Anacystis Mutants with Different Tolerances to DCMU-Type Herbicides Show Differences in Architecture and Dynamics of the Photosynthetic Apparatus, Depending on Site and Mode of the Amino Acid Exchanges in the D 1 Protein*

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Dedicated to Prof. Dr. A. Trebst on the occasion of his 60th birthday

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Mutants of *Anacystis* R 2 with different amino acid exchanges in positions 255 and/or 264 in copy I of the *psb*A gene, leading to different tolerances to DCMU-type herbicides, are compared with the respective wild type concerning pigmentation and incorporation of ^{35}S into the D 1 protein upon growth in the presence of [^{35}S]methionine. All mutants have shade-type appearance compared to the wild type, although to different extents depending on site and mode of the amino acid exchange in the D 1 protein. Except for 3 mutants, there is no correlation between shade-type appearance on one hand and resistance towards a certain inhibitor on the other hand.

Not only the molar ratio of phycocyanin (PC) to chlorophyll (Chl) is higher in all mutants compared to the respective wild type, but also the rate of synthesis of the D 1 protein. On the background of different levels of total ^{35}S incorporation within 18 min, D 1 synthesis can be related to shade adaptation. Degradation of the D 1 protein remains to be thoroughly studied in this context.

No reproducible differences in whole chain electron transport were observed between mutants and wild type.

Introduction

Tolerance to DCMU-type herbicides has been found to map within the *psb*A gene [1–7] which encodes the PS II reaction center polypeptide D 1 thought to bind Q_B, the secondary plastoquinone electron acceptor of photosystem II [8–10]. Binding of Q_B to D 1 is impaired by DCMU-type inhibitors to different extents. In contrast to green algae and higher plants, cyanobacteria have more than one *psb*A gene. In *Anacystis* two of the three copies remain wild type, when copy I of the family mutates to herbicide resistance [6, 7, 11]. Several different amino acid exchanges in the D 1 protein can cause tolerance to DCMU-type herbicides in organisms with two photosystems (for references see [12–14]).

Among other amino acids in positions 211 [13], 219, 251, and 275, Phe-255 and Ser-264 are frequently exchanged [7]. In many higher plants [15] the exchange of Ser-264 *versus* Gly, leading to a high resistance towards atrazine, was shown to induce a decrease in the ratio of chlorophyll *a* to chlorophyll *b*, together with increases in both, the antenna size of photosystem II (PS II) and in grana stacks. This was also demonstrated for otherwise isogenic lines of *Brassica napus* [16].

Traits characteristically developed during growth in low light intensity have also been observed in herbicide-resistant cyanobacteria, the most prominent feature being a shift in the ratio of phycocyanin (PC) to chlorophyll (Chl), *i.e.* a relative enlargement of the PS II antenna [17–19] characteristic for naturally shade adapted cells and for cells grown in high light intensity in the presence of DCMU-type inhibitors [17, 18, 20–22].

Abbreviations: Atrazine, 2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine; D 1, reaction center polypeptide, carrying the secondary plastoquinone electron acceptor Q_B of photosystem II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; metribuzine, 4-amino-6-(1,1-isopropyl)-3-methyl-thio-1,2,4-triazine-5-one; PCC, Pasteur Culture Collection; PS II, photosystem II; *psb*A gene, gene encoding the reaction center polypeptide D 1; Q_A and Q_B, primary and secondary plastoquinone electron acceptors of PS II, respectively; terbutryn, 2-(*tert*-butylamino)-4-(ethylamino)-6-methyl-thio-*s*-triazine.

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During the present investigation it became obvious, that different amino acid exchanges in the D 1 protein in context with mutation to herbicide tolerance lead to different degrees of shade-type appearance in *Anacystis* as judged from the PC/Chl ratio. Moreover, different rates of synthesis of the D 1 protein were observed in mutants and wild type under identical culture conditions.

In green algae and higher plants the D 1 protein is known to be characterized by light intensity dependent synthesis and degradation, the rate of which can be slowed down by DCMU-type inhibitors (for literature see [3, 23]). Also for cyanobacteria it could be shown that presence of DCMU-type inhibitors prevents the degradation of D 1 in strong light [24] or leads to a slow rate of D 1 synthesis in the wild type [21]. Low rates of D 1 synthesis have been also demonstrated to be characteristic for shade-grown *Anacystis* [21].

If shade-type appearance in *Anacystis* were generally connected to low rates of D 1 synthesis and breakdown, one could also expect a slower breakdown of the D 1 protein in the herbicide-tolerant mutants, as they appear shade adapted to different extents compared to the wild type. There is preliminary evidence, however, that just the opposite is the case in all herbicide-resistant *Anacystis* mutants tested so far [25]. In the present paper not breakdown but instead, rate of D 1 synthesis will be compared to the wild type. The attempt will be made, to find a correlation between mode of amino acid exchange on one hand and herbicide tolerance, shade-type appearance and dynamics of the D 1 protein on the other hand.

Materials and Methods

Cyanobacteria, *Anacystis nidulans* R 2 (*Synechococcus* R 2, PCC 7942) and mutants of this strain (kindly provided by Dr. J. Hirschberg, The Hebrew University, Jerusalem) with reduced binding capacities for DCMU-inhibitors (Table, ref. [11, 26–28]), were grown in Allen's media [29] at 32 °C in continuous white light (110 $\mu E\ m^{-2}\ s^{-1}$) [21]. Initial optical density of the cultures was adjusted to 0.280 at 800 nm. Batch cultures were analyzed or harvested after 6 days growth. Routine absorption spectra were recorded with a Kontron Uvikon 810; light scattering effects were minimized. Molar ratios of phycocyanin (PC) to chlorophyll (Chl) were calculated from the absorption spectra of whole cells using the equations and coefficients given by Myers [30]. Results (Fig. 1, Table) are given in molar ratios of chromophores. Whole chain electron transport was measured as described before [18] with or without 2,5-dimethyl-benzoquinone or phenyl-*p*-benzoquinone as electron acceptors.

Pulse labelling (^{35}S) experiments, preparation of samples for electrophoresis and electrophoretic separation of whole cell proteins were described earlier [20, 21]. Samples containing equal numbers of cells were used as starting material. Lanes in Fig. 2 are comparable with respect to cell number and concentration of [^{35}S]methionine during the experiment. X-ray films were scanned and evaluated with a Desaga densitometer CD 60.

Results and Discussion

Concerning pigmentation, all mutants appear shade adapted compared to the wild type, although to different extents (Fig. 1, Table). Deepest shade character, expressed in molar ratio of phycocyanin (PC) to chlorophyll (Chl) (0.615), is observed in double mutant D 5 (Tyr-255, Ala-264), while single mutants Di 1 (Phe-255, Ala-264) and Tyr 5 (Tyr-255, Ser-264) and are found to be intermediate (0.540 and 0.504, respectively) (Fig. 1, Ta-

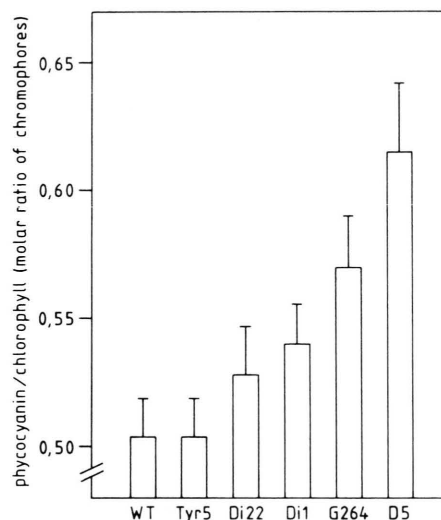


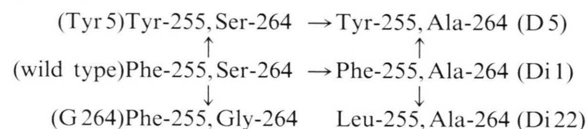
Fig. 1. Comparison of *Anacystis* R 2 wild type and herbicide-tolerant mutants Tyr 5, Di 22, Di 1, G 264 and D 5 (Table) with respect to the ratio of phycocyanin to chlorophyll. Molar ratio of chromophores is given on the ordinate.

Table. Comparison of wild type *Anacystis* R 2 with herbicide-tolerant mutants obtained *via* exchange of amino acids in positions 255 and 264 of the D 1 protein [11, 26–28].

Parameters	Herbicide-tolerant mutants D 5	G 264	Di 1	Di 22	Tyr	Wild type
Amino acid in position 255	Tyr	Phe	Phe	Leu	Tyr	Phe
264	Ala	Gly	Ala	Ala	Ser	Ser
Rel. resistance to						
diuron	167	10	100	1500	1.5	
metribuzine	2000		5000	175	0.8	
atrazine	200	2000	30	1.3	25	
terbutryn	670	450	17	1.3	13	
(confer refs. [11, 26–28])						
Phycocyanin/chlorophyll (ratio of chromophores)	0.615	0.570	0.540	0.528	0.504	0.504
Mean errors	0.027	0.020	0.016	0.019	0.015	0.015

ble). In fact, this sequence also corresponds to the sequence in atrazine resistance: While mutant Tyr 5 has nearly wild type character with respect to atrazine resistance, mutant D 5 is highly tolerant to this triazine derivative, and mutant Di 1 takes a somewhat intermediate position [11, 26–28].

Leaving the system of wild type and mutants Tyr 5, Di 1 and D 5 (confer 28) and exchanging Phe-255 for Leu instead of the aromatic amino acid Tyr in mutant D 5 (Tyr-255, Ala-264), leads to a double mutant Di 22 (Leu-255, Ala-264) with an extremely high resistance to diuron [27], a low resistance to atrazine and intermediate shade-type appearance (Table). In contrast, mutant G 264 (Phe-255, Gly-264) which has the amino acid exchanges originally only found in atrazine resistant green algae and higher plants, is highly atrazine resistant (J. Hirschberg, personal communication), but has a lower PC/Chl ratio than the corresponding double mutant D 5 with Tyr in position 255 (Table).



Apparently, there is no correlation between the resistance towards a certain herbicide on one hand [11, 26–28] and shade-type appearance on the other hand (Fig. 1, Table) except for atrazine and the three mutants Tyr 5, Di 1 and D 5 mentioned above.

Concerning the dynamics of the D 1 protein, the results are more than unexpected: *Anacystis* grown in low light (shade) in the absence or in strong light in the presence of DCMU-type inhibitors is characterized by low rates of D 1 synthesis [21]. Therefore, low rates both, of synthesis and also of breakdown (confer [24]) of the D 1 protein would be expected in the apparently shade adapted mutants. However, there is preliminary evidence from experiments of Amir-Shapira *et al.* [25], that the D 1 protein in the herbicide-tolerant *Anacystis* mutants investigated so far is less stable than the wild type; degradation of the altered D 1 in these mutants was observed to be faster than in the wild type. It was also reported that all of these mutants had a reduced stability of Q_B^- [31], thus, possibly inferring a shorter binding period to D 1. According to Trebst [10] on the other hand, D 1 is only stable when the binding pocket is occupied by either a quinone or a stabilizing herbicide of the DCMU-type. In the present investigation it could be shown, that the obviously accelerated breakdown of D 1 in the mutants appears to be compensated by a higher rate of synthesis. Fig. 2 clearly demonstrates that the rate of D 1 synthesis in most of the mutants – Di 1, Di 22, G 264 and D 5 – is higher than in the wild type. On the gel, equal numbers of cells of the different mutants are compared with respect to ^{35}S incorporation into their polypeptides. Again, as with the PC/Chl ratios (Fig. 1) there are gradual differences between the mutants, double mutant D 5 (387 arbitrary units) being the

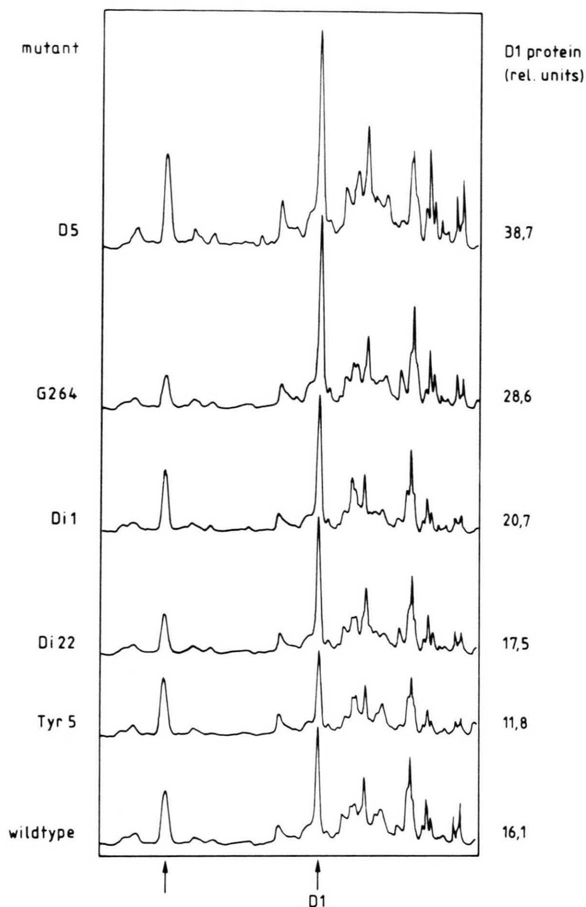


Fig. 2. Comparison of *Anacystis* R 2 wild type and herbicide-tolerant mutants (Table) with respect to the rate of D 1 synthesis. Distribution of radioactivity in the polypeptide pattern of whole cells after 18 min exposure to [^{35}S]methionine. Densitometric scans of fluorograms.

fastest while single mutant Di 1 (207) has an intermediate position between wild type (161) and mutant D 5 (confer [20]). For D 1 synthesis, there is a clear correlation between shade-type appearance on one hand and D 1 quantity in an equal number of cells after 18 min exposure to [^{35}S]methionine on the other hand, starting from wild type (161 arbitrary units) over Di 22 (175), Di 1 (207) and G 264 (286) to D 5 (387), a series which, this time, does not exclude double mutant Di 22 and single mutant G 264.

In part, these data have to be discussed on the background of differences in ^{35}S incorporation. Highest incorporation of [^{35}S]methionine into whole cell proteins was observed in mutant D 5 (1626 arbitrary units), followed by G 264 (880), Di 1 (677), Di 22 (666) and Tyr 5 (616), wild type level was 790. Under the certainly critical assumption that wild type and all mutants had reached the same total amount of label in their proteins after 18 min exposure to [^{35}S]methionine, a different sequence of mutants emerges, starting from mutant G 264 (1.60) over Di 1 (1.50), Di 22 (1.29) and D 5 (1.17) to Tyr 5 (0.94); wild type level being set 1.0. The same sequence is found, independent on the total amount of radioactivity incorporated, when the percentage of D 1 among all the polypeptides synthesized after 18 min is compared: G 264 (32.5) – Di 1 (30.6) – Di 22 (26.3) – D 5 (23.8) – wild type (20.4) – Tyr 5 (20.1).

It should also be mentioned that different patterns of polypeptide synthesis can be observed in different mutants (Fig. 2), the most striking difference being the amount of a low molecular weight component yet unidentified. Concerning the amount of this polypeptide freshly synthesized within 18 min, a different sequence of mutants emerges, starting from D 5 (211 arbitrary units) over Di 1 (131), Tyr 5 (124) and Di 22 (76) to G 264 (64), wild type (120) being similar to mutant Tyr 5, or in percent of total protein synthesis: wild type (20.4) – Tyr 5 (20.1) – Di 1 (19.3) – D 5 (13.0) – Di 22 (11.5) – G 264 (7.3).

From the comparison of the present data on herbicide-tolerant mutants grown under identical conditions and data on wild type *Anacystis* grown under different conditions of light intensity and herbicide binding [21] the conclusion has to be drawn, that shade-type appearance in herbicide-tolerant mutants is not related to D 1 *synthesis* in a simple way. It can, however, be well imagined that a delicate balance between synthesis and degradation of this protein gives rise to shade-type appearance in *Anacystis*. For this reason, rate of D 1 breakdown needs to be studied more thoroughly in comparison with rate of synthesis in mutants and wild type.

If shade-type appearance in herbicide-tolerant mutants were correlated with a reduction in PS II electron transport as a consequence of the altered primary structure of the Q_B binding protein (D 1), the mutants with the highest PC/Chl ratios should be severely impaired compared to the wild type. In fact, in all mutants mentioned in this paper, the

stability of Q_B^- was observed to be lower than in the wild type [31]. In the present investigation, no clear reproducible difference in whole chain electron transport, measured as light dependent O_2 evolution with or without 2,5-dimethylbenzoquinone or phenyl-*p*-benzoquinone as electron acceptors, was observed between wild type and mutants. These observations on *Anacystis* correspond well to some results with *Chenopodium*, where also the alteration in the primary structure of the D1 protein, leading to tolerance to DCMU-type herbicides, does not impair whole chain electron transport. In contrast, other higher plant mutants have a reduced photosynthetic capacity, some of them reaching wild type levels in saturating light intensity. A triazine-resistant mutant of *Phalaris paradoxa* even shows a higher photosynthetic capacity (V_{max}) than the wild type [34, 35] and there is an early report by Erickson [2] on herbicide-tolerant mutants with impaired and others with unimpaired electron transport: High resistance to atrazine is usually paralleled by altered electron transport. Astier *et al.* [36] found electron transfer efficiency between Q_A and the plastoquinone pool decreased in their herbicide-tolerant mutants of *Synechocystis*.

Electron transport remains to be reinvestigated in *Anacystis* wild type and mutants described in this paper, in order to make a decision to which extent shade-type appearance of the mutants is a consequence of a possibly impaired electron transport and to which extent the dynamics of the D1 protein itself, whose primary structure is altered in the herbicide tolerant mutants described [11, 26–28], is responsible for the organization of the photosynthetic apparatus.

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