Amino Acid Substitutions in the D 1 Protein of Photosystem II Affect Q_B^- Stabilization and Accelerate Turnover of D 1

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Isogenic strains of *Synechococcus* PCC 7942 were genetically engineered so that copy I of the gene *psbA* was mutated at specific sites. These mutations resulted in replacements of Ser 264 by Gly or Ala and of Phe 255 by Tyr or Leu in the D 1 protein. The mutants were resistant to herbicides inhibiting electron transfer in photosystem II. All mutants exhibited alterations in the stability of Q_B^- as demonstrated by a temperature downshift, to various extents, of the *in vivo* thermoluminescence emission. Measurements of the light-dependent turnover of D 1 showed a marked decrease in the $t\,1/2$ of this protein in the mutants as compared to wild-type, under low to medium light intensities. A correlation was found between the degree of perturbation in the Q_B^- stability and the rate of acceleration in the turnover of D 1. These data provide a direct evidence for the overlapping binding sites for the plastoquinone B and herbicides in the D 1 protein. In addition these data indicate a close link between Q_B^- destabilization in reaction center II and the mechanism controlling the light-dependent turnover of D 1. Based on these results and previous work we suggest that destabilization of the semireduced quinone, facilitates a light-induced damage in D 1 which triggers its degradation.

Introduction

The photosystem II reaction center (RC II) is comprised of at least five polypeptides: D 1, D 2 (a heterodimer), cytochrome b_{559} and the 4.8 kDa *psb*I gene product, and contains bound chlorophylls, pheophytines, β-carotene, quinones and a non-heme iron. Two plastoquinones, Q_A and Q_B, are the primary and secondary stable electron acceptors, respectively. The apoprotein of Q_B is the 32 kDa polypeptide, D 1, known also to bind herbicides that inhibit electron transfer in PS II (reviewed in [1]). These herbicides displace Q_B from its binding site in D 1 thus blocking electron transfer from Q_A to Q_B. D 1 is encoded by the chloroplast gene *psb*A. Mutations in that gene leading to amino acid substitutions in D 1 were found to confer herbicide resistance (for review see [2]). As a result of the alterations in D 1, the binding affinity for herbicides was affected in the mutants and in certain cases was accompanied also by a lower quantum yield for electron transfer from Q_A to Q_B. D 1 turns over rapidly in the light [3] at a rate that is proportional to the light intensity. Since light of a broad spectrum influences D 1 turnover it was suggested that different photosensitizers may be involved [4]. The reason for the rapid light-dependent turnover of D 1 and the detailed mechanism which regulates this process are not yet completely understood. It has been proposed that a light-induced damage to the D 1 protein is the primary signal for its degradation [5–6]. A light-induced change in RC II expressed as a reduction in the stability of the secondary electron acceptor, Q_B^- was recently demonstrated in *Chlamydomonas* cells *in vivo* [6]. This alteration of the Q_B binding site was detected as a downshift in the temperature of thermoluminescence (TL) emission [6] generated from the charge recombination of $S_2-Q_B^-$ pair (the "B band" [7–9]. More recently the initial stages of the process of light induced inactivation of RC II (photoinhibition) could be resolved into two phases: a reversible phase, characterized by a change in the Q_B^- stability as detected by the temperature downshift of the TL curve, followed by

Abbreviations: Atrazine, 2-(ethylamino)-4-chloro-6-isopropylamino)-*s*-triazine; diuron (DCMU), 3-(3,4-dichlorophenyl-)-1,i-dimethylurea; metribuzin, 4-amino-6-*tert*-butylmethylthio)-as-triazin-5(4H)-one; ioxynil, 4-hydroxy-3,5-di-iodobenzonitrile; PS II, photosystem II; Q_B, the second plastoquinone in PS II; RC II, reaction center II; TL, thermoluminescence; wt, wild-type.

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an irreversible phase characterized by a covalent modification of the D 1 protein [8, 9]. Recovery of RC II activity after photoinhibition was correlated with degradation of the irreversibly modified D 1 protein and replacement by newly synthesized molecules [8, 9]. We report here the results of TL measurements and rate of turnover of the D 1 protein in isogenic herbicide-resistant mutants of *Synechococcus* PCC 7942. We conclude that the higher turnover rate of D 1, found in the mutants compared to the wt, is linked to the destabilization of Q_B^- possibly by mediating a damage to the D 1 protein.

Materials and Methods

Synechococcus PCC 7942 cells were grown on BG 11 medium as described before [10]. Herbicide-resistant mutants in this organism have been generated either by random chemical mutagenesis [11] or by site-specific *in vitro* mutagenesis [12, 13]. Isogenic strains except for the mutations in the gene *psb*A I, were constructed by transformation of wild-type cells with cloned *psb*A I genes carrying different mutations (described in detail in ref. [12]). Homologous recombination, involving a double crossing-over event between the foreign DNA and the bacterial chromosome, replaced *psb*A I in the transformed cells by the mutated gene. Colonies of transformants were obtained by selecting for herbicide resistance. When propagated in liquid media, cultures were occasionally exposed to the appropriate herbicide to select against non-transformed cells or revertants. Cultures at the final propagation step were grown in the presence of herbicides. Measurements of electron transfer in photosystem II were carried out in isolated membranes as described before [12, 13]. Thermoluminescence measurements were carried out using whole cells as previously described [6]. Cells were harvested, resuspended in a buffer containing 0.2 M sucrose, 0.3 M Na-citrate, 0.5 M Na-phosphate (pH 7.4) and sonicated 3 times, 5 sec each with 10 sec intervals, in an ice bath with a tip sonicator operated at 50% power. This procedure did not result in cell breakage but was found to increase the yield of emitted thermoluminescence signal. For recording TL signals, cells (60–80 μg chlorophyll), treated as above, were suspended in 10 mM MES buffer (pH 7.0) containing 2 mM CaCl, 5 mM MgCl, 20 mM NaCl, and 30% (v/v) glycerol. Dark-adaptated samples were cooled to −40 °C and charge separation was achieved either by continuous illumination (orange filter) for 30 sec, or by a saturating single-turnover flash excitation [6, 8]. Measurements of D 1 protein turnover were performed by prelabeling the cells with [^{35}S]sulfate (500 μCi/μmol) at a final concentration of 0.01 μmol/ml in the growth medium. Cells suspensions (30–40 μg chlorophyll/ml) were labelled at 25 °C for 2 h under light intensity of 500 $W \cdot m^{-2}$. The radioactively labelled cells were washed free of radioactive sulfate, resuspended in fresh growth medium and further incubated at various light intensities for up to 8 h. Samples were taken at times as indicated and cytoplasmic membranes prepared as described [13]. The polypeptide pattern of the isolated membranes was resolved by SDS-PAGE, and the dried gels were autoradiographed for detection of the residual radioactivity in the D 1 protein band. The location of the D 1 polypeptide was identified by immunoblotting with antibodies against D 1, as described before [14]. The radioactivity of the D 1 polypeptide band was quantitated by scanning the autoradiograms. Equal amounts of membrane proteins were loaded for each sample and the data were normalized to the radioactivity of polypeptide bands which did not change during the chase period. In all experiments white light was provided by a tungsten-halogen lamp.

Results

The mutants employed in this study have been previously described [2, 11–13]. They were all isogenic with the wt strain except for the mutations in the gene *psb*A I. This feature is crucial in order to ascribe any differences between wt and mutants to the specific mutations in *psb*A I. The specific amino acids replacements in the D 1 protein and the resulting resistance to various herbicides are summarized in Table I. Autotrophic growth curves of cells in liquid cultures indicated a slower growth rate of all the mutants, compared to the wt strain (data not shown). The TL glow curves resulting from a continuous light excitation were asymmetric in all the mutants due to presence of residual TL emission at the same temperature as that of the wt. They showed a peak or a shoulder at a temperature lower than that of the wt cells which exhibited a symmetric TL glow curve. Examples of such

Table I. Properties of the herbicide-resistant mutants used in this work.

Strain	Mutation	Relative resistance (I_{50} mut./wt)			
		Atrazine	Diuron	Metribuzine	Ioxynil
1) Di1*	Ser264–Ala	30	100	5000	0.4
2) Tyr5**	Phe255–Tyr	25	1.5	0.8	4.0
3) G264**	Ser264–Gly	2000	10	1500	0.4
4) D5***	Ser264–Ala Phe255–Tyr	200	160	2000	0.5
5) Di22***	Ser264–Ala Phe255–Leu	1.3	1500	175	5.0

Mutations were obtained by one step chemical random mutagenesis (*), site directed mutagenesis, (**), and two steps chemical random mutagenesis (***). All strains are isogenic transformants. I_{50} values were calculated from measurements of photosystem II activity in isolated cytoplasmic membranes as described before [12, 13]; the I_{50} values for the wild type were $3 \times 10-7$ M for atrazine; $3 \times 10-8$ M for diuron; $2 \times 10-7$ M for metribuzin and $4 \times 10-7$ M for ioxynil.

glow curves are shown in Fig. 1. In contrast, the glow curves of cells excited by a single flash were symmetric. A possible explanation for the asymmetric shape of the glow curves in the mutants excited continuously for 30 sec, could be the presence of a small amount of form II of D1, the product of genes *psb*A II and *psb*A III [15], which is not mutated and may be integrated in reaction centers excited more efficiently under these conditions. Although *psb*A I contributes to most of the *psb*A mRNA [15, 16], it is possible that low expression of *psb*A II and *psb*A III produces some amount of D1 assembled into PS II complexes. The peak temperature or the TL curve of the wt and the mutants are given in Table II. It should be noted that these data represent the TL of whole cells. Only slightly different peak TL temperatures

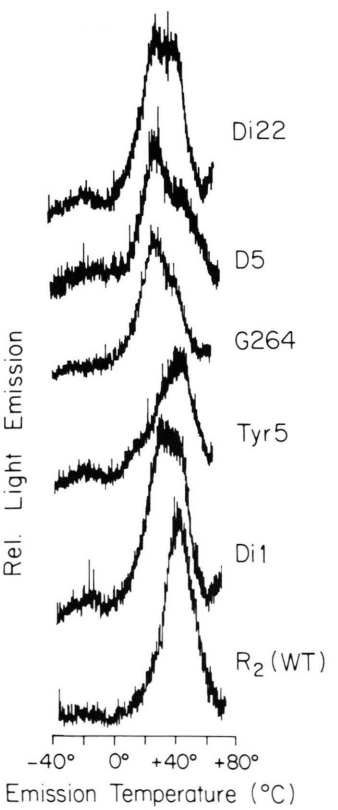

Fig. 1. Recorder traces of the glow curves of wild-type and mutant cells *in vivo* following excitation by continuous illumination at −40 °C.

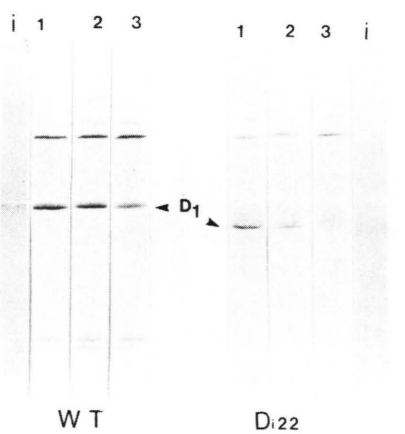

Fig. 2. Autoradiograms of wild-type and mutants, G264 (A) and Di22 (B) showing loss of D1 protein radioactivity following chase in medium light. Cells were labelled with 35 S- sulfate as described in Materials and Methods. D1, localization of the D1 protein by immunoblotting (i); 1, 2, 3, samples taken at the end of the pulse and following 4 h and 8 h chase.

Table II. TL emission temperature and $t\,1/2$ values for D 1 turnover in wt and mutant cells at various light intensities.

Cells	Mutation	TL 0 C	$t\,1/2$ [h] Low light	Medium light	High light
wt		40–42	8.3–7.5	4.5	1.9
Di 1	264 Ser–Ala	34	4.0	n.d.	2.0
Tyr 5	255 Phe–Tyr	38	5.0	3.6	1.9
G 264	264 Ser–Gly	24	3.4	2.8	1.9
D 5	255 Phe–Tyr 264 Ser–Ala	31	4.3	n.d.	2.0
Di 22	255 Phe–Leu 264 Ser–Ala	32–33	3.7–2.5	2.5	1.8

TL emission temperatures were measured following single flash excitation *in vivo;* the low, medium and high light intensities were 100–200 $W \cdot m^{-2}$, 300 $W \cdot m^{-2}$ and 2000 $W \cdot m^{-2}$, respectively. Incubation was in a 3 cm diameter glass tube; white light intensity provided by a tungsten-halogen lamp and filtered through 3 cm of water was measured with a radiometer (Kettering, Yellow Spring); for additional details of D 1 turnover measurements see Materials and Methods.

were found in isolated membranes from the same mutants (see Gleiter *et al.* [17]). An example of the autoradiograms used for measuring the D 1 protein turnover is shown in Fig. 2. The $t\,1/2$ of the D 1 protein *in vivo* in mutants exposed to relatively low light intensity (100–200 $W \cdot m^{-2}$) was lower than that of the wt cells (Fig. 3, Table II). The differences in the $t\,1/2$ values of D 1 protein in the various mutants, as compared to the wt cells, were maintained at intermediate light intensities (300 $W \cdot m^{-2}$) but not in cells exposed to high light intensity (2000 $W \cdot m^{-2}$). In this case the $t\,1/2$ value for all cells was reduced to about 2 h (Table II). No significant turnover of the D 1 protein was observed, neither in the mutants, nor in the wt cells incubated in the dark (data not shown).

Discussion

It has been previously demonstrated that specific amino acid substitutions in the D 1 protein reduced the binding affinity of certain herbicides (for review see [2]). As shown in Table I, different amino acid replacements in residues 255 and 264 of the D 1 protein, conferred a distinctive degree of resistance towards triazines, ureas and phenolic-type herbicides. This phenomenon implies that al-

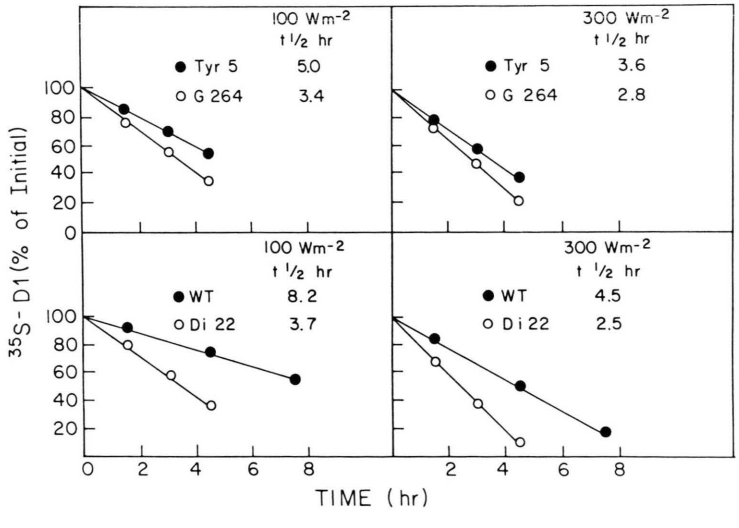

Fig. 3. Loss of D 1 radioactivity as a function of time in wild-type and mutants exposed to low and medium light intensities; for experimental details see Materials and Methods.

though these herbicides bind to D 1 in the same polypeptide domain, each one of them has a slightly different binding niche due to unique interactions with amino acid residues in the binding pocket. While binding affinities of herbicides have decreased dramatically in the mutants (up to three orders of magnitude in some cases), it is apparent that Q_B binding was not completely abolished since they can still grow autotrophically. The TL measurements presented in this work, as well as in the accompanying paper by Gleiter *et al.* [17], show that replacement of amino acid residues Phe 255 and Ser 264 in the D 1 protein affect also the Q_B-stability. This is a direct demonstration of the involvement of these residues in binding and/or stabilizing the second plastoquinone, Q_B, in RC II. It also confirms previous predictions of overlapping binding sites in RC II of herbicides and Q_B [12]. Similar effects on the TL signal have been observed in herbicide resistant mutants in other systems [18, 19], yet the comparison was made between two non-isogenic strains where the molecular basis for the resistance has not been identified. The data presented here, together with previous analyses of the mutants [12, 13] provide additional evidence to the similarities in the Q_B binding sites in RC II and in the RC in purple bacteria. Thus, Phe 255 and Ser 264 of D 1 are equivalent to Phe 216 and Ser 223, respectively, in the L subunit of the *R. viridis* reaction center [20–22]. The interaction of the latter ones with herbicides and Q_B have been described in detail by X-ray crystallography [21–23]. It is very likely that residues Phe 255 and Ser 264 in the D 1 are structurally oriented towards the Q_B binding niche similarly to their counterparts in the L subunit. The changes in the Q_B binding site induced by the mutations, are expected to affect the electron flow from Q_A to Q_B and to the plastoquinone pool. This could lead to a damping of the oscillation pattern of the TL response induced by a train of excitation flashes [7]. Indeed, similar downshifts in the TL emission temperature in isolated thylakoids from the same mutants used in this work, were found to be accompanied by a significant damping of the oscillation pattern, as expected [17].

The same alterations in the D 1 that affect Q_B binding were found also to induce an increase in the rate of light-dependent turnover of D 1. This finding is significant and could implicate the mechanism of regulation of the latter process. As already mentioned, light-induced modification of RC II precedes a covalent conformational change in the pathway of degrading D 1 [8, 9]. There are two main possibilities by which structural changes in D 1 in the mutants can affect the rate of its degradation: 1. Direct enhancement of the degradation process. Since all the mutations are in close vicinity to the postulated cleavage site in D 1 [24], they could induce conformational changes in this domain thus increasing D 1 accessibility to the putative enzymes involved in the cleavage mechanism. A herbicide resistant mutation in *Synechocystis* 6714, possibly affecting the degradation of D 1, has been recently reported [25]. 2. Enhancement of the photoinduced triggering of the process. This could be due to the alteration of the Q_B binding site that facilitates the photoinduced damage. An interesting correlation has been found between the degree of destabilization of Q_B^- in RC II and the rate of turnover of D 1 (Table II). Most notably is the fact that substitution of Phe 255 (in Tyr 5) has smaller effects on both processes than mutations in Ser 264 (Di 1, G 264). This correlation favors the second possibility namely, that changes in the Q_B^- stability in RC II are linked to the initial steps of degradation of D 1. The mechanism by which this can be achieved is yet unknown. Our results indicate that the alteration ("damage") of the D 1 protein in the light leads to the recently reported covalent modification in the D 1 polypeptide [9] which is then degraded and replaced. A modified form of D 1 detected by a change in its electrophoretic mobility was found to appear transiently in the light in *Spirodella* [26]. The question arrises whether this finding is related to the covalent light-induced modification generating a trypsin resistant fragment in D 1 isolated from light-treated cells [8, 9] which could be due to the formation of an internal cross-link between loops II and III of D 1 in *Chlamydomonas* [8]. The occurrence of a covalent modification for a given reaction center is a random event with a calculated probability of 10^{-4} to 10^{-5} photon absorbed [27]. Based on the results presented here we propose that the probability for the irreversible covalent modification of D 1 to occur is increased in RC II of the mutants due to the mutation effect on Q_B^-. It is possible that Q_B^- is the damaging species that occasionally can modify D 1. Change in the equi-

librium constant between Q_A^-/Q_B and Q_A/Q_B^-, as a result of the mutations in D 1 [17], can increase the possibility of damaging D 1 [8, 9].

As expected, the $t1/2$ of D 1 decreased proportionally to the increasing light intensities in all the examined strains. However, the difference in D 1 turnover rate in the various mutants relative to that in the wt was highest at low light intensity and diminished completely at a very high light (Table II). This could be explained if one considers that degradation of the D 1 polypeptide is an enzymatic process triggered by the light-induced modification (damage) of the D 1 within RC II. The rate of alteration of D 1 (and the covalent modification that follows) could be proportional to the photon flux over a wide range of light intensities while the rate of the enzymatic cleavage process could reach a plateau when the amount of accessible substrate (modified D 1) is no longer rate limiting. The $t1/2$ of D 1 in *Chlamydomonas* cells at light intensities of 1550–2000 $W \cdot m^{-2}$ was found to be comparable to that of *Synechococcus* PCC 7942 namely, about 1.5 h [14]. Alteration of the electron flow due to the effects on Q_B binding could also be the reason for the "shade type" appearance of the mutants analyzed here [28]. It has recently been reported [29] that the light-dependent synthesis of D 1 in the mutants described here is faster than that in the wt cells. The light-dependent synthesis of D 1 was highest in mutant G 264. This mutant showed also the highest shade-adapted appearance. These findings are in agreement with our data indicating that this mutation has the highest effects on both TL and rate of D 1 degradation. Since degradation and synthesis of D 1 are related [8] the mutations in the D 1 protein described here provide an useful tool for the investigation of the mechanism of D 1 protein turnover.

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