An Application of Thermoluminescence to Herbicide Studies

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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 QA. QA is the reductant for plastoquinol formation at a special domain, the QB binding site, which is located between transmembranal helices 4 and 5 of the intrinsic 32 kDa polypeptide D1 [2]. The QB binding site not only binds the endogenous plastoquinone but also a number of different chemicals.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; atrazine, 2-(ethylamino)-4-chloro-6-(isopropylamino)-s-triazine; D1, 32 kDa (QB or herbicide binding) protein; PS II, photosystem II; TL, thermoluminescence; QA, special bound plastoquinone, first stable acceptor of photosystem II; QB, plastoquinone bound to the QB binding site; PQ, plastoquinone pool on the acceptor side of PS II; K+Pi, K+HPO4.

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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 QA at its binding site thereby blocking the electron transfer from QA 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 D1 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 D1 on herbicide binding was demonstrated [9, 10].

Different techniques are available to study the effects of single or double amino acid exchange in polypeptide D1 within the region of the QB-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.
side of PS II after illumination. The Q- and B-bands are assigned to the recombination of $S_2 Q_A$ and $S_2/S_Q B$, 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 Phe255 and Ser264 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 KA1 and $S_2 C_3$ (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 DNAs (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 *psbA* genes [8], that code for two forms of D1 proteins. D1 form I encoded by *psbA* I differs in 25 out of 360 amino acids from form II which is encoded by *psbA* II/III. It has been shown that any one of the three *psbA* 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 *psbA* 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 $S_2 C_3$ and KA1 in which *psbA* I and *psbA* 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 $S_2 Q_B$ recombination is about double of that due to $S_2 Q_B$ 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 $S_2 C_3$ and 1.4 for KA1. 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.
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 probabilities between wild type, $S_2C_3$ and $K\Delta 1$. The higher TL emission in mutants leads to the assumption that the quantum yield of TL in strains $S_2C_3$ and $K\Delta 1$ differs from those of wild type thylakoids. This might be explained by structural differences of the gene products of the three copies of psbA 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 $S_2C_3/K\Delta 1$ thylakoids might be explained by the assumption that the assembly of wild type PS II is influenced by the expression of psbA 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 $K\Delta 1$ after the 2nd flash ($Y_2$). 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 $Q_{A}Q_{B}$ 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 $D1$ and G264 exhibit a marked downshift of the B-band by 10 °C to +30 °C, whereas the B-band of Tyr 5 is still peaking at about +40 °C. The flash induced oscillation patterns of TL and $O_2$ evolution are strongly damped for $D1$ and G264, whereas Tyr 5 exhibits a wild type like
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 $Q_A Q_B / Q_A Q_B$ from 16 to 3.5. Our data confirm these findings and demonstrate the structural importance of the amino acid residue Ser264 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 $psbA I$.

Although mutant Tyr 5 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 Phe255 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 $psbA I$ gene product for PS II assembly in this mutant might be blocked and only the D1 proteins encoded by the unmutated $psbA II/III$ genes can be incorporated.

To address this problem we also investigated two mutants in which $psbA II/III$ were deleted by insertion of genes coding for spectinomycin and chloramphenicol resistance. These mutants were denoted G264S2C3 and Tyr5S2C3 with additional single site mutations in $psbA I$ accordingly. As can be seen in Fig. 5 and 6, no significant differences

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**Fig. 3.** Flash oscillation patterns and glow curves after one flash (insert) of (---) wild type and mutants (---) Di1, (---) G264 and (---) Tyr 5.

**Fig. 4.** Flash oscillation patterns of $O_2$ evolution of (O) wild type and mutants (●) Di1, (△) G264 and (▲) Tyr 5.

**Fig. 5.** Normalized flash oscillation patterns and glow curves after one flash (insert) of mutants (---) G264S2C3 and (---) Tyr5S2C3.
Fig. 6. Flash oscillation patterns of O$_2$ evolution of mutants (○) G264S$_2$C$_3$ and (●) Tyr5S$_2$C$_3$.

between G264 and G264S$_2$C$_3$ 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, Tyr5S$_2$C$_3$ shows a downshifted B-band and strong damping of both flash patterns. Obviously the psbA II and psbA III gene products are preferred in Tyr5 for the assembly of PS II, which therefore displays a wild type like behavior. The shoulder of the B-band of Tyr5 may be due to some contamination from PS II complexes formed with the mutated psbA I gene product.

In strains KA1 and S$_2$C$_3$ 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 D1, (b) differences in amino acid residues on the lumen exposed part of D1 between proteins coded by copy I or II/III of psbA, (c) structural changes of PS II induced by an assembly with a heterogeneous mixture of gene products of all psbA 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 KA1 and S$_2$C$_3$ 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 PCC7942 mutants. Fig. 7 shows a double reciprocal plot for binding of $[^{14}$C]atrazine. Wild type and S$_2$C$_3$ thylakoids have the same total number of binding sites (about 250 Chl/bound atrazine) and the same binding constants of $K_B = 5 \times 10^{-8}$ M. For all strains with mutations of only one amino acid (G264, D1, Tyr5) 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 G264, D1 and Tyr5 are heterogeneous with respect to the content of gene products of the three psbA genes, with mutated psbA I gene products having a low and psbA II/III gene product having a high affinity binding site for $[^{14}$C]atrazine. Interestingly, $[^{14}$C]atrazine binding to T yr5S$_2$C$_3$ was too low to be detected, indicating a strong resistance to triazines. This finding is in perfect agreement with the fact that Tyr5S$_2$C$_3$ shows the most drastic changes in electron transport activity.
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 D1 in Synechococcus PCC7942 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 O₂ evolution. The damping and the downshift of peak temperature is consistent with a lower redox potential of the couple Q₀/ Q₁ giving rise to a change of the equilibrium constant between Q₀/ Q₁ and Q₂/ Q₃ and therefore increased miss factor.

The conclusion on the existence of a comparatively stable S₂ state is in line with a previous report of Vermaas et al. [23] showing that S₂ 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 Phe₂₅₅ and Ser₂₆₄ not only for the binding of Q₀ 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 psbA II/III in the psbA I-mutated Tyr5. However, these differences, although not completely understood, might provide a new possible insight into the fact that there are three psbA genes in Synechococcus PCC7942, 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 psbA 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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