to oxygen and thereby promote the formation of reactive oxygen species.

Thus, under physiological conditions which limit electron transfer through PSI, PSII will also be a target of these stress conditions because of damage caused by PSI. The extent of damage will be determined by the physiological status of the electron sink beyond PSI. We propose that more reducing conditions would lead to the formation of reactive oxygen species in donor and acceptor side mutants. Thus when the formation of reactive oxygen species exceeds the scavenging capacity of the system, photo-oxidative damage is inevitable.

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3 Discussion

3.1 The Model Chlamydomonas reinhardtii and the methods deployed

In recent years the development of powerful techniques in classical and molecular genetics in conjunction with the Chlamydomonas genome project have greatly increased the potential of this system for the analysis of photosynthetsis, organelle biogenesis and many other processes. A back-breaking overview on *C. reinhardtii* is given in Harris, 1989 and Rochaix, Goldschmidt-Clermont, and Merchant, 1998. *C. reinhardtii* is a heterothallic chlorophyte algae that has been termed "the green yeast" (Rochaix *et al*., 1995). Cells of both mating types (+) and (-) can be grown vegetatively. Under certain stress conditions gamete formation is induced which leads in the presence of two opposite mating-types to zygotes, which undergo meiosis and finally result in a tetrad of haploid progeny cells. Like land plants *C. reinhardtii* contains three distinct genetic systems located in the nucleus, chloroplast and mitochondria. Nuclear genes follow a typical Mendelian 2:2 segregation whereas chloroplast and mitochondrial mutations are predominantly inherited from the maiting-typ (+) and (-), respectively.

An important feature of *C. reinhardtii* concerning the dissection of bioenergetic processes in chloroplasts by classical and reverse genetics is the fact that photosynthesis is dispensable for growth and survival when a carbon source such as acetate is added to the medium.

Numerous nuclear and chloroplast photosynthetic mutants have been isolated taking advantage of acetate requirement or altered fluorescence emission kinetics. Especially non invasive methods like video fluorescence imaging have helped in large-scale screening projects to distinguish between WT and mutant phenotypes (Fenton *et al*., 1990; Bennoun *et al*., 1997; Fleischman *et al*., 1999; Kruse *et al*., 1999).

In this work I took advantage of a set of existing point and deletion mutants altered in the PsaC subunit (point mutation: PsaC-K35E, triple mutation PsaC-I12V/T15K/Q16R: FB_1) (Fischer *et al*., 1998; 1999), a PsaF-deficient mutant (3bF) (Farah *et al*., 1995) and PsaA or PsaB deficient strains (ΔPsaA, ΔPsaB) (Redding, 1998).

3.1.1 Methods

In this work a set of molecular biology, biochemical and biophysical methods were used. This chapter gives a brief overview over the methods used in this work.

3.1.1.1 Molecular biology techniques

In the first mutagenesis approach that was aimed to analyze the PS1 core subunit PsaB, a combinatorial site directed mutagenesis was performed to achieve a first picture of residues important for the interaction of pc or cyt c_6 with the core of PS1. This technique has already successfully been probed for specifying the interaction site of ferredoxin to PsaC (Fischer *et al*., 1998). The degenerate oligonucleotides (up to four changes per oligonucleotide) allowed a permutated set of 32 altered *psaB* sequences respectively. In spite of varying PCR conditions only 7 different mutants could be generated (single and double mutations).

Chloroplast transformation is well established in *C. reinhardtii* (Boynton *et al*., 1988; Sanford *et al*., 1993). In this work the altered *psaA* and *psaB* genes were transformed into deletion strains of ΔPsaA and ΔpsaB, respectively. The plasmids pKR154 and pKR162 which were used for the transformation experiments contained the aadA-cassette which confers resistance to spectinomycin/streptomycin (Goldschmidt-Clermont, 1991). Screening was performed on plates containing spectinomycin which were exposed to light at 100 µE $m^{-2}s^{-1}$ or were kept in darkness. A total transformation efficiency of 10 - 100 colonies per µg DNA was acchived.

Other commonly used molecular biology techniques were performed as described (Sambrook *et al.,* 1989).

3.1.1.2 Biochemical techniques

For isolation, quantification and detection of thylakoids, proteins and protein complexes standard techniques were deployed, such as cell rupture, membrane solubilization, differential centrifugation, anion exchange and size exclusion chromatography, gel electrophoresis, immuno-detection using polyclonal antibodies, spectroscopy and estimation of ROS generation by measuring thio

barbituric acid reactive species (TBARS) (see for e.g. Sommer *et al*., 2002; 2003).

3.1.1.3 Biophysical techniques

Flash induced spectroscopy is an ideal technique to measure fast electron transfer reactions. This technique is of excellent use in photosynthetic research since most electron or excitation transfer reactions occur within chromatophores which change their spectral properties upon excitation or redox-status. Dark adaptation of photosystems prior to a flash ensured that the flash triggered oxidation of all photosystems present in the cuvette at the same time which allows the investigation of transient reactions.

The measurement of fluorescence transients and F_v / F_m values is an easy to perform measurement and offers the advantage of a noninvasive method.

Analysis of the more complex electron transfer kinetics were performed using global analysis (Holzwart, 1996). For the evaluation of the kinetic traces obtained at the presence of high concentrations of $MgCl_2$ (> 30 mM), where the donor dependent and independent reduction rates and amplitudes were hard to distinguish, a global fit analysis was performed. In this case a constant ratio of the amplitudes was assumed, that was derived from measurements at concentration below 30 mM $MgCl_2$. A global fit analysis has also been performed to determine the amplitude A1 at low donor concentrations. In this case the lifetime for the fast kinetic phase was fitted to the same value over the range of donor concentrations. All fits were performed at least on two independent sets of measurements.

3.2 The lumenal recognition site of PS1 for pc and cyt c_6

3.2.1 Influence of the lumenal side of PS1 on PsaF

Results from investigations on PsaJ, a small hydrophobic PS1 subunit with a single transmembrane domain, suggested a requirement for the correct orientation of the PsaF subunit in a way that allows the proper binding of the electron donors (see chapter 1.4.4.2).

Site directed mutagenesis of highly conserved residues within the inter-

helical-region of the lumenal loop *j*, that connects transmembrane helices IX and X of the PsaB protein in *C. reinhardtii*, resulted in the mutant strain E613K. It is strongly impaired in electron transfer between PS1 and the two donor proteins as investigated by flash-induced absorption spectroscopy (Sommer *et al.*, 2002). The electron transfer rates for the mutant E613K PS1 and the donors pc or cyt c_6 were about two orders of magnitude lower as compared to WT. These values are comparable to values obtained for electron transfer between PS1 from the PsaF-deficient mutant, 3bF, and the two donors.

Interestingly, mutation of E613 to N of PsaB resulted in a clear acceleration of electron transfer rates as well as improved binding of both donors to PS1 as compared to WT. How can this discrepancy be explained? From the structural data it seems to be clear that the negative charge provided by residue E613 is exposed on the surface of PS1 (see Fig. 9). Thus, a change of E to N could lead to a tighter binding of the donors to PS1 in mutant E613N because this mutation decreases the electrostatic repulsion between the negatively charged donors and PS1 which leads to a slower unbinding of the donors from PSI (see Fig. 7C) (Sommer *et al.*, 2002). Fig. 9 shows that the corresponding E613 of *C. reinhardtii* (E617 in *S. elongatus*) is in a close distance to the N-terminus of the PsaF subunit of the cyanobacterial PS1. Because the N-terminal domain of PsaF in *C. reinhardtii* is even larger, it can be rationalized that mutation E613K may cause a distortion of the positively charged N-terminal domain of PsaF by charge repulsion from the positively charged K at position 613 (see Fig. 7D).

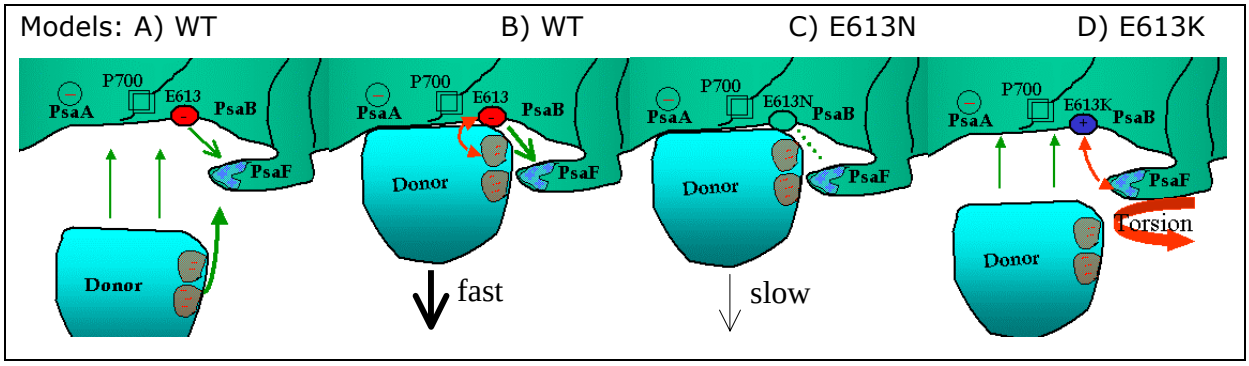

Figure 7: Model of the interaction between residue E613 and the basic patch of PsaF: (A) and (B) show the situation for WT PS1 where long range electrostatic interaction leads to a tight docking of the donor to PS1, electron transfer takes place in the transient complex and unbinding is facilitated by repulsion of acidic patches on the donor and E613 and electrostatic interaction is regained between E613 and PsaF. In (C) depicting PS1 E613N where unbinding of the donors is slowed down upon loss of electrostatic repulsion whereas in PS1 E613K (D) electrostatic repulsion between E613K and PsaF lead to a distorted PsaF no more able to promote tight docking of the donors.

Such a disorientation may have a strong effect on binding and electron transfer of both donors to PS1 since the electrostatic interaction with the N-terminal domain of PsaF would be strongly impaired, which is indeed manifested in the strong impairment of electron transfer between both donors and the altered PS1 E613K. It may also explain why mutations E613N and E613K have opposite effects on binding and electron transfer between the altered PS1 particles and the two donors.

The effect of a slower release of the donors from the altered PS1 E613N was further probed by using a steady state system. $NADP^+$-photoreduction by PS1 E613N in presence of small concentrations of the donors (1.5 µM) yielded a higher photoreduction rate compared to wild type whereas in presence of higher amounts (30 µM) the reduction rate became strikingly smaller compared to WT. This effect can be explained by assuming that the PS1 centers are either open or closed after unbinding/binding of the donors, respectively. In the case that release of the electron donors from PS1 after electron transfer limits steady-state electron transfer an increasing donor concentrations would slow down forward electron transfer, which is observed in Fig. 8. This results points to the same direction as discussed above, namely that unbinding of pc is three times slower as compared to WT and that this limits forward electron transfer from pc to $P700^+$. It should be noted that the lumenal pc concentration was estimated to about 400 µM (Whitmarsh, 1986) which would mean at a determined K_D of 7.7 µM for the pc-PS1 E613N complex that nearly all centers would be occupied. Drepper *et al.*, (1996) proposed that release of oxidized pc from PS1 is limiting the linear electron transfer between the cyt b_6f complex and PS1. Therefore a decrease in the unbinding rate may slow down the electron transfer between cyt b_6f complex and PS1 *in vivo*. The light-sensitivity of the

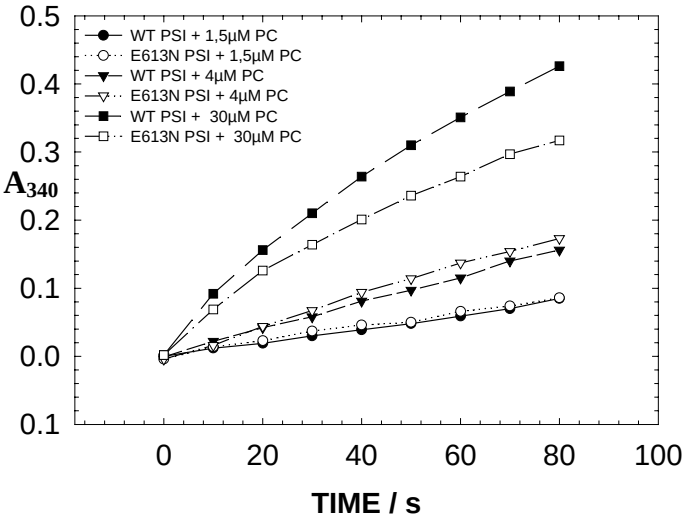

Figure 8: At high concentrations of pc $NADP^+$ photo-reduction becomes slower with PsaB E613N PS1-particles when compared to PS1-particles isolated from WT. The cuvette contained PS1 particles at a concentration of 5 µg/ml chlorophyll, 15 mM Tricine-buffer (pH 7.0), 0.03% dodecyl-β-maltoside, 2 mM sodium ascorbate, 0.5 mM NADP, 3 µM fd, 0.5 µM fd:NADP-oxidoreductase (from spinach) and pc in concentrations of 1.5, 4 and 30 µM; the probe was illuminated continuously, absorption was monitored at 340 nm.

PsaB-E613N strain supports this interpretation. The rate of cyt f oxidation was measured *in vivo* and compared to the rate in WT, and it is indeed slowed down by a factor of about 1.5 (Finazzi and Hippler, personal communication). This measurement demonstrates that the PsaB-E613N mutation limits electron transfer between cyt b_6f complex and PS1 *in vivo*, although binding and forward electron transfer appears to be more efficient as WT *in vitro*.

Taken together these data indicate that residue E613 supports the release of the two donors from PS1 by electrostatic repulsion and orientates the positively charged N-terminal domain of PsaF in a way that it allows efficient binding of the donors to PS1.

3.2.2 The hydrophobic recognition site for pc and cyt c6 on PS1

For eukaryotic organisms site directed mutagenesis of pc and analysis of binding and electron transfer between the altered pc and PS1 (Redinbo *et al.*,

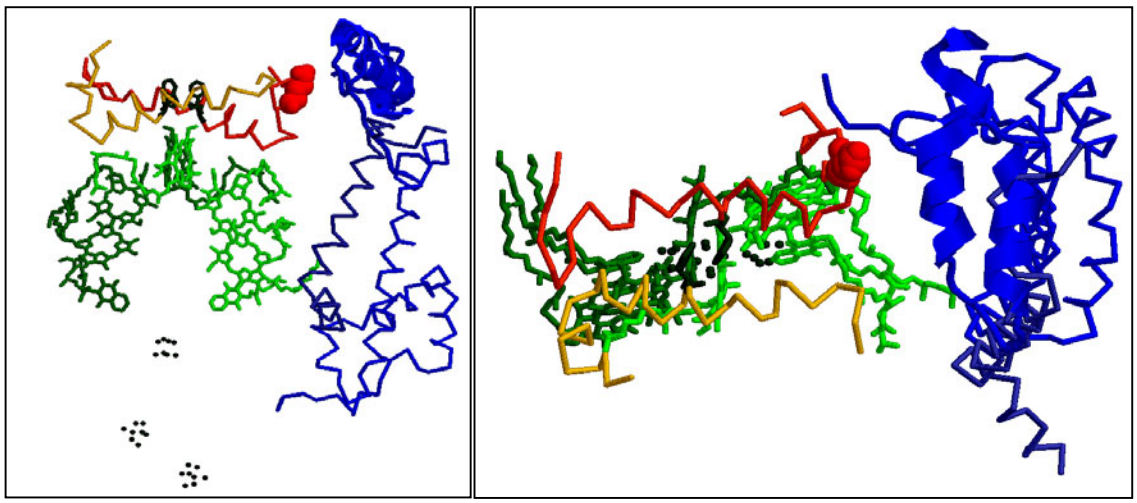

Figure 9: Crystal structure of *S. elongatus* PS1 at a resolution of 2.5 Å (Jordan *et al.*, 2001) seen in a open view along the membrane (left) or from the lumenal side(right). N-terminal part of PsaF in blue, helix l in red (PsaB) and orange (PsaA), the corresponding E613 of *C. reinhardt* is in red spacefilled and the Trp-dimer is shown in dark green, the internal electron transport chain is in darker and lighter green. The structure suggests *i)* interaction of negative charged E613 with the positively charged PsaF in *C. reinhardtii* and *ii)* participation of PsaB-W627 and PsaA-W651 in binding of the electron donors.

1993; Nordling *et al.*, 1991; Haehnel *et al.*, 1994; Hippler *et al.*, 1996) suggested that beside the long-range electrostatic interaction between the positively charged PsaF and the negative patches of pc, a second recognition site is required which brings the flat hydrophobic surface of pc in close contact with the core of PS1 in order to allow efficient electron transfer from copper *via* the "northern face" of the

molecule to $P700^+$ (see Fig. 9). According to the three-dimensional structure of PS1, P700 the primary photosynthetic electron donor is localized near the lumenal surface.

The lumenal surface of PS1 is mainly build up of loop regions that connect transmembrane α-helices of subunits PsaA and PsaB. Part of the loops *j'/j* of PsaA/B form two α-helices *l'/l* which are in parallel to the membrane containing two conserved W residues: PsaA655 (*C. reinhardtii* W651) and PsaB631 (*C. reinhardtii* W627) (Fig. 9). It is assumed that these loops could be involved in the docking of the soluble donors pc and cyt c_6 (Jordan *et al*., 2001; Schubert *et al* 1997; Sommer *et al*, 2002; manuscript; Sun *et al*., 1999). Sun and coworkers (1999) generated site-directed mutants in the lumenal loop *j* of the PsaB protein from *Synechocystis sp.* PCC 6803 and consistent with previous hypothesis, the double mutant W622C/A632R was strongly affected in the interaction between the altered PS1 and the electron donors.

In *C. reinhardtii* PsaB-W627F and PsaA-W651F mutants (corresponding to W631 W655 in *S. elongatus*) were generated by site-directed mutagenesis (Sommer *et al*., 2002; Sommer *et al*., manuscript). The fact that the mutation PsaB-W627F and the corresponding mutation of PsaA-W651 to F abolished the formation of an intermolecular electron transfer complex between the altered PS1 and pc indicates that the W-dimer of helices *l'/l* is part of the suggested recognition site required for binding of pc to the core of PS1. While cyt c_6 was still able to form a complex, the dissociation constants for binding of cyt c_6 to PS1 from PsaB-W651F and PsaA-W651F were 2-fold and about 6- to 10-fold increased compared to WT indicating that these two residues constitute an essential binding site for cyt c_6 as well as for pc (Sommer *et al*., manuscript). This interpretation is strongly supported by the high resolution structural data of PS1 (Jordan *et al*., 2001). Fig. 9 shows that W631 (corresponding to W627 in *C. reinhardtii*) from α-helix *l* of PsaB together with W655 (corresponding to W651 in *C. reinhardtii*) from α-helix *l'* of PsaA, form a sandwich complex, having their indole groups stacked at van der Waals distance (Jordan *et al*., 2001), directly situated above P700. Thus binding of pc or cyt c_6 to this structural element would possibly allow electron transfer from His87 of pc resp. ring D of the porphyrin cofactor of cyt c_6 *via* the π-electron system of the aromatic W residues directly to $P700^+$. The mutation of W to F at positions PsaB627 or PsaA651 decreases the hydrophobic surface that is exposed into the putative binding site from a value of about 255 to 210 ($Å^2$) (Sommer *et*

al., 2002). This change is apparently too large to allow a hydrophobic contact sufficient for the stable formation of an electron transfer complex between the northern face of pc and α-helix *I* of the mutated W627F PS1 or the resp. change of PsaA-W651F.

In line with an increase of K_D, the rate constant for electron transfer between cyt c_6 and PS1 from PsaA-W651F was about 3 times slower than for the electron transfer with PS1 from PsaB-W627F and a mutation of PsaA-W651 to S had an even larger impact on the dissociation constants as well as the electron transfer rates (Sommer *et al*., manuscript). A similar correlation of K_D and electron transfer rate was already observed for the interaction of cyt c_2 and the reaction center of *Rb. sphaeroides* (Tetreault *et al*., 2001) and proofed also for the reactions of pc or cyt c_6 with the altered PS1 PsaB-E613N (Sommer *et al*., 2002). In contrast the opposite effect on the electron transfer rates for the reaction of pc with the altered PS1 was observed which increased 3- and 5-fold with PS1 from PsaA-W651F and PsaA-W651S resp. as compared to the rate constant measured for PS1 from PsaB-W627F. This indicates that alterations of PsaB-W627 or PsaA-W651 have a different impact on binding and electron transfer between the PsaA or PsaB mutant PS1 and pc or cyt c_6 and suggest that in WT pc binds more tightly to the PsaB-W627 whereas cyt c_6 interacts more directly with PsaA-W651.

Interestingly the change of residue PsaA-W651 to the hydrophilic S restored slightly the formation of the electron transfer complex between pc and the altered PS1 not observed for the interaction of pc with PS1 from PsaA-W651F or PsaB-W627F. It may be conceived that a hydrophilic interaction between the S residue at position PsaA-W651 and an appropriate residue on the surface of pc eventually H87 on the "northern" hydrophobic interaction site of pc results in an equivalent gain of binding energy that compensates the loss of hydrophobic interaction surface.

The electron transfer between cyt c_6 and the altered PsaB-W627F PS1 was not as much disturbed for pc and cyt c_6 was still able to form a transient complex with the altered PS1 (Sommer *et al*., 2002). Interrestingly cyt c_6 is still capable for complex formation with mutants PS1 PsaA W651F/S where the electron transfer to PS1 was reduced to a much higher extend for cyt c_6 as for pc (Sommer *et al*., manuscript). How can this be explained? In cyanobacteria site-directed mutagenesis of pc or cyt c_6 from *Synechocystis sp.* PC 6803 and *Anabaena*

PCC 7119 and analysis of binding and electron transfer between the altered donors and the cyanobacterial PS1 revealed that a positively charged amino acid located at the "northern face" of either pc or cyt c_6 is crucial for the interaction with the reaction center (De la Cerda *et al*., 1999; Molina-Heredia *et al*., 1999; 2001). Interestingly, an equivalent positively charged amino acid is present in cyt c_6 from *C. reinhardtii* (Kerfeld *et al*., 1995) but absent in pc of *C. reinhardtii* and other eukaryotic pc structures (see Fig. 5). This implies that this positively charged amino acid is an important structural motif that promotes binding of cyt c_6 from *C. reinhardtii* to the core of PS1. The presence of this additional recognition site in cyt c_6, that is absent from pc, may therefore explain why complex formation of cyt c_6 to the mutant PsaB-W627F, PsaA-W655F and PsaA-W655S PS1 is less affected as compared to pc. The binding mechanism of cyt c_6 from *C. reinhardtii* to PS1 can be regarded as an evolutionary intermediate between cyt c_6 from *S. elongatus* and pc from *C. reinhardtii* (Hippler *et al*., 1999).

The rate of electron transfer within the intermolecular complex between cyt c_6 or pc and the mutated PS1 is about 5 to 10 times slower as compared to WT. This implies that the orientation and/or conformation of the donor-PS1 complex is slightly altered in comparison to WT, since the rate of electron transfer is highly sensitive to changes in distance between electron transfer partners (Marcus *et al*., 1985). The change of residues close to the special chlorophyll pair P700 could alter the P700 redox midpoint potential. A change in the P700 redox midpoint potential would in turn change the free energy of the electron transfer reaction. Measurements of the P700 redox midpoint potentials indicated only slight change compared to WT and could account for decrease of the half life of the intra complex electron transfer reaction by about 0.5 µs using an approximation for electron transfer from (Moser *et al*., 1995) and a reorganisation energy λ of 545 mV as estimated by (Ramesh *et al*., 2002). The data rather indicate that an alteration of the hydrophobic binding site induces a change in distance between the redox centres of the donors and P700 which is supposed to be in the range of 0.5 – 1 Å according to the estimations mentioned above. Still it can not be excluded that a disturbation of the π-electron system bridging the proposed electron transfer pathway of the donor acceptor pair may have an effect on the rate constant of electron donation by lowering the electronic coupling and donation probability.

In summary, it can be deduced that W627 of PsaB together with W651 of

PsaA, form the specific hydrophobic recognition site of the core of PS1 that is required for effective binding and electron transfer as well as stable complex formation between pc or cyt c_6 and PS1. It has also been shown that pc and cyt c_6 bind differently to the core of PS1, since both donors are affected in a distinct way concerning their binding affinities towards the two tryptophan amino acid residues as highlighted by the reverse genetic experiments.

3.2.3 Influence of altered electron exit/entry at PS1 on the electron transfer chain

Light energy is required for photosynthetic activity, however, excess light can damage the photosynthetic apparatus and its cellular environment. The process that causes a decrease of photosynthetic efficiency under excessive illumination is called photoinhibition. The primary target of photoinhibition is often considered to be PS2. However, PS1 is also a target of photoinhibition and is as sensitive as PS2 in isolated thylakoid membranes (Inoue *et al*., 1986; 1989). The light-induced inactivation of PS1 is probably caused by active oxygen species which are produced by reduction of O_2 to O_2^- from the terminal iron-sulfur clusters of PS1 itself (Inoue *et al*., 1989). To protect PS1 from oxidative damage and to scavenge active oxygen produced by PS1 a Cu, Zn-superoxide dismutase appears to be localized in close vicinity of PS1 (Ogawa *et al*., 1995). The scavenging of active oxygen species in intact chloroplasts involves a complex network of redox reactions which result in the photoreduction of O_2 to H_2O in a process called the water-water cycle (Asada, 2000). If the production of superoxide exceeds the scavenging capacity, O_2^- and H_2O_2 can diffuse to stromal protein targets and inactivate them. It has been shown that H_2O_2 can react with the photoreduced (4Fe-4S) clusters of PS1 to generate •OH radicals (Asada, 1999). The acceptor side of PS1 can also become a target of active oxygen species since the (4Fe-4S) clusters are extremely sensitive to oxidation (Jakob *et al*., 1996). Interestingly, the selective excitation of PS1 in isolated thylakoids in the presence of reductants appears not only to damage PS1 but also PS2, indicating that active oxygen species produced by PS1, found as superoxide and hydrogen peroxide, may diffuse to PS2 and cause damage (Tjius *et al*., 1998). In addition, when the acceptor side is fully reduced, recombination between the radical pairs $P700^+/A_0^-$ or $P700^+/A_1^-$ can generate the P700 triplet state which can react with oxygen leading to the formation of highly toxic singlet oxygen. It has

been speculated that the increased PS1 photoinhibition observed at low temperature is due to reduced carbon assimilation that would lead to the accumulation of electrons in the terminal acceptors of PS1 and to the effects described above. Taken together, it appears that PS1 is able to generate reactive oxygen species, that PS1 can be photoinhibited by such oxygen species and that scavenging mechanisms exist to protect PS1.

Acceptor side mutants where the affinity of PS1 to ferredoxin is either strongly decreased, as in the case of the PsaC-K35E, or strongly increased in the case of the FB_1 triple mutant PsaC-I12V/T15K/Q16R, are photosensitive as they are unable to grow on acetate-containing medium under high light intensities (Fischer *et al*., 1999; Naver *et al*., 1998; Sommer *et al*., 2003). The FB_1 triple mutant is the most affected strain *in vivo* and is unable to grow when illuminated with more than 80 $\mu E\ m^{-2}\ s^{-1}$ (Fischer *et al*., 1999; Sommer *et al*., 2003). To evaluate the impact of the acceptor side mutations on photosynthetic electron transfer, gross oxygen evolution and consumption of the PsaC-K35E and of the FB_1 triple mutant as well as of the PsaF-deficient mutant have been measured at increasing light intensities by using mass spectrometry (Sommer *et al*, 2003). The acceptor side mutations limit linear electron transfer *in vivo* even under saturating carbon dioxide conditions as they cause an about ten-fold increase of the compensation point for oxygen evolution and an about two-fold decrease of overall oxygen evolution (= evolution - uptake) as compared to WT. This is in sharp contrast to the PsaF-deficient mutant in which gross oxygen evolution and uptake follow saturation curves under high CO_2 conditions (Sommer *et al*, 2003). In the acceptor side mutants electron transfer between PS1 and fd is considerably diminished. While mutant PsaC-K35E shows a strong limitation in fd binding in the FB_1 mutant unbinding is extremely slow. However the fluorescence transients as well as PS2 recovery rates differ for both strains and suggest a greater electron flux through PS2 in strain PsaC-K35E which is in line with a faster PS2-recovery and a slightly higher oxygen evolution compared to the FB_1 strain. This seems to be in contrast with the extreme light sensitivity of PsaC-K35E compared to strain PsaB-E613N which shows similar values in the PS2-fluorescence measurements but is less affected in growth properties. Thus where do the electrons go? Looking at the generation of ROS by measurements of TBARS the limitation of fd binding in strain PsaC-K35E displays the biggest effect. It seems possible that the strong

photosensitive phenotype of the PsaC mutant is directly linked to PS1 photoinhibition and is due to electron donation from PS1 to oxygen, thus generating excess O_2^- which can no longer be neutralized by the scavenging enzymes and which may lead to the formation of •OH radicals (see also Fig. 10B). Alternatively, charge recombination events could lead to singlet oxygen formation if electron exit from PS1 is hindered which could be the case in strain FB_1 where the acceptor side is strongly occupied by fd leading to an electron jam. Therefore it is possible that under increasing light intensities which induce high electron flow into PS1, additional charge separations could fully reduce the iron-sulfur clusters, thus leading to recombination between the radical pairs $P700^+/A_0^-$ or $P700^+/A_1^-$ and to the generation of P700 triplet and singlet oxygen (see also Fig 10A). The role of oxygen in the photoinhibitory process is clearly revealed by the restored growth capacity of the PsaC mutants under anaerobic conditions.

The potential danger of electron escape from the photo-reduced iron-sulfur clusters of the acceptor side of PS1 to oxygen could be reflected in the reversed order of the redox potentials of the terminal electron acceptors in PsaC, which may serve to protect the cells from excessive damage. In the absence of ferredoxin, the electron would be localized preferentially on F_A, the proximal iron-sulfur cluster to F_X, and thus less exposed to the solvent than F_B. The observed light sensitivity under aerobic conditions of the PsaC-K52S/R53A mutant in which F_B is preferentially photoreduced could be due to higher rates of electron escape from F_B to oxygen and is consistent with the above hypothesis (Fischer *et al*., 1997). In this respect it is interesting to note that F_B is the preferentially reduced cluster in *C. limicola*, an organism which is strictly anaerobic.

The photosensitivity of nuclear and chloroplast donor side mutants from *C. reinhardtii* have been analyzed (Hippler *et al*., 2000; Sommer *et al*., 2002). The electron transfer between the soluble electron donors and PS1 is largely affected in the PsaF-deficient mutant and the PsaB mutants W627F and E613K, as a consequence mutant cells become light-sensitive under photoautotrophic conditions when the light intensity is higher than 400 $\mu E\ m^{-2}\ s^{-1}$. How can this photosensitivity been rationalized? It is established that *in vivo* the limiting step in linear photosynthetic electron transfer is the oxidation of PQH_2 at the Q_o site of the cyt b_6f complex (Stiehl *et al*., 1969).

Spectrophotometric measurements of intact cells of *Chlorella* showed that this transfer is accelerated to 1.2 ms under high light (Joliot *et al*., 1998). The electron transfer between the cyt b_6f complex and $P700^+$ in the PsaF-deficient strain was measured to be 1.2 ms, 20 times slower than in the WT (Farah *et al*., 1995). Thus under these conditions the electron transfer between pc and PS1 lacking PsaF may become limiting. However, Finazzi *et al*. (1999; 2001) proposed that under high-light conditions, State 2 conditions, no linear electron transfer but only cyclic photosynthetic electron transfer occurs in *C. reinhardtii*. This was concluded from their finding that although the cyt b_6f turnover was the same under State 1 and State 2, the turnover was completely inhibited by the addition of the PS2 inhibitor DCMU, whereas no effect of this inhibitor was observed in State 2. According to these results it is possible that a slow down of the electron transfer to the oxidizing site of PS1 due to a mutation becomes indeed limiting for cyclic photosynthetic electron transfer under these high light conditions, which would lead to an over-reduction of the electron transfer components and overexcitation of the antennae resulting in the production of reactive oxygen species.

The observation that the PsaF-deficient strain survives under high light conditions when oxygen is removed indicates that the formation of reactive oxygen species under high light in aerobic conditions is most likely responsible for the cell damage. This view is further compatible with the fact that the stability of PS1 isolated from WT and the PsaF-deficient strain is comparable in high light (Hippler *et al*., 2000). It should be of note that *in vitro* measured electron transfer between PsaB mutant E613K and pc or cyt c_6 is as slow as measured for the same reaction using the PsaF-deficient PS1 particles (Sommer *et al*., 2002). In addition the PsaB mutant strain becomes also sensitive to higher light intensities (Sommer *et al*., 2002). This substantiates the view that a less efficient electron transfer between the electron donors and PS1 leads to photo-oxidative damage of the cells.

What is the source of reactive oxygen species produced in the donor side mutants under high light? Mass spectroscopy measurements of gross oxygen evolution under high light ($>$1800 $\mu Em^{-2}s^{-1}$) showed that oxygen evolution declines in the PsaF-deficient 3bF strain, but not in the WT (Hippler *et al*., 2000). The decrease of photosynthetic electron transfer activity in 3bF in high light could be due to damage of the photosynthetic machinery under

these conditions. However under saturating concentrations of carbon dioxide, the gross oxygen evolution remains stable in 3bF when stressed with high light (Sommer *et al*., 2003). These results suggest that when the PS1 electron sink is saturated, charge recombination between the acceptor side of PS1 and $P700^+$ may occur and lead to the formation of the P700 triplet state which can react with O_2 to form the highly toxic singlet oxygen. This recombination reaction would be favored in the donor side mutants since the re-reduction of $P700^+$ is much slower in 3bF or the PsaB mutants than in the wild type (see also Fig 10C). In this context it is of note that PsaB mutant E613N becomes photosensitive in very high light (> 700 $\mu Em^{-2}s^{-1}$, (Sommer *et al*., 2002)). In this mutant the electron transfer between the donors and the mutant PS1 is faster and the dissociation constants are lower as compared to WT. These properties result in a tighter binding of the donors, but as a consequence leads also to a 3 times slower unbinding of pc and cyt c_6 from PS1. The rate of unbinding of pc from PS1 has been suggested to be the rate-limiting step in the electron transfer between the cyt b_6f complex and PS1 (Drepper *et al*., 1996). Thus the slower unbinding of both donors from PS1 would result in a slow-down of the *in vivo* electron transfer between the cyt b_6f complex and PS1, which apparently is sufficient to induce photo-oxidative stress under high light (see also Fig. 10D). Substantiated is this view from the findings that in this strain cyt b_6f turnover is slowed down by the factor of about 1.5 (Finazzi and Hippler, personal communication).

In summary it is obvious that limitations at the donor and at the acceptor side of PS1 have deleterious effects for the respective strains at least under high light intensities. Different limitation sites may have different mechanisms but all leading to the generation of ROS damaging PS1 and have also effects on PS2. Thus electron transfer reactions involving transient complex formation as is the case for $P700^+$ reduction by pc or cyt c_6 as well as for ferredoxin reduction by F_B at the stromal side of PS1 need to be well balanced. Limitation in binding of the reaction partners (PsaC-K35E, 3bF, PsaB-E613K) as well as for unbinding (FB_1 or PsaB-E613N) lead to harmful side products, which are most likely reactive oxygen species.

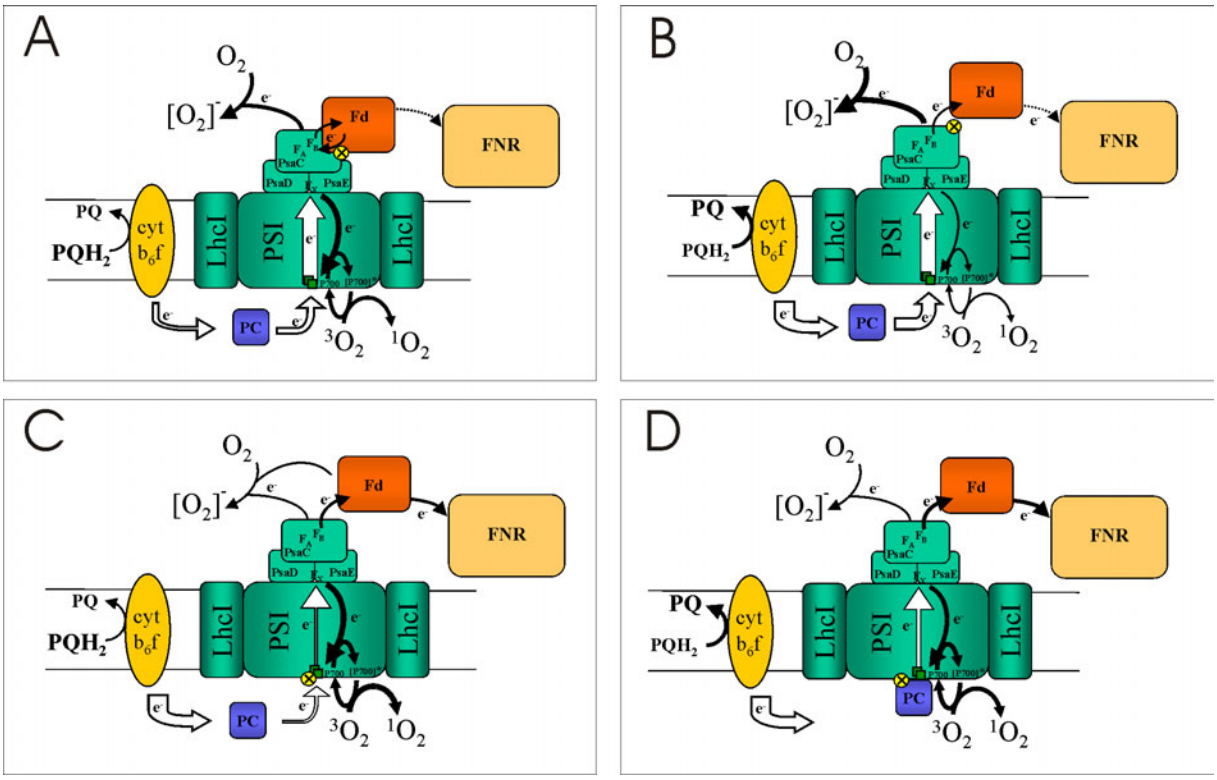

Figure 10: Proposed correlation between limitation of electron transfer through PS1, formation of ROS ($^1O_2$ upon spin recombination with exited ^{3}[P700] generated from charge recombination within PSI or O_2^- by reduction of O_2 as an alternative electron acceptor) and light sensitivity. Strong arrows represent preferred electron pathways, the crossed circles indicate the site of mutations. Differential preference for the different ROS generating pathways is proposed in the different mutants. Electron transfer properties of PS1 from the different mutant strains 3bF, PsaB,-E613K, PsaB-E613N, PsaB-W627F, PsaC-K35E and PsaC-FB_1 mutant strains was reported (Hippler *et al.*, 1997; Fischer *et al.*, 1998, 1999; Sommer *et al.*, 2002). The PsaC mutants are affected on the stromal side of the complex (panels A and B). The PsaC-K35E mutation (see panel B) diminishes the electrostatic interaction between fd and PSI, abolishing fd-PSI complex formation and reducing the electron transfer rate from PSI to fd 70 to 90-fold (Fischer *et al.*, 1998). The FB_1 triple mutation (see panel A) has the opposite effect as fd has a very high affinity for this complex. In this case, although the k_{off} rate of fd could not be measured, it seems likely that release of fd from PS1 is limiting electron flow (Fischer *et al.*, 1999). Binding and electron transfer between pc and PS1 are affected in the donor side mutants (panels C and D). Loss or disorientation of the PsaF subunit in PS1 in the 3bF resp. PsaB-E613K mutants prevents the formation of a pc-PS1 complex on the lumenal side (see panel C) and leads to a 80-fold reduction of the electron transfer rate from pc to PS1 (Hippler *et al.*, 1997; Sommer *et al*., 2002). The PsaB mutation E613N leads to a stronger binding of pc to PS1, which results in a 3-fold slower off-rate of the oxidized pc from the PS1 complex (Sommer *et al.*, 2002). Thus, it appears that in the 3bF, PsaB-K35E and PsaB-E613N mutants, electron input into PS1 is slowed down, whereas electron exit is diminished in the PsaC mutants.

4 Summary

In order to shed more light on the interaction site of the electron donors pc and cyt c_6 to the core subunits PsaA and PsaB of PS1, the chloroplast encoded *psaA* and *psaB* genes were modified by site directed mutagenesis and transformed into *C. reinhardtii* strains lacking the respective genes. The generated strains are listed in table 1.

In comparision to WT growth properties and PS1 content of the generated mutant strains together with strains that were altered in PsaF and PsaC were tested (table 1). Most of the mutant strains displayed an increased photosensitivity under aerobic but not under anerobic conditions, indicating that oxidative stress is one of the main causes for the observed light sensitivity. In addition the phenotype can be ascribed in some strains by a strongly (> 50 %) decreased PS1 content. Such a decrease in PS1 content is probably due to a severe destabilisation of PS1 upon the introduced changes in the respective PS1 subunits.

Table 1:
Cells from liquid cultures were spotted on plates containing TAP or HSM medium for heterotrophic or photoautotrophic growth respectively and placed under different atmospheric and light conditions (n.d. not determined).

Strain	TAP 60µE/m²s	HSM 60µE/m²s	TAP 700µE/m²s	HSM 700µE/m²s	TAP 700µE/m²s	HSM 700µE/m²s	PS1 Content
	AEROB				ANAEROB		
WT	++	++	++	++	++	++	100%
3bF	+	+	-	-	++	++	>50%
PsaB-E613N	++	++	+	-	++	++	>50%
PsaB-E613K	++	+	-	-	n.d.	n.d.	>50%
PsaB-W627F	++	+	-	-	-	-	>50%
PsaA-W651F	++	++	+	-	++	++	>70%
PsaA-W651S	++	++	+	-	++	++	>70%
PsaB-E613K/W627F	+	-	-	+	n.d.	n.d.	>20%
PsaB-D624K	+	-	-	+	n.d.	n.d.	<20%
PsaB-E613K/D624K	+	-	-	+	n.d.	n.d.	<10%
PsaB-D624K/W627F	+	-	-	+	n.d.	n.d.	<10%
PsaC-K35E	++	-	-	-	++	++	>50%
PsaC FB_1	++	-	-	-	+	+	>50%

Mutant strains that showed a PS1 content of > 50% as compared to WT were further analysed and the properties of electron transfer and interaction between PS1 and the electron carriers pc, cyt c_6 and fd were investigated *in vitro*, using flash absorption spectroscopy, $NADP^+$ photoreduction measurements and chemical cross linking experiments. For the strains that employed an altered kinetic of intra complex electron transfer the $P700/P700^+$ midpoint potential was determined as well. Obtained data are listed in table 2.

Table 2:
$P700/P700^+$ midpoint potentials and electron transfer properties from pc or cyt c_6 to PS1 isolated from WT, 3bF and different strains mutated either on PsaA or PsaB.

	Plastocyanin		**Cytochrome c_6**		**Midpoint-potential**
	k_2 [$s^{-1}*10^6$]	K_D [µM]; (*f*)	k_2 [$s^{-1}*10^6$]	K_D [µM]; (*f*)	E_m [mV]
WT	90	83 (f=0.67)	34	81 (f=0.68)	473.1
3bF	1.3	-	2.5	-	-
PsaB-E613N	110	29 (f=0.71)	8.2	7.7	-
PsaB-E613K	2.2	-	3.4	-	-
PsaB-W627F	6.4	-	16	177 (f=0.66)	463.9
PsaA-W651F	19	-	5.5	510 - 1087 (f=0.38-0.68)	463.9
PsaA-W651S	31	254 - 554 (f=0.38-0.67)	2.1	1310 - 2560 (f=0.38-0.68)	477

From the determined data, the following conclusions were derived:

- The lumenal helix *I* of PsaB together with helix *I'* of PsaA is important for modulating electron transfer between pc or cyt c_6 and PSI of *C. reinhardtii*.

- E613 of PsaB has two functions (i) to facilitate the fast release of pc and cyt c_6 from PS1 after electron transfer and (ii) to orientate the N-terminal domain of PsaF so that it can form the specific recognition site for docking of both donors to PS1.

- W627 of PsaB is essential for interaction and efficient electron transfer between pc and PS1 and is a key residue of the hydrophobic pc recognition site formed by the core of PS1. The data indicate that this recognition site

is required for binding of pc to PS1 but is less essential for binding of cyt c_6.

- Changes of residue W651 in PsaA have a strong effect on electron transfer between the electron donors and PS1 indicating involvement of this residue in forming the hydrophobic recognition site of the PS1 core. The effects are more pronounced for cyt c_6 than for pc indicating a differential selectivity of the both donors toward the tryptophan dimer PsaB-W627/PsaA-W651.

- The change of the intermolecular electron transfer rate in the complex between cyt c_6 or pc and the altered PsaB-W627 or PsaA-W651 PS1 by a factor of about 10 suggests that change of residues W627 and W651 lead to conformational changes within the inter-molecular electron transfer complex in the range of 0.5 – 1 Å thus indicating that the proper binding to these residues is essential for the correct positioning within this bimolecular complex.

For strains that are mutated either at the donor or acceptor side of PS1, excess light leads to a decrease of PS1 and PS2 protein subunits whereas ATPase and cyt b_6f complexes remained constant. These data suggest that an impaired electron transfer to or from PS1 has also a strong impact on PS2. Fluorescence induction measurements and PS2 recovery rates suggest in the same line limitation of electron entry to PS1 as well electron exit from PS1 result in PS2 inactivation.

Oxygen evolution experiments indicate that *in vivo* overall photosynthetic electron transfer can be limited by an impairment of the acceptor side whereas mutation of the donor side limited $NADP^+$ photoreduction *in vitro.*

4.1 Zusammenfassung

Zur Untersuchung der Wechselwirkungen der Elektronendonoren pc und cyt c_6 mit den Kernuntereinheiten PsaA und PsaB von PS1 wurden die im Chloroplasten codierten Gene *psaA* und *psaB* mittels gerichteter Mutagenese verändert und in Zellinien von *C. reinhardtii* eingebracht, denen die entsprechenden Gene fehlten. Die so erzeugten Zellinien sind in Tabelle 1 aufgelistet.

Die genannten Zellinien wurden gemeinsam mit Zellinien welche hinsichtlich der Proteine PsaF oder PsaC verändert worden waren auf Wachstumseigenschaften und PS1 Gehalt relativ zu WT untersucht Tabelle 1. Die meisten der veränderten Zellinien zeigten eine erhöhte Lichtsensitivität unter aeroben Bedingungen nicht jedoch unter anaeroben, was darauf hinweist, daß oxidativer Streß eine der Hauptursachen für die erhöhte Lichtsensitivität ist. Zusätzlich kann ein stark (> 50 %) verminderter PS1 Gehalt den Phänotyp erklären. In diesen Zellinien könnte eine starke Destabilisierung des PS1 durch Austausch strukturell wichtiger Aminosäuren vorliegen.

Tabelle 1:
Zellen wurden auf TAP- oder HSM-Platten für heterotrophes- bzw. photoautotrophes Wachstum gezogen und verschiedenen atmospherischen Bedingungen und Lichtintensitäten ausgesetzt.

Zellinie	TAP 60µE/m²s	HSM 60µE/m²s	TAP 700µE/m²s	HSM 700µE/m²s	TAP 700µE/m²s	HSM 700µE/m²s	PS1 Gehalt
	AEROB				ANAEROB		
WT	++	++	++	++	++	++	100%
3bF	+	+	-	-	++	++	>50%
PsaB-E613N	++	++	+	-	++	++	>50%
PsaB-E613K	++	+	-	-	n.d.	n.d.	>50%
PsaB-W627F	++	+	-	-	-	-	>50%
PsaA-W651F	++	++	+	-	++	++	>70%
PsaA-W651S	++	++	+	-	++	++	>70%
PsaB-E613K/W627F	+	-	-	+	n.d.	n.d.	>20%
PsaB-D624K	+	-	-	+	n.d.	n.d.	<20%
PsaB-E613K/D624K	+	-	-	+	n.d.	n.d.	<10%
PsaB-D624K/W627F	+	-	-	+	n.d.	n.d.	<10%
PsaC-K35E	++	-	-	-	++	++	>50%
PsaC FB_1	++	-	-	-	+	+	>50%

Veränderte Zellinien die > 50 % PS1 verglichen mit WT aufwiesen wurden weitergehend untersucht. Elektronentransfereigenschaften und die Wechselwirkungen zwischen PS1 und den Elektronenüberträgern pc, cyt c_6 und fd wurden *in vitro* mit Hilfe von zeitaufgelöster Laserblitz-Spektralphotometrie, $NADP^+$-Photoreduktion und chemischem Vernetzen analysiert. Für die Zellinien, welche eine veränderte Zeitabhängigkeit der Elektronenübertragung im bimolekularen Komplex aufwiesen wurden auch die elektrochemischen $P700/P700^+$ Mittelpunktspotentiale bestimmt. Die gefundenen Daten wurden in Tabelle 2 zusammengefaßt.

Tabelle 2:
$P700/P700^+$ Mittelpunktspotentiale und ermittelte Konstanten der Elektronenübertragung von pc oder cyt c_6 auf PS1 aus WT, 3bF und Zelllinien mit verändertem PsaA oder PsaB.

	Plastocyanin		Cytochrom c_6		Mittelpunkts-potential
	k_2 [$s^{-1}*10^6$]	K_D [µM]; (*f*)	k_2 [$s^{-1}*10^6$]	K_D [µM]; (*f*)	E_m [mV]
WT	90	83 (f=0.67)	34	81 (f=0.68)	473.1
3bF	1.3	-	2.5	-	-
PsaB-E613N	110	29 (f=0.71)	8.2	7.7	-
PsaB-E613K	2.2	-	3.4	-	-
PsaB-W627F	6.4	-	16	177 (f=0.66)	463.9
PsaA-W651F	19	-	5.5	510 - 1087 (f=0.38-0.68)	463.9
PsaA-W651S	31	254 - 554 (f=0.38-0.67)	2.1	1310 - 2560 (f=0.38-0.68)	477

Aus diesen Daten können folgende Schlüsse abgeleitet werden:

- Die zum Lumen weisenden Helices *l* von PsaB und *l'* von PsaA sind wichtig für die Regulierung der Elektronenübertragung von pc oder cyt c_6 auf das PS1 aus *C. reinhardtii*.
- Aminosäure PsaB-E613 erfüllt zwei Funktionen: (i) Sie ermöglicht pc und cyt c_6 ein schnelles Abbinden vom PS1 nach der Elektronenübertragung; (ii) Sie richtet die basische N-terminale Domäne von PsaF so aus, daß eine spezifische Erkennungsstelle für die Bindung der beiden Elektronendonoren entsteht.

- Aminosäure PsaB-W627 ist essentiell für die Bindung und die Elektronenübertragung zwischen pc und PS1. Sie bildet einen Teil der hydrophoben Erkennungsstelle für pc am Kern von PS1. Die Daten lassen auch den Schluß zu, daß diese hydrophobe Erkennungsstelle für die Bindung von pc notwendig ist, jedoch weniger wichtig für die Bindung von cyt c_6.
- Auch die Änderung der Aminosäure PsaA-W615 zeigte einen starken Einfluß auf die Elektronenübertragung von pc oder cyt c_6 auf PS1, was darauf hinweist, daß sie Bestandteil der hydrophoben Erkennungsstelle für die Elektronendonoren am Kern von PS1 ist. Hier waren die beobachteten Effekte für cyt c_6 stärker als für pc was darauf hinweist, daß die beiden Elektronendonoren eine unterschiedliche Selektivität für die Tryptophane des Dimers PsaA-W651/PsaB-W627 besitzen.
- Eine leichten Konformationsänderung (mit einer Abstandsänderung von etwa 0.5 – 1 Å) im bimolekularen Komplex zwischen cyt c_6 oder pc und den veränderten PS1 (PsaA-W651 und PsaB-W627) kann die Erniedrigung der Elektronenübertragungsrate um den Faktor 10 erklären. Eine exakte Bindung der Elektronendonoren scheint daher sehr wichtig für die genaue Ausrichtung des Komplexes.

Übermäßige Anregungsenergie durch Licht führte bei den Zellinien, die and der Akzeptor- oder Donorseite von PS1 mutiert waren, zu einer Abnahme von PS1 und PS2 wohingegen die Menge an ATPase und cyt b_6f Komplex konstant blieb. Diese Ergebnisse und Messungen der Fluoreszenzinduktion sowie der PS2 Fluoreszenz nach starker Bestrahlung zeigen, daß eine verlangsamte Elektronenübertragung auf PS1 und von PS1 weiter auch PS2 hemmen kann.

Messungen der Sauerstoffentwicklung *in vivo* zeigen, daß Änderungen an der Akzeptorseite von PS1 limitierend auf den gesamten photosynthetischen Elektronenfluß wirken können. Messungen der $NADP^+$-Photoreduktionzeigten, daß Änderungen an der Donorseite *in vitro* eine Verlangsamung des Elektronenflusses bewirken.

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2003 Gordon conference on photosynthesis, 22nd – 27th June, Bristol, USA.
Efficient comlex formation and electron transfer between and photosystem 1 is mediated by the lumenal loop *j* of PsaB.
(Poster price and young investigators talk)

2002 Botanikertagung 22nd – 27th September Freiburg, Germany.
The lumenal helix *l* of PsaB is essential for recognition of plastocyanin or cytochrome c_6 and fast electron transfer to photosystem 1 in *Chlamydomonas reinhardtii*.
(Poster)

2001 12th International Congress on Photosynthesis, 18th - 25th August, Brisbane, Australia.
The lumenal α-helical loop *j* of photosystem I (PSI) reaction center subunit PsaB modulates electron transfer between PSI and plastocyanin or cytochome c_6 and is important for proper orientation of PsaF.
(Poster)

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The lumenal helix *I* of PsaB is essential for recognition of plastocyanin or cytochrome c_6 and fast electron transfer to photosystem 1 in *Chlamydomonas reinhardtii*.
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Ehrenwörtliche Erklärung

Hiermit erkläre ich, daß ich die vorliegende Arbeit selbstständig und nur unter der Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe. Es wurde weder die Hilfe eines Promotionsberaters noch die Hilfe Dritter, nicht in dieser Arbeit erwähnter Personen, in Anspruch genommen. Die Arbeit wurde weder in dieser noch in ähnlicher Form bei einer anderen Hochschule als Dissertation oder Prüfungsarbeit eingereicht.

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