An Application of Thermoluminescence to Herbicide Studies

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Thylakoids of genetically engineered species of *Synechococcus* PCC 7942 were investigated by thermoluminescence (TL), polarographic and herbicide-binding studies. The results were as follows: 1) Deletion of either copy I or copy II/III of the *psb*A gene family resulted in modified oscillation patterns of flash induced TL and O_2 evolution compared to wild type cells. 2) Replacement of Ser_{264} in *psb*A I by Ala or Gly inducing strong herbicide resistance leads to a downshift of the TL peak temperature of B-band by 10 °C and alters the oscillation pattern of flash induced TL and O_2 evolution significantly to exhibit a pattern with comparatively high O_2 yield after the 2nd flash. Additional deletion of *psb*A II/III does not lead to significant changes compared to the single mutated strains. 3) Contrary to Ser_{264} mutants, replacement of Phe_{255} by Tyr in *psb*A I alone, which also induces herbicide resistance, is not accompanied by corresponding changes in TL peak temperature and/or oscillation pattern of flash-induced TL and O_2 evolution. However, strongly altered properties are observed after additional deletion of *psb*A II/III. Based on these data it is inferred that expression of either *psb*A I or *psb*A II/III genes only gives rise to PS II complexes slightly different in their function. Site-directed mutagenesis of *psb*A I gene modulates the expression ratio between copies I and II/III. The possible implications are discussed.

Introduction

The key steps of photosynthetic water cleavage take place in a membrane-bound protein complex, referred to as photosystem II (PS II), containing several proteins of molecular masses between 5 kDa and 47 kDa (for a recent review see ref. [1]). After light induced formation of the primary radical pair $P680^+Pheo^-$, the charge separation is stabilized by electron transfer from $Pheo^-$ to a specifically bound plastoquinone molecule Q_A. Q_A is the reductant for plastoquinol formation at a special domain, the Q_B binding site, which is located between transmembranal helices 4 and 5 of the intrinsic 32 kDa polypeptide D 1 [2]. The Q_B binding site not only binds the endogenous plastoquinone but also a number of different chemicals.

Information about the mechanism of this non-covalent interaction with the acceptor side on PS II is of great relevance for pesticide science since many commercially used herbicides of triazine and diuron type act by replacing Q_B at its binding site thereby blocking the electron transfer from Q_A to the plastoquinone pool PQ ([3, 4] for a review see [5]). Much effort has been put forward to clarify the functional and structural properties of the interactions of these herbicides with protein D 1 and to unravel the mechanism of herbicide resistance in many plant mutants. Recently, the development of procedures for site directed mutagenesis enabled the engineering of herbicide resistant mutants of many species [6, 7]. Various single and double point mutations have been induced in *Synechococcus* PCC 7942 using chemical or site-specific mutagenesis [7, 8]. Additivity of the effects of point mutations at positions 255 and 264 of D 1 on herbicide binding was demonstrated [9, 10].

Different techniques are available to study the effects of single or double amino acid exchange in polypeptide D 1 within the region of the Q_B-site. Among them thermoluminescence (TL) has been demonstrated to be a promising tool. TL is known to originate from recombination of positive and negative charges stored on the donor and acceptor

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; atrazine, 2-(ethylamino)-4-chloro-6-(isopropylamino)-*s*-triazine; D 1, 32 kDa (Q_B or herbicide binding) protein; PS II, photosystem II; TL, thermoluminescence; Q_A, special bound plastoquinone, first stable acceptor of photosystem II; Q_B, plastoquinone bound to the Q_B binding site; PQ, plastoquinone pool on the acceptor side of PS II; K-Pi, K_2HPO_4.

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side of PS II after illumination. The Q- and B-bands are assigned to the recombination of $S_2Q_A^-$ and $S_2/S_3Q_B^-$, respectively [11, 12]. In dark adapted samples illuminated by a flash train, the oscillatory pattern of the Q- and B-bands gives insight into the reaction properties of Q_A and Q_B as well as of the redox states S_i of the catalytic site of water oxidation [13]. Furthermore, as peak temperature and shape of the glow curves depend on the activation free energy of the recombination reaction, these parameters provide a measure of the redox potential difference between the involved redox couples [14, 15]. Altered properties of the B-band in herbicide-resistant strains compared to susceptible wild types have been clearly demonstrated [16].

In order to get more information about the structural and functional importance of the amino acid residues Phe_{255} and Ser_{264} of D1 for Q_B and herbicide binding to PS II, we examined various mutants of *Synechococcus* PCC 7942 by means of TL, polarographic detection of oxygen evolution and herbicide binding studies.

Materials and Methods

Synechococcus PCC 7942 mutants were raised by the method previously described in [6, 7]. Mutants KΔ1 and S_2C_3 (ref. [17]) were kindly given to us by Dr. R. Haselkorn, University of Chicago.

Cyanobacterial cells were grown in BG 11 medium at 30 °C (ref. [18]). After one wash with 50 mM K-Pi buffer (pH 6.8), cells (0.25 mg Chl/ml) were treated with lysozyme (1.5 mg/ml) in 50 mM K-Pi (pH 6.8)/0.4 M mannitol/2 mM EDTA at 35 °C for 2 h. After one wash with the same buffer, the cells were disrupted by dilution with a large amount of 20 mM Hepes (pH 7.0)/10 mM $MgCl_2$, followed by addition of an equal volume of 60 mM Hepes (pH 7.0)/2 M sucrose/10 mM $MgCl_2$ and 3 drops of DNAse (Sigma). Thylakoids were then pelleted by centrifugation and resuspended in 50 mM Hepes (pH 7.0)/1 M sucrose/10 mM $CaCl_2$ (3 to 4 mg Chl/ml) after two washes with the same buffer. Thylakoids were stored in liquid N_2 until measurement.

Flash induced oxygen evolution was measured with a Joliot-type oxygen electrode [19]. Dark-adapted (5 min) thylakoid suspension (1 mg Chl/ml) was applied on the platinum electrode and illuminated at room temperature with saturating Xenon flashes (4 μs, 2 J) at 2 Hz.

Thermoluminescence was measured with a previously described setup [20] using a heating rate of 1.0 °C/s. In order to ensure a reproducible distribution of Q_B^-/Q_B and S_0/S_1 for all measurements, samples were preilluminated with strong continuous white light for 1 min and relaxed in darkness at room temperature for 10 min before further treatment.

[^{14}C]-Atrazine binding was measured according to Tischer and Strotmann [21] with a dark equilibration time of 5 min in darkness followed by centrifugation.

Results and Discussion

Synechococcus PCC 7942 contains three *psb*A genes [8], that code for two forms of D1 proteins. D1 form I encoded by *psb*A I differs in 25 out of 360 amino acids from form II which is encoded by *psb*A II/III. It has been shown that any one of the three *psb*A genes can sustain photosynthetic growth [8]. Although light influences the ratio of the two forms of D1 (ref. [22]), it was found that in wild type cells under various light intensities *psb*A I is preferentially expressed [8, 22]. In order to analyze the effects of gene expression pattern on photosynthetic electron transport, we comparatively investigated the wild type and two mutants S_2C_3 and KΔ1 in which *psb*A I and *psb*A II/III, respectively, are deleted [17].

Fig. 1 shows the TL glow curves and their flash induced oscillation patterns for all three types of cells. They exhibit a strong B-band at +40 °C which oscillates with period four over several cycles. In contrast to this similarity there are significant differences regarding the TL pattern. The peak ratio T2/T1 of TL emission in samples illuminated with two or one flashes, respectively, is 2.0 for wild type thylakoids. This value fits nicely with the often observed fact that the quantum yield of TL due to $S_3Q_B^-$ recombination is about double of that due to $S_2Q_B^-$ recombination [11]. In the two mutants, on the other hand, the TL intensity on Chl basis is significantly higher, and the ratio T2/T1 is altered: 2.5 for S_2C_3 and 1.4 for KΔ1. There are two possible explanations: (a) a high miss and/or double hit factor which desynchronizes the S-state cycle after 1 or 2 flashes already or (b) a change of the quantum yield of TL

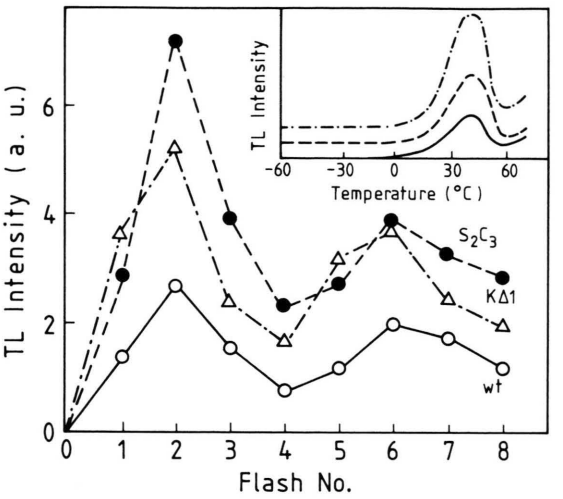

Fig. 1. Flash oscillation patterns and glow curves after one flash (insert) of (—○—) wild type and mutants (·—·△—·—) KΔ1 and (– –●– –) S_2C_3.

due to $S_2Q_B^-$ and $S_3Q_B^-$ recombination and/or of the initial ratio of Q_B^-/Q_B.

To distinguish between these alternatives we measured the O_2 yield pattern (Fig. 2). All three thylakoid preparations show clear maxima on the 3rd and 7th, and minima on the 5th and 9/10th flashes. These oscillation patterns do not favor drastic differences of the double hit and/or miss

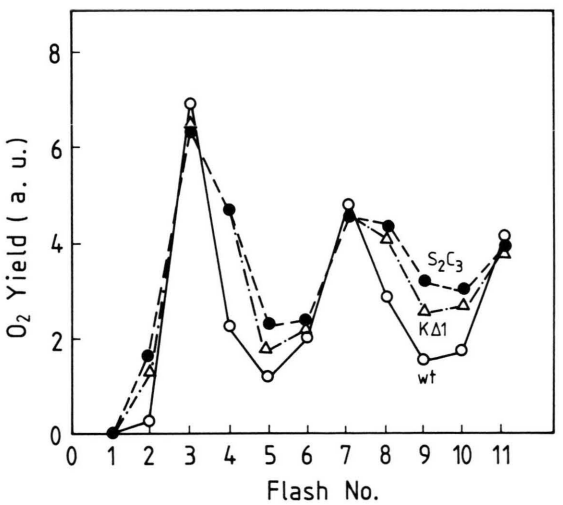

Fig. 2. Flash oscillation patterns of O_2 evolution of (○) wild type and mutants (△) KΔ1 and (●) S_2C_3.

probabilities between wild type, S_2C_3 and KΔ1. The higher TL emission in mutants leads to the assumption that the quantum yield of TL in strains S_2C_3 and KΔ1 differs from those of wild type thylakoids. This might be explained by structural differences of the gene products of the three copies of *psb*A in *Synechococcus* PCC 7942 in the neighborhood of the reaction center, affecting the yield of P680* singlet formation by charge recombination or the efficiency of non radiative decay processes of P680*. The variation in T2/T1 ratio in mutants may also be explained by different Q_B^-/Q_B ratios in the initial dark adapted state, since a lower Q_B^- content will be expected to give a higher T1 with lower T2. This may also be attributable to structural differences, affecting the decay of Q_B^- during dark relaxation. The differences between wild type and S_2C_3/KΔ1 thylakoids might be explained by the assumption that the assembly of wild type PS II is influenced by the expression of *psb*A II/III genes.

Analogous arguments as for the T2/T1 ratio are pertinent to understand the high O_2 yield of S_2C_3 and KΔ1 after the 2nd flash (Y2). This phenomenon can be explained by the assumption of a significant initial S_2 population. The existence of a very slowly decaying S_2 state has previously been reported for triazine resistant *Brassica napus* leaves [23]. As will be mentioned later, this phenomenon can be observed in all our investigated mutants of *Synechococcus* PCC 7942.

Demeter *et al.* [16] reported altered properties of the B-band of herbicide resistant strains (*i.e.* downshift of 15 °C in *Erigeron canadensis* at pH 7.5). This finding and other observations lead to the conclusion that in many herbicide resistant strains of plants and cyanobacteria the equilibrium between $Q_A^-Q_B$ and $Q_AQ_B^-$ is shifted towards $Q_A^-Q_B$ and consequently this high population of closed reaction centers causes a high miss factor for stabilizing the primary charge separation in PS II. Fig. 3 shows the glow curves of three different herbicide resistant D1-mutants of *Synechococcus* PCC 7942. Regardless of the varying TL quantum yield Di1 and G264 exhibit a marked downshift of the B-band by 10 °C to +30 °C, whereas the B-band of Tyr5 is still peaking at about +40 °C. The flash induced oscillation patterns of TL and O_2 evolution are strongly damped for Di1 and G264, whereas Tyr5 exhibits a wild type like

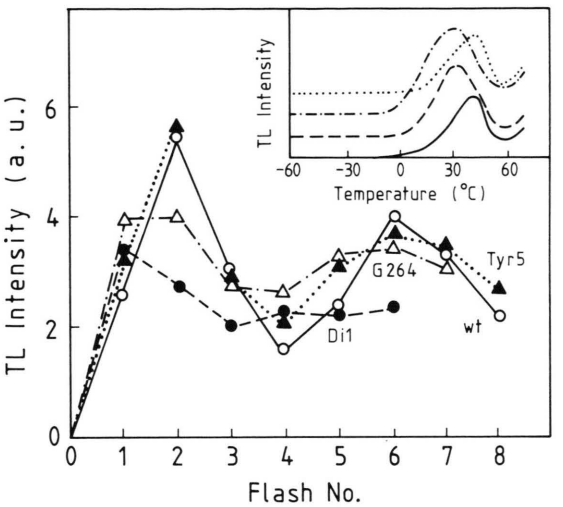

Fig. 3. Flash oscillation patterns and glow curves after one flash (insert) of (—○—) wild type and mutants (– –●– –) Di1, (·–·△·–·) G264 and (···▲···) Tyr5.

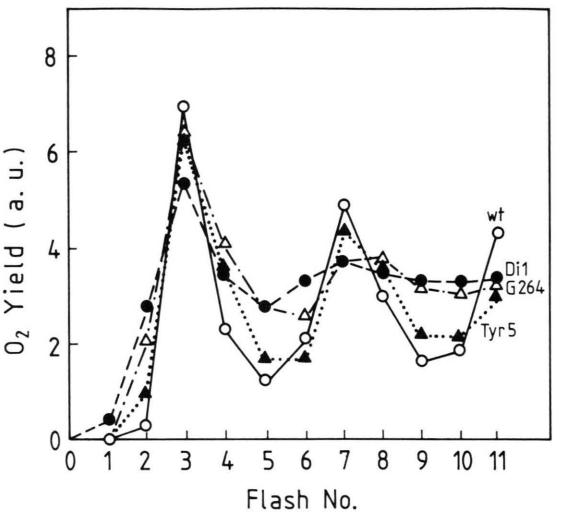

Fig. 4. Flash oscillation patterns of O_2 evolution of (○) wild type and mutants (●) Di1, (△) G264 and (▲) Tyr5.

behavior. The high damping in mutants Di1 and G264 is in agreement with the downshifted B-band according to [16]. From a downshift of the B-band of about 15 °C, Demeter *et al.* [16] calculated a shift of the equilibrium constant K for $Q_AQ_B^-/Q_A^-Q_B$ from 16 to 3.5. Our data confirm these findings and demonstrate the structural importance of the amino acid residue Ser_{264} for the functional integrity of the Q_B-binding pocket. They also indicate that in Di1 and G264, PS II contains preferentially the gene product of mutated *psb*A I.

Although mutant Tyr5 is susceptible to diuron, this strain has been reported to be resistant against various herbicides [9]. However, our data depicted in Fig. 3 and 4 do not show drastic differences in TL band shape (except for a small shoulder of the B-band at lower temperature) and in the flash induced oscillation pattern of TL and O_2 evolution compared to the wild type. These findings might be explained by the assumption that the change from Phe_{255} to Tyr has no influence on Q_B-binding and the electron transport through PS II, but hinders the binding of specific herbicides to the Q_B-binding site. As an alternative model one has to consider the possibility that the use of *psb*A I gene product for PS II assembly in this mutant might be blocked and only the D1 proteins encoded by the unmutated *psb*A II/III genes can be incorporated.

To address this problem we also investigated two mutants in which *psb*A II/III were deleted by insertion of genes coding for spectinomycin and chloramphenicol resistance. These mutants were denoted G264S_2C_3 and Tyr5S_2C_3 with additional single site mutations in *psb*A I accordingly. As can be seen in Fig. 5 and 6, no significant differences

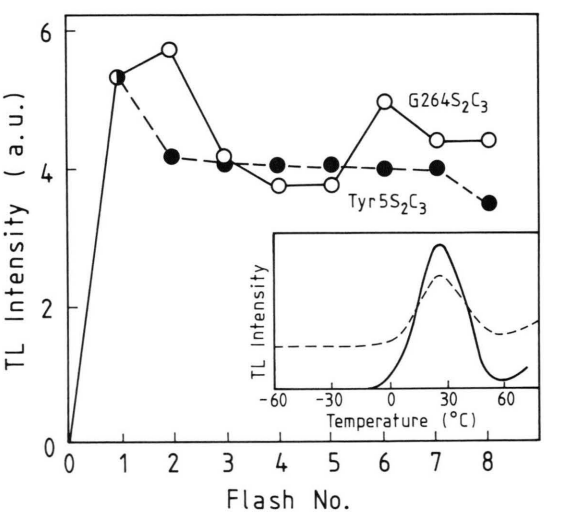

Fig. 5. Normalized flash oscillation patterns and glow curves after one flash (insert) of mutants (—○—) G264S_2C_3 and (– –●– –) Tyr5S_2C_3.

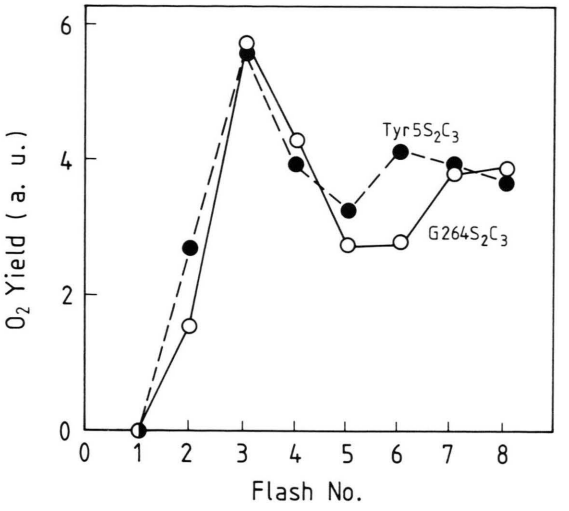

Fig. 6. Flash oscillation patterns of O_2 evolution of mutants (○) G 264 S_2C_3 and (●) Tyr 5 S_2C_3.

between G 264 and G 264 S_2C_3 could be observed. Beside the altered TL quantum yield, the band shape and peak temperature of TL as well as the oscillation patterns of O_2 evolution and TL are similar. Surprisingly, Tyr 5 S_2C_3 shows a down-shifted B-band and strong damping of both flash patterns. Obviously the *psb*A II and *psb*A III gene products are preferred in Tyr 5 for the assembly of PS II, which therefore displays a wild type like behavior. The shoulder of the B-band of Tyr 5 may be due to some contamination from PS II complexes formed with the mutated *psb*A I gene product.

In strains KΔ1 and S_2C_3 as well as in all herbicide resistant strains we observed a very high O_2 yield after the 2nd flash, which can be explained by a relatively high initial S_2 population as discussed earlier [23]. This phenomenon can be attributed to (a) long range effects of acceptor side modifications on the donor side of D 1, (b) differences in amino acid residues on the lumen exposed part of D 1 between proteins coded by copy I or II/III of *psb*A, (c) structural changes of PS II induced by an assembly with a heterogeneous mixture of gene products of all *psb*A copies. Although the first explanation seems to be less likely in the light of latest findings about the absence of allosteric effects of the Q_B site on the $P680^+$ reduction rate [24], we cannot totally rule out any of the possibilities (a) to (c). However, possibility (c) seems to fit our observations with KΔ1 and S_2C_3 strains and might be of some importance for herbicide-resistant mutants, too.

To support our conclusions based on TL and O_2 yield measurements, the properties of atrazine binding have been determined for the investigated *Synechococcus* PCC 7942 mutants. Fig. 7 shows a double reciprocal plot for binding of [^{14}C]atrazine. Wild type and S_2C_3 thylakoids have the same total number of binding sites (about 250 Chl/bound atrazine) and the same binding constants of $K_B^{-1} = 5 \times 10^{-8}$ M. For all strains with mutations of only one amino acid (G 264, Di 1, Tyr 5) changes are observed not only in the apparent binding constants but also in the total number of binding sites, which were smaller than in wild type thylakoids. These data suggest the existence of at least two populations of PS II with binding constants differing by several orders of magnitude. In this case, only the high affinity site will be detected. They further support our hypothesis that PS II protein complexes from G 264, Di 1 and Tyr 5 are heterogeneous with respect to the content of gene products of the three *psb*A genes, with mutated *psb*A I gene products having a low and *psb*A II/III gene product having a high affinity binding site for [^{14}C]atrazine. Interestingly, [^{14}C]atrazine binding to Tyr 5 S_2C_3 was too low to be detected, indicating a strong resistance to triazines. This finding is in perfect agreement with the fact that Tyr 5 S_2C_3 shows the most drastic changes in electron transport activity.

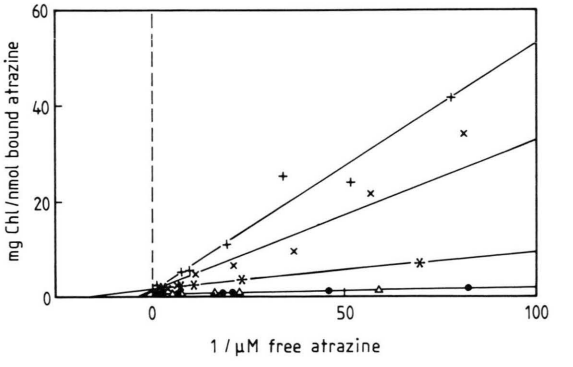

Fig. 7. Double reciprocal plot for binding of [^{14}C]atrazine to thylakoids of *Synechococcus* PCC 7942 mutants: (●) wild type, (△) S_2C_3, (+) Di 1, (*) G 264, (×) Tyr 5.

Concluding Remarks

In this study we confirm and extend previous reports on altered properties of electron transport activity in herbicide resistant species. We show that single point mutations at positions 255 and 264 of protein D 1 in *Synechococcus* PCC 7942 induce characteristic modifications of the PS II reactions: a downshift of the B-band of TL from +40 °C to about +30 °C and strongly damped oscillations of flash induced yield of TL and O_2 evolution. The damping and the downshift of peak temperature is consistent with a lower redox potential of the couple Q_B/Q_B^- giving rise to a change of the equilibrium constant between Q_A^-/Q_B and Q_A/Q_B^- and therefore increased miss factor.

The conclusion on the existence of a comparatively stable S_2 state is in line with a previous report of Vermaas *et al.* [23] showing that S_2 in a small fraction of PS II centers decays extremely slowly in triazine-resistant *Brassica napus*. We extend this idea also to our investigated mutants, although we cannot offer a straightforward explanation in this case. Further experiments with various species are required to clarify this problem.

In this study we clearly demonstrated the importance of amino acid residues Phe_{255} and Ser_{264} not only for the binding of Q_B and analog herbicides but also for the assembly of PS II. Until now we cannot offer a mechanistic explanation for the preferential PS II assembly with gene products of *psb*A II/III in the *psb*A I-mutated Tyr 5. However, these differences, although not completely understood, might provide a new possible insight into the fact that there are three *psb*A genes in *Synechococcus* PCC 7942, which are differentially expressed under different light intensities [22]. The mechanism of this regulation is not known. It has been found that the herbicide resistant mutants are more "shade-adapted" as compared to wild type cells [25]. It is possible that the mechanism which regulates the differential expression of members of the *psb*A gene family and the process of shade adaptation are both under control of the rate of electron flow in PS II.

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