Photosystem II electron transfer in highly herbicide resistant mutants of *Synechocystis* 6714

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Three mutants of *Synechocystis* 6714 have been selected for their high relative resistance to DCMU and to atrazine (×300 for DCMU, ×67 for atrazine). Measurements of chlorophyll fluorescence have been used to monitor electron transfer from Q_A (the primary electron acceptor of photosystem II) to Q_B (the secondary electron acceptor). The initial electron transfer is not slowed down in the resistant strains; the second phase of the fluorescence decay is somewhat slower. The data are interpreted to indicate that a mutation giving rise to a high level of resistance does not induce a large alteration of the Q_B binding site for mutants resistant to atrazine and/or to DCMU.

Photosystem II *Herbicide resistance* *Fluorescence*

1. INTRODUCTION

The first herbicide resistant mutants studied were triazine resistant weeds occurring naturally in the field [1]. They were still sensitive to diuron but 50-times less sensitive to atrazine than the wild type. The mutation resulted in a drastic decrease of the electron transfer rate on the acceptor side of photosystem II [1,2].

The molecular basis for such resistance is a change in the *psb*A gene coding for the Q_B protein.

The first reported amino acid substitution in the *psb*A gene was Ser 264 to Gly in atrazine resistant higher plant species [3] and to Ala in a DCMU resistant mutant of *Chlamydomonas reinhardii* [4]. Then with a collection of herbicide resistant mutants from *C. reinhardii* [5], it was shown that the resistance could be due to three distinct changes in one amino acid (Val 219, Phe 255, Ser 264) in the *psb*A gene [6]. These results were in accordance with our proposal of different binding sites for DCMU, atrazine and Q_B [7]. In *C. reinhardii* when the substituted amino acid was not Ser 264, the relative resistance induced by the mutation was small (maximum 15) and in that case the electron transfer between Q_A and Q_B remained unaltered [5]. When the relative resistance was higher and due to the substitution of Ser 264, the electron transfer was slowed down in *C. reinhardii* and in higher plants.

In the present report we show that among a large number of herbicide resistant mutants of *Synechocystis* 6714, we selected three mutants with high relative resistance to one or both herbicides but which had still an efficient electron transfer between Q_A and Q_B. Therefore we demonstrate first that there are indeed different binding sites for different herbicides and second that a mutation giving a high level of resistance (up to ×300) compared to the wild type does not necessarily induce a large alteration of the Q_B binding site.

The recent paper of Golden and Hazelkom [8] showing the possibility to transform wild type cells of *Anacystis nidulans* R_2 to herbicide resistant with a mutated *psb*A gene proved that in this case the mutation within the *psb*A gene is responsible for that phenotype. The same experiment with the *psb*A genes of the three mutants of *Synechocystis* is in progress in our laboratory.

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2. MATERIALS AND METHODS

The strain *Synechocystis* 6714 given by Dr R.Y. Stanier was of the American type culture collection no ATCC 27178.

2.1. *Growth conditions*

The minimal medium (MM) for growth was that defined by Herdman et al. [10] with twice the concentration of nitrate. For the solid medium, $NaHCO_3$ was omitted and 1.5% agar autoclaved separately was added. Standard photosynthetic growth was achieved by incubation in a Gallenkamp rotatory shaker at 34°C under 2500 lux in a CO_2 enriched atmosphere, the flasks being constantly shaken. The generation time was 6 h.

2.2. *Isolation of mutants*

To isolate herbicide resistant mutants, the wild type *Synechocystis* 6714 was grown in liquid minimal medium up to a cell concentration of 10^8 cells/ml, then herbicide (DCMU 10^{-5} M or atrazine 10^{-4} M) was added. Most of the cells died, the spontaneous mutants were grown to a cell concentration of 10^7–10^8 cells/ml, and then transferred onto solid medium containing the same herbicide concentration in order to isolate the clones.

The selection of mutants was also achieved by adding the herbicide (DCMU 10^{-5} M, atrazine 10^{-4} M) only to the solid medium on which the wild type cells are layered. Several independent selections of the two types were performed. Each of the selected clones was then isolated and classified in terms of growth sensitivity in the presence of various DCMU or atrazine concentrations as described in [11]. To screen rapidly a great number of clones, a microbiological test was used. All clones were grown in minimal medium at the exact same cell concentration (10^7/ml) and calibrated drops (50 μl) were layered into a series of Petri dishes containing various concentrations of the appropriate herbicides. One clone of each differfent phenotype was kept.

2.3. *Photosystem II activity assays*

Fluorescence under continuous illumination was measured as described [13]. The fluorescence was excited with a tungsten lamp through a monochromator and a 4–96 Corning filter. The fluorescence was detected in the red region through a 2–64 Corning filter and a Wratten 90 filter. The recording was done through a multichannel analyzer. The cell suspension contained about 0.5 μg chlorophyll/ml.

Fluorescence decay was measured after excitation by a series of saturating flashes. The fluorescence decay was monitored by detection of the fluorescence excited by a train of non-actinic detecting flashes synchronized with the address advance of a datalab connected to a multichannel analyzer. 5–10 accumulations were needed for a good signal to noise ratio. The luminescence artefact was substracted from the overall signal [4].

With dichlorophenol indophenol (DCPIP) as an electron acceptor, photosystem II activity was measured at pH 6.8 by the Hill reaction assay from the absorption change at 580 nm using a Cary 14 spectrophotometer with side illumination.

3. RESULTS

After independent selection of a large number of mutated colonies, we discarded cell mutants which retained their sensitivity to the herbicides in isolated thylakoids. In table 1, 9 of the cloned herbicide resistant mutants have been classified. We indicate the mode of selection, the I_{50} concentration needed for half inhibition of the growth rate (column 2), Hill activity (column 3) for two herbicides DCMU and atrazine, and relative resistance to both herbicides (column 5).

From these results we decided to focus our attention on three mutants: (i) DCMU-II_B (line 1) very resistant to DCMU and still atrazine sensitive; (ii) A_Z-IV (line 8) resistant to both herbicides; (iii) A_Z-V (line 9) derived from the latter, which has regained a better sensitivity to DCMU while becoming more resistant to atrazine.

In a fluorescence induction curve obtained under continuous illumination there are three important fluorescence levels: F_0 the initial fluorescence mostly due to the fluorescence of the antennae when all photosystem (PS) II centers are opened, F_i the level reached at the end of a fast photochemical rise indicative of the rate of electron transfer between Q_A and Q_B and F_m which is the F_i level reached at the end of the fast photochemical rise when the electron transfer be-

Table 1

Strain	Mode of selection	Growth rate (molar concentrations)		PS II activity (molar concentrations)		PS II activity	
		I_{50} DCMU	I_{50} atrazine	I_{50} DCMU	I_{50} atrazine	Relative resistance to DCMU	Relative resistance to atrazine
DCMU-II$_R$	derived from a previous mutant screened in liquid medium by plating on solid medium with DCMU 10^{-5} M present	3×10^{-4}	5×10^{-6}	5×10^{-5}	7×10^{-6}	300	5
DCMU-III	screened from the wild type on solid medium with DCMU 10^{-5} M present	9×10^{-5}	10^{-4}	1.5×10^{-5}	2×10^{-5}	88	13
DCMU-IV	independently screened in liquid medium with DCMU 10^{-5} M present	1.2×10^{-4}	4×10^{-4}	8×10^{-6}	10^{-5}	47	7
DCMU-V		1.8×10^{-4}	1.6×10^{-4}	8×10^{-6}	1.5×10^{-5}	47	10
A$_Z$-I	screened from the wild type on solid medium with atrazine 10^{-4} M present	10^{-6}	8×10^{-5}	6×10^{-7}	2×10^{-5}	3.5	13
A$_Z$-II		1.2×10^{-6}	10^{-4}	6×10^{-7}	10^{-5}	3.5	7
A$_Z$-III		2×10^{-5}	2×10^{-5}	6×10^{-6}	3×10^{-5}	35	20
A$_Z$-IV		1.3×10^{-5}	6.5×10^{-5}	2×10^{-5}	5×10^{-5}	118	33
A$_Z$-V	mutant derived from Atz 1 by plating on solid medium with atrazine 2.5×10^{-4} M present	10^{-6}	2×10^{-4}	10^{-6}	10^{-4}	5.8	67
Wild type		5×10^{-7}	3×10^{-6}	1.7×10^{-7}	1.5×10^{-6}		

tween Q_A and Q_B is inhibited by either DCMU and/or atrazine at saturating concentrations.

In table 2, the values for the $\frac{F_i - F_0}{F_m - F_0} \times 100$ ratio are indicated for different experiments with three strains in whole cells and thylakoids.

There is a larger difference in thylakoids between the wild type and the herbicide resistant mutants but in whole cells the ratio is only slightly larger for the resistant strains as compared to the wild type. A better analysis of the rate of electron transfer between Q_A and Q_B is done when a saturating flash produces a maximal concentration of Q_A^- and when the fluorescence decay corresponding to the reoxidation of Q_A^- is followed in the dark after the flash.

Fig.1 shows the fluorescence decay after the first and the second flash (spaced at 0.5 s) for whole cells, wild type, DCMU-II$_B$, A$_Z$-IV and A$_Z$-V. The fluorescence decay after the first flash was analyzed in terms of a two exponential decay. (The fitting with one exponential decay was not

Table 2

Values of the ratio of $(F_i - F_0)/(F_m - F_0) \times 100$

Strain	2.2.	A$_Z$-V	DCMU-II$_B$
Whole cells			
Expt 1	8	18	18
Expt 2	11	15	20
Expt 3	9	13	16
Thylakoids	28	50	49

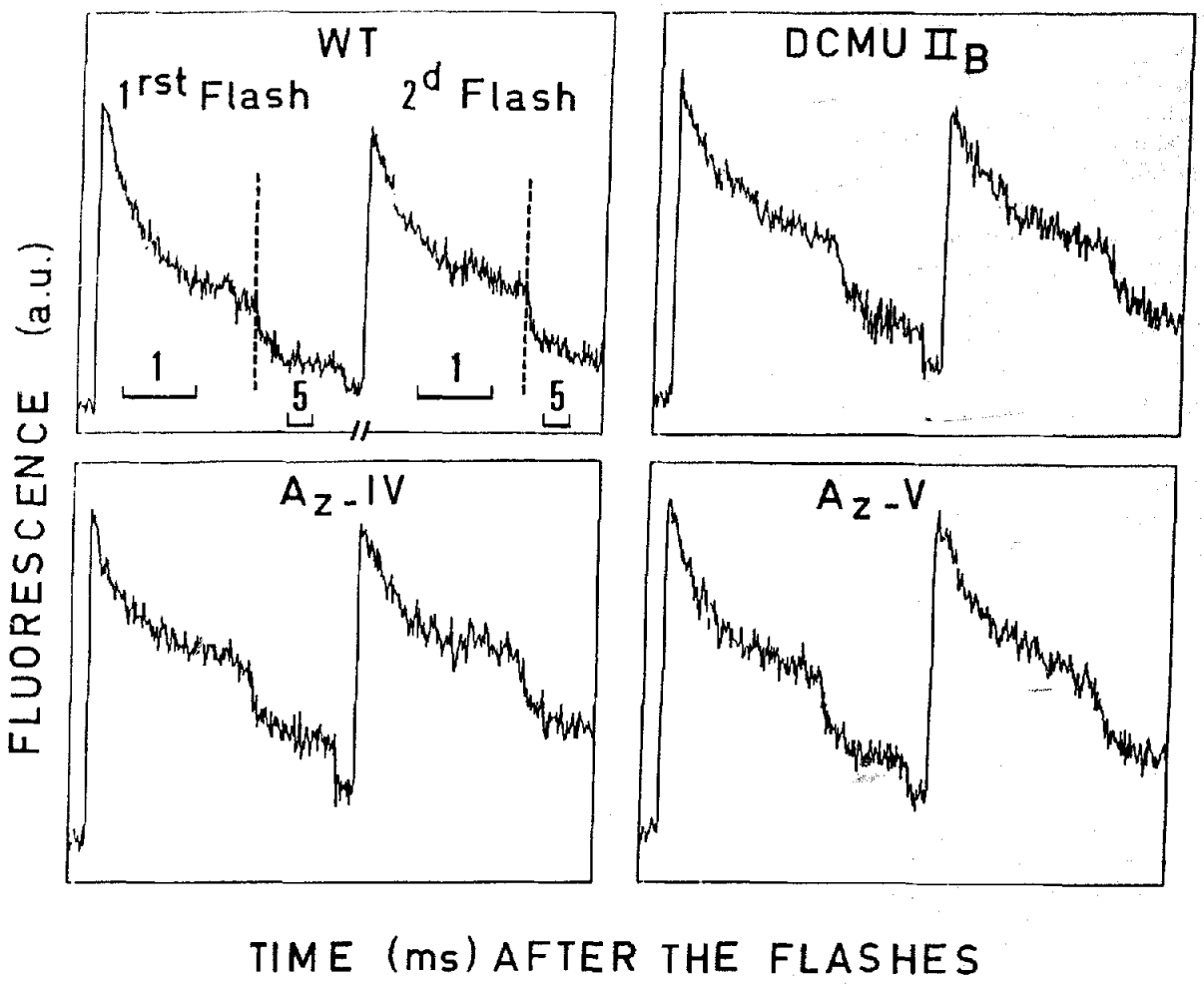

Fig.1. Decay of fluorescence following 2 short-saturating flashes spaced at 0.5 s. Cells were dark adapted for 15 min and fluorescence was detected at times between 100 μs and 14 ms as described in section 2. F_0 values were measured with the detecting beam before the actinic flashes; chlorophyll concentration used was 2 μg/ml.

Table 3
Decomposition in two exponentials of the fluorescence decay

Strain	First exponential		Second exponential	
	Coeffi-cient	Half time (ms)	Coeffi-cient	Half time (ms)
Wild type	48	0.41	52	3.45
DCMU-II_B	37	0.41	63	6.9
Az-IV	28.3	0.20	72	6
Az-V	40	0.31	60	6.1

satisfactory.) Table 3 summarizes the results of this analysis.

The sequential events which occur can be described by the following set of equations:

$$Q_A^- + \text{plastoquinone} \underset{}{\overset{K_0}{\rightleftharpoons}} Q_A^- Q_B \overset{K_1}{\rightleftharpoons} Q_A Q_B^-$$

$$Q_A^- Q_B^- \overset{K_1'}{\rightleftharpoons} Q_A Q_B^{2-} \overset{K_2}{\rightleftharpoons} Q_A Q_B$$

The exact values for the affinity constants between the Q_B site and Q_B in its different redox

states (Q_B, Q_B^-, Q_B^{2-}) are not known. The equilibrium constants between Q_A, Q_B^-, and Q_A, Q_B^{2-} are different [15]. Therefore it is premature to interpret the significance of the fluorescence two exponential decay. The fast phase is indicative of the initial reoxidation rate of Q_A^- when the Q_B site is occupied by either Q_B or Q_B^-. The slower phase has to do with the apparent affinity constant of Q_B for its site (if the site remains empty, Q_A^- will not be readily reoxidized). In the mutant strains, the second phase is slowed by a factor of less than two.

4. DISCUSSION AND CONCLUSION

We have selected mutants of *Synechocystis* 6174 highly resistant to atrazine and/or to DCMU and studied three of them: DCMU-II_B, DCMU resistant, atrazine sensitive; A_Z-IV, resistant to both herbicides; A_Z-V, atrazine resistant, DCMU sensitive.

Despite their different phenotypes, their fluorescence kinetics show that the consequences of three different mutations are similar for the mechanism of electron transfer between Q_A and the plastoquinone pool. The electron transfer efficiency is slightly decreased in the three mutants. Our interpretation is that the apparent affinity of Q_B for its site is somewhat decreased and after Q_A^- formation some sites remain empty for a longer time with as a result a slower overall reoxidation of Q_A^-. When Q_B is in its site the electron transfer between Q_A and Q_B occurs at the normal rate.

So for the first time, we have clear results showing that it is possible to obtain by mutation highly herbicide resistant mutant strains which retain a photosynthetic activity close to normal. We have now to characterize the mutated genes and to check if the phenotypes can be induced in the wild type cells by transformation with the *psb*A genes from the mutants.

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