Structural Analysis of the Q_B Pocket of the D 1 Subunit of Photosystem II in *Synechocystis* PCC 6714 and 6803

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Various herbicides inhibit photosynthesis by displacing the second electron acceptor Q_B from its binding site at the D 1 protein. Different amino acid substitutions within this binding site have been found to reduce herbicide affinities, thereby conferring herbicide resistance. In *Synechocystis* PCC 6714 we have selected 7 single mutants and 6 double mutants resistant to various herbicides due to amino acid substitutions at different positions in the Q_B pocket. Characterization of these mutants consists in molecular determination of the mutations in the *psb*AI genes and in transformation of *Synechocystis* PCC 6803 by the cloned mutated genes to analyze the role of the mutations in the mutant phenotypes. Comparison with equivalent *Chlamydomonas* mutants is presented. These studies allow us to specify the interactions of several amino acid residues with herbicides and Q_B and with each other. Furthermore some *Synechocystis* mutants present additional characteristics such as an increased sensitivity to photoinhibition, or resistance to formate or modification of the oscillatory pattern of oxygen evolution. Among the 6 point-mutations giving herbicide resistance, only those located at the limit of the loop and the parallel helix produced additional effects on photosystem II function.

Introduction

Different classes of herbicides have been known for many years to inhibit photosynthesis at the level of photosystem II by blocking electron transfer between the primary electron acceptor Q_A and the secondary acceptor Q_B. Competition between the herbicide molecules and the secondary electron acceptor Q_B, in the "Q_B pocket" of the D 1 protein, have been demonstrated [1, 2]. Different amino acid substitutions in the D 1 protein have been shown to reduce herbicide binding in plants, green algae and cyanobacteria (see [3] for a review). These results have allowed prediction of the folding of this polypeptide [4]. In *Synechocystis* PCC 6714 we have selected several single and double mutants resistant to various herbicides. They have been characterized for their phenotypes (herbicide resistances and electron transfer) and their genotypes. These mutants have allowed us to determine the interactions of several amino acid residues with herbicides and Q_B [5–11].

In this paper, using these mutants, we looked for the interactions between several amino acids according to Horovitz *et al.* [12]. These authors have demonstrated that when two amino acids contribute to the binding of herbicides in an additive manner, there is an apparent lack of interaction between these two amino acids. As we have several double mutants and the corresponding single mutants, the interactions between amino acids at position 211–251, 251–266, 255–264 and 264–266 were studied.

In addition to affecting herbicide binding, the alteration of some amino acid residues, located in the same area, modify certain functional properties of photosystem II such as sensitivity to high light, sensitivity to formate and oxygen evolution.

Comparison of *Synechocystis* mutants with equivalent mutants in *Chlamydomonas reinhardtii* points out several differences in D 1 conformations in these two organisms.

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Materials and Methods

Cyanobacterial strains

Synechocystis PCC 6714 and PCC 6803 were grown photoautotrophically. The mineral medium was that described in ref. [13] with twice the concentration of nitrate. For the solid medium, 1.5% agar autoclaved separately was added. Standard growth was achieved by incubation in a Gallenkamp rotatory shaker at 34 °C in a CO_2 enriched atmosphere, under 70 $mE \cdot m^{-2} \cdot s^{-1}$.

Measurements of herbicide resistance

In whole cells: the inhibition of photosystem II electron transport between Q_A and Q_B by herbicides was measured by the changes in chlorophyll fluorescence as in [8]. It is known that chlorophyll fluorescence yield is controlled by the redox state of Q_A. In conditions where the photosystem I is preferentially excited, there are very few PS II centers in the Q_A^- state and the fluorescence yield is low. Addition of herbicide, blocking electron transfer between Q_A and Q_B, produced an increase of the fluorescence quasi proportional to the number of PS II centers blocked in the Q_A^- state by the herbicide. The apparatus was previously described in [14]. Cell suspensions contained 1 µg chlorophyll per ml.

In thylakoids: photosystem II activity was measured at pH 6.8 by the Hill reaction assay from the absorption change at 580 nm using a Cary 2300 spectrophotometer with side illumination in the presence of dichlorophenolindophenol as an electron acceptor.

DNA preparation, polymerase chain reaction and sequencing

Genomic DNA from *Synechocystis* 6714 was isolated from exponentially growing cultures as in ref. [5]. DNA fragments (4.5 to 5.5 Kb) containing only the copy 1 of the *psb*A gene were obtained by digestion with *Eco*RI. Polymerase Chain Reaction (PCR) was performed with the DNA amplification reagent kit (Gene Amp) from Perkin Elmer Cetus. Two primers were used: one corresponding to nucleotides 441 to 458 and one corresponding to nucleotides 1048 to 1072 with an additional *Hind*III site on the 5′ end. The PCR product was digested by *Kpn*I and *Hind*III and the 531 bp fragment was inserted in Bluescript plasmid. Sequencing was done by dideoxy chain termination according to [15], using a Sequenase kit from USB. Oligonucleotide primers were synthesized on a Milligen 7500 DNA synthesizer.

Transformation of Synechocystis 6803 wild type

0.5 ml at 2.10^8 cells per ml of wild type *Synechocystis* 6803 growing exponentially were mixed in 2 ml top agarose and plated on mineral medium. About 3 µg of cloned DNA (in 10 µl) were dotted onto this lawn of recipient cells. After 16 h incubation in light at 34 °C, selective herbicide was added by the underlying technique.

Results

Determination of the herbicide resistances

All the mutants presented in Table I are spontaneous mutants which have been selected for their

Table I. The *psb*A mutations and the herbicide resistances (R/S) of *Synechocystis* 6714 mutants. Mutants were names as follows: name of the wild type amino acid in single letter code, followed by the number of the amino acid and then the single letter code of the mutated amino acid. For the double mutants, the first residue corresponds to the mutation of the single mutant from which the double mutant has been selected. R/S is the ratio of the I_{50} of the mutant to that of the wild type. I_{50} values were determined in whole cells as the inhibitor concentration which blocks half of the variable fluorescence (see Materials and Methods).

N°	Herbicide used for selection	Mutation(s)	DCMU	Atrazine	Metribuz	Ioynil	Ref.
1	DCMU	S264A	1000	100	1000	0.8	[5]
2	DCMU	F255L	10	0.5	0.5	7.5	(a)
3	atrazine	F211S	1	15	15	3	[6]
4	ioxynil	N266T	0.8	1	6.5	10	[7]
5	ioxynil	N266D	0.7	1		4	[9]
6	metribuzin	I248T	2	1	30	1	(b)
7	metribuzin	A251V	1	25	200	1	(b)
8	-DCMU	S264A–F255L	1000	1.5	1000	3	[5]
9	-DCMU	N266T–S264A	1000	100	~4000	4.5	[10]
10	-atrazine	F211S–A251V	2.5	150	800	15	[5]
11	-atrazine	N266T–A251V	2	100	1500	15	(a)
12	-DCMU	F255L–X (c)	50	0.1	0.5	7.5	(a)
13	-ioxynil	N266T–Y (c)	1	2.5	14	30	(a)

(a) Present work; (b) Kirilovsky *et al.*, in preparation; (c) X and Y represent unknown mutations not located in *psb*A gene.

resistance to one herbicide (listed in the second column). After their purification, their herbicide cross-resistances were determined in whole cells by fluorescence measurements and in thylakoid membranes by Hill reaction measurements (see Materials and Methods). Only in two cases (double mutants N° 12 and 13) were the R/S (ratio of the I_{50} of the mutant to that of the wild type) determined *in vivo* and *in vitro* different. This indicates that the second mutations are not in genes coding for thylakoid components.

The *psb*AI gene of each mutant was sequenced. In *Synechocystis* 6714, we have previously shown that only *psb*AI, which is the majoritarily expressed copy, bears mutations [13]. Sequence results are presented in the third column. One point-mutation was found in each single mutant and an additional point-mutation was found in all the double mutants except in the double mutants N° 12 and 13 described above. To confirm that the determined mutations are responsible for the observed phenotypes, the cloned *psb*A or part of the *psb*A gene was used to transform wild type cells. Phenotypes of the transformant cells were analyzed. In all cases the transformant phenotype was similar to that of the mutant which the DNA came from, except in the mutants N° 12 and 13.

Determination of amino acid interactions

Horovitz *et al.* have proposed that when two amino acids contribute to the binding of herbicides in an additive manner, there is an apparent lack of interaction between these two amino acids. As we have several double mutants and the corresponding single mutants, the interactions between amino acids at position 211–251, 251–266, 255–264 and 264–266 were studied. The following equation was used:

$$\frac{\text{R/S wild type}}{\text{R/S mutant 1}} = \frac{\text{R/S mutant 2}}{\text{R/S mutant 1–2}}.$$

R/S of the double mutants were calculated for four herbicides, DCMU (D), atrazine (A), metribuzin (M) and ioxynil (I) and compared to the R/S measured (Table I). In Fig. 1, we present the relationship between measured and calculated R/S for each couple of amino acids. A good correlation between experimental and predicted R/S values was found for the couple 264–266 and 211–251 reflecting an apparent lack of interaction between the amino acids 264 and 266 and between the amino acids 211 und 251. In contrast, for the couples 251–266 and 264–255 some calculated values did not fit the experimental results, suggesting that these amino acids interact in herbicide binding.

Mutation effects on photosystem II functioning

Electron transfer between Q_A and Q_B

In all our mutants, there is only a slight modification of the electron transfer between Q_A and Q_B, therefore the different amino acid substitutions do not significatively modify the quinone affinity [11].

Formate-bicarbonate reversible effects

Bicarbonate has been shown to stimulate electron transfer in photosystem II depleted of bicarbonate by treatment with formate. It has been suggested that formate behaves as a competitive analogue of bicarbonate and blocks the proton uptake that stabilizes Q_B^- and in this way inhibits electron transfer [14]. It is also assumed that formate binding and Q_B binding are independent of each other, *i.e.* formate must not be considered as an herbicide. Nevertheless, mutations affecting herbicide binding also affect formate binding. Resistance of cells to formate treatment is in the following order: F211S–A251V > F211S ≈ wild type > S264A [15].

Oxygen evolution

Two metribuzin resistant mutants (N° 6 and 7) present a modified oscillatory pattern of oxygen emission, the maximum of emission being on the fourth instead of on the third flash ([16] and Kirilovsky *et al.* in preparation).

Light sensitivity

Photoinhibition was studied in several mutants. The mutants having the Ala 251 modified to Val present an increased sensitivity to light. It was proposed that residue 251 plays a role in the degradation of the D1 protein ([6, 17] and Kirilovsky *et al.* in preparation).

Comparison with mutants of Chlamydomonas reinhardtii

Herbicide binding

Przibilla *et al.* [18] have constructed a double mutant S264A–N266T by site-specific mutagen-

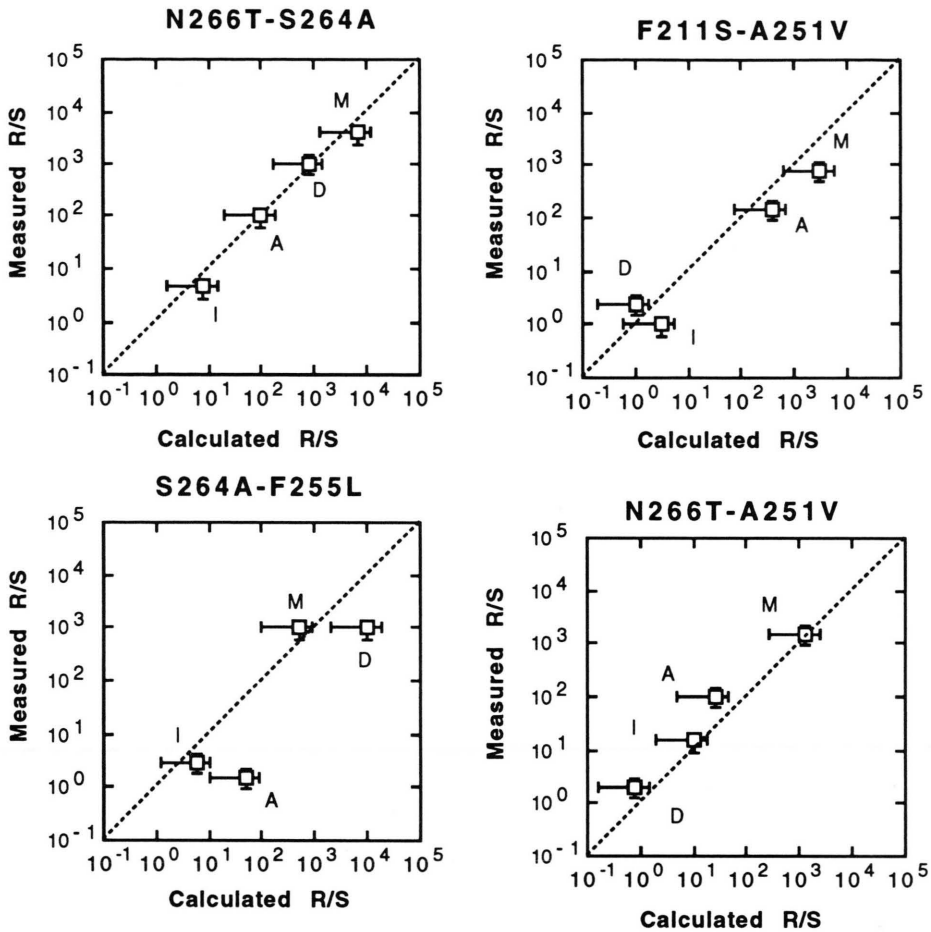

Fig. 1. Correlation between experimental and predicted R/S values of the different herbicides with respect to the four double mutants N266T–S264A, F211S–A251V, N266T–A251V and S264A–F255L. Abbreviations for herbicides are according to the first letter of their commun name as given in Table I. The average experimental error in R/S is about 40%.

esis of wild type *Chlamydomonas reinhardtii* chloroplasts. This mutant was selected by resistance to metribuzin. The mutation N266T in addition to the mutation S264A led to an increased sensitivity *i.e.*, to a partial reversal of the tolerance for metribuzin, atrazine, and DCMU induced by the substitution at position 264 alone. According to Horovitz *et al.*, prediction for the phenotype of the single mutant N266T is increased sensitivity to metribuzin, atrazine, and DCMU with respect to the wild type and only a slight resistance to ioxynil. Recently, Przibilla and Yamamoto [19] selected this single mutant using an unrelated resistance marker conferring resistance to spectinomycin for cotransformation with mutated *psb*A. The authors only report resistance to phenol-type herbicides but not to classical herbicides. These results were different from those obtained with equivalent single and double mutants in *Synechocystis*.

Quinone-binding

Erickson *et al.* [20], Etienne *et al.* [11], and more recently Govindjee *et al.* [21] have analyzed electron transfer from Q_A^- to $Q_B^{(-)}$ by measuring decay of chlorophyll fluorescence yield after one saturating flash in various *Chlamydomonas* mutants. It was concluded that mutations at 219, 255 and 275 are of marginal importance for this electron transfer and for Q_B binding but that mutation at 264 and 256 are of major importance. The mutants

S264A and G256D had an altered forward electron transfer, an altered apparent equilibrium for the $Q_A^- Q_B \leftrightarrow Q_A Q_B^-$ reaction and an abnormally high ratio of the slow to the fast centers. In contrast in the *Synechocystis* S264A mutant, only slight modifications were observed [11].

Formate effect

From transient fluorescence modifications induced by formate treatment, Govindjee *et al.* [22] determined that resistance of *Chlamydomonas* cells to formate followed the order: L275F > A251V >> wild type ~ G256D ~ F255Y ~ V219I >> S264A. It is the same order of resistance as in the *Synechocystis* equivalent mutants [15].

Oxygen evolution

Except for the mutant A251V which has "an as yet incompletely characterized defect on the water-splitting side" [23], nothing is known about oxygen evolution in *Chlamydomonas* mutants modified in the Q_B pocket.

Discussion

Selection of various herbicide-resistant mutants in *Synechocystis* has allowed us to apply the method of Horovitz *et al.* for evaluating interactions between amino acids in binding of an inhibitor. Single and double mutants were tested for their resistance to four different herbicides, DCMU (phenylurea family), atrazine (triazine family), metribuzin (triazinone family) and ioxynil (phenol family). Fig. 1 shows the correlation between the measured and the calculated values of the relative herbicide resistances for four double mutants. Clearly residues at position 266 and 264 contribute independently to herbicide binding, and therefore an apparent lack of interaction between these two amino acid residues can be assumed. The same is true for amino acids at position 211 and 251. In contrast, the agreement between experimental and predicted values is poor for the couple 266 and 251 and especially for the couple 264 and 255. The aminoacids of these two pairs interact in binding of herbicides. The "interactions" assumed in these cases are difficult to specify. The three dimensional model of Tiejen *et al.* [24] proposed a location of the parallel helix so that amino acids 264 and 255 are not far from each other, but in this model the distance between 251 and 266 seems too large to allow direct interaction between these residues.

The increased sensitivity to light of mutants altered at position 251 led to the idea that this amino acid is a hinge-residue between the parallel helix and the sequence between the parallel helix and helix IV. This part of the sequence, sometimes called the extra-loop, is larger in D1 than in the L polypeptide and therefore cannot be easily modelized. It is also supposed to be the target of proteolysis occurring during photoinhibition. Folding of this part of the D1 sequence and interaction with the equivalent part of the D2 protein has been discussed by Trebst [25]. Changes of the residue at 251 or at proximity might cause partial unfolding, leading to a less stable D1 conformation and this conformational change might be responsible for the modified oxygen evolution pattern.

Comparison of *Synechocystis* mutants altered at positions 264 and/or 266 with equivalent mutants in *Chlamydomonas* points out differences both in Q_B binding and in herbicide binding. In [10] we proposed that this was due to the presence of three different amino acid residues in the "extra-loop" (residues 233, 235 and 238). In the model of Tiejen *et al.* [24], the side chain of tyrosine 237 is placed next to the side chain of histidine 252 and interactions are assumed between the hydroxyl of tyrosine 237 and the residues at 264 and 266. This pro-

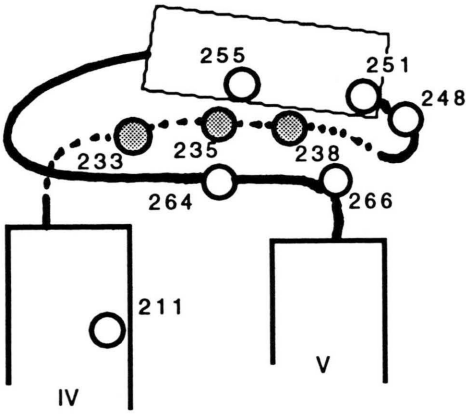

Fig. 2. Schematic representation of part of D1 protein. Amino acids residues changed in single and double herbicide resistant mutants of *Synechocystis* PCC 6714 are represented by empty circles. Amino acids residues of the loop which are different in *Chlamydomonas reinhardtii* and *Synechocystis* PCC 6714 are represented by shadowed circles. Boxe or parts of boxes represent the parallele helix and parts of helices IV and V.

posed arrangement is in good agreement with our hypothesis that the nature of the three amino acids at position 233, 235 and 238 can influence the conformation of the Q_B and herbicide binding pocket.

In Fig. 2 we have schematized the above hypothesis. In particular we have tried to represent the postulated interactions between various amino acid residues by locating them in close proximity.

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