Metribuzin-Resistant Mutants of *Chlamydomonas reinhardii*

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Herbicide resistance in *Chlamydomonas reinhardii* cells was induced by mutagenesis with 5-fluorodeoxyuridine and ethylmethanesulfonate. Four mutant strains were isolated and analyzed for resistance against DCMU-type or phenolic inhibitors of photosynthetic electron transport.

The mutants were different in both the extent and the pattern of their resistance: the R/S value, *i.e.* the ratio of I_{50} values of the inhibition of photosynthetic electron transport in isolated resistant and susceptible thylakoids, varied for metribuzin from 10 000 to 36. The mutant MZ-1 was resistant against metribuzin, atrazine and DCMU, whereas the mutant MZ-2 showed resistance mainly against metribuzin and atrazine. The mutant MZ-3 was similar to MZ-1, but showed a lesser extent of resistance against DCMU. The mutant MZ-4 showed resistance against metribuzin, but not against atrazine. These results demonstrate that the resistance against one herbicide of the DCMU-type (metribuzin) must not be accompanied by similar resistance against te other inhibitors. Binding studies with radioactively labeled herbicides, [^{14}C]metribuzin, [^{14}C]atrazine and [^{3}H]DCMU, and isolated thylakoids supported these observations.

Phosphorylation of thylakoid membrane proteins was studied with wild-type cells and resistant mutants under *in vivo* conditions in the light. The ^{32}P-labeled main proteins bands were in the molecular weight range of 10–14 kDa, 26–29 kDa, 32–35 kDa and 46–48 kDa. The pattern and the extent of incorporation of ^{32}P were similar for the mutants and the wild-type cells.

Introduction

Herbicide resistance of photosynthetic electron transport is a useful tool for selecting a variety of organisms, which have altered properties of the herbicide binding protein (Q_B-protein). The importance of this protein as the major target for commercial herbicides and as part of the electron transport chain ($Q_A \rightarrow Q_B$) promoted the establishment of its genetics with the subsequent determination of the DNA sequence [1, 2]. The procedure for identification of the "active sites" (binding sites for plastoquinone and for the diverse herbicide molecules) with the related questions as to whether they are common, overlapping or completely different, is a major goal for achieving a better understanding of the basic mechanism of herbicide action [3]. Within this framework, therefore, we isolated a series of metribuzin-resistant mutants and analyzed and characterized their resistance patterns in order to have a genetic stock of different mutants with a common wild-type as a source for protein analysis. The characterization of their resistance properties was achieved by binding studies with labeled inhibitors, which yielded the number of specific binding sites and binding constants, and by determination of the pI_{50} values.

Since it was reported that the Q_B-protein can be phosphorylated in chloroplasts [4, 5], and that the dephosphorylation of the Q_B-protein alters the binding affinity for DCMU [5], it was of special interest to investigate, whether the mutation of this protein affects its phosphorylation. The intact cells were incubated in light with ^{32}Pi to study the phosphorylation *in situ*. Before this treatment the light grown cells were dark-adapted, and the ^{32}Pi was added with the onset of illumination. To avoid significant losses due to dephosphorylation the thylakoids were rapidly processed in the presence of 100 mM sodium fluoride.

Abbreviations: Atrazine, 2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; DNSJ, 2-iodo-4-nitro-6-isobutylphenol; DTT, dithiothreitol; ioxynil, 4-hydroxy-3,5-diiodobenzonitrile; metribuzin, 4-amino-6-isopropyl-3-methyl-thio-1,2,4-triazin-5-one; Q_A, primary quinone electron acceptor of photosystem II; Q_B, secondary quinone electron acceptor; SDS, sodium dodecyl sulphate.

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Results and Discussion

Isolation of mutants

The induction of mutagenesis was carried out with 5-fluorodeoxyuridine and ethylmethane sulfonate [6] in cells of *Chlamydomonas reinhardii* strain 2137 mt +, isolated by Spreitzer and Mets [7].

The screening for metribuzin resistance was performed on plates with 10^{-5} M of this herbicide in the light. Those clones were selected, which showed normal growth and pigmentation. Four mutants (MZ-1, MZ-2, MZ-3, and MZ-4) were isolated in different mutation experiments.

Characterization of mutants

The photosynthetic rate ($H_2O \rightarrow CO_2$) of the mutant cells was similar to the wild-type cells and varied between 90–100 μmol O_2/mg Chl × h. The chlorophyll (Chl) content of the cells and the chlorophyll *a*/*b* ratio was also similar. The analysis of the thylakoid membrane proteins, dissolved by SDS and separated by gel electrophoresis [8], showed no significant differences between mutant and wild-type cell thylakoids.

Resistance and electron transport inhibition

Thylakoids were isolated after cell fractionation in a Ribi cell fractionator (Sorvall) and subsequently purified by sucrose density gradient centrifugation [6]. The incubation of the thylakoids in presence of the inhibitors occurred for 2 min in the dark. Photosynthetic electron transport was measured as DCPIP reduction with H_2O as electron donor. The pI_{50} values for the electron transport inhibition in the thylakoids of the mutant cells and the wild-type cells were determined at different concentrations of the inhibitor and extrapolated to 50% inhibition, keeping constant the amount of chlorophyll in all the assays.

As shown in Table I, the *R*/*S* value (*i.e.* the ratio of I_{50} values for resistant and susceptible thylakoids) for the MZ-1 thylakoids was 10 000 (metribuzin), 126 (atrazine), and 200 (DCMU). Similar values were obtained for mutant MZ-3: 4570 (metribuzin), 100 (atrazine), and 126 (DCMU). Mutant MZ-2 was more susceptible: 910 (metribuzin), 25 (atrazine), and 2.5 (DCMU). Mutant MZ-4 was completely different as compared to the others with a *R*/*S* value of 36 (metribuzin), and was not resistant against the other herbicides. It is interesting to note that mutants MZ-1, MZ-3, and MZ-4 are more susceptible against phenolic electron transport inhibitors such as DNSJ [10] and ioxynil, whereas mutant MZ-2 was significantly resistant against ioxynil (Table I).

Table I. Determination of the resistance of the herbicide mutants.

Strain	Inhibitor	pI_{50}	pK_B	x_t
Wild-type	Metribuzin	7.5	7.6	0.98
	Atrazine	6.7	6.3	2.93
	DCMU	7.7	7.4	1.78
	DNSJ	6.8	6.8	2.74
	Ioxynil	6.3	6.3	3.46
MZ-1	Metribuzin	3.5	no specific binding	
	Atrazine	4.6	5.0	(8.68)
	DCMU	5.4	5.0	(23.7)
	DNSJ	7.4	6.9	3.27
	Ioxynil	6.2	6.2	3.55
MZ-2	Metribuzin	4.5	no specific binding	
	Atrazine	5.3	5.3	6.08
	DCMU	7.0	7.0	1.75
	DNSJ	6.6	6.6	5.56
	Ioxynil	4.7	5.9	6.08
MZ-3	Metribuzin	3.8	no specific binding	
	Atrazine	4.7	5.4	5.39
	DCMU	5.6	6.5	2.06
	DNSJ	7.5	6.7	3.74
	Ioxynil	6.3	6.3	4.27
MZ-4	Metribuzin	5.9	5.8	1.75
	Atrazine	6.6	6.2	3.48
	DCMU	7.0	6.5	2.77
	Ioxynil	6.7	6.4	3.43
	DNSJ	7.0	6.8	3.08

Resistance and inhibitor binding

The purified thylakoids were incubated with either [^{14}C]metribuzin, [^{14}C]atrazine, [^{3}H]DCMU, [^{14}C]ioxynil or [^{3}H]DNSJ [10] in separate experiments. The specific binding was determined according to the method of Tischer and Strotmann [9]; the binding constants (K_B) and the number of binding sites (x_t) were calculated from Scatchard plots. The results, as presented in Table I, show that no specific binding could be observed with mutants MZ-1, MZ-2, and MZ-3, but in the experiments with mutant MZ-4 a specific binding with a binding constant pK_B of 5.8 could be detected. This is in good agreement with the higher pI_{50} value of 5.9. Despite the complete resistance against metribuzin, the thylakoids could still specifically bind atrazine and DCMU, although with lower pK_B values.

It can be concluded that the mutagenic alteration of the Q_B-protein affects the binding site for metri-

Table II. Phosphorylation of thylakoid proteins.

Strain	cpm 32–35 kD × 100 / cpm tot. thylakoid	cpm 10^{-3} 32–35 kD / mg chlorophyll	cpm 10^{-3} LHCP / mg chlorophyll	cpm 32–35 kD / cpm LHCP
Wild-type	0.23	12.0	58.9	0.20
MZ-1	0.22	10.1	46.7	0.22
MZ-2	0.22	10.1	35.6	0.28
MZ-3	0.19	6.7	30.6	0.22
MZ-4	0.29	14.2	61.8	0.23

cpm = counts per minute.

buzin in a way that a complete loss is observed. An interesting aspect of the differences in the binding sites for "DCMU-type" inhibitors could be obtained by comparing the binding of DCMU or metribuzin for the mutant MZ-2. The binding constants (MZ-2: $pK_B = 7.0$; WT: $pK_B = 7.4$), and the number of specific binding sites (MZ-2: $x_t = 1.75$; WT: $x_t = 1.78$) are comparable. Since no specific binding was observed for metribuzin, it can be assumed that the binding sites for DCMU and for metribuzin are at least partially different. Furthermore, the binding behaviour of atrazine was similar to metribuzin, but not to DCMU.

In vivo-phosphorylation of metribuzin resistant mutants

Light grown, P_i starved cells (wild-type, MZ-1, MZ-2, MZ-3, and MZ-4) were incubated in presence of $^{32}P_i$ (6×10^5 Bq $^{32}P_i$/mg Chl; 0.1 mg Chl/ml) in light (2.2×10^6 erg/cm^2 × s) at 25° for 30 min. The cells were washed after incubation with 20 mM tricine buffer, pH 7.8, containing 100 mM NaF, resuspended in the same buffer and sonicated five times for 15 s. The thylakoids were purified by a discontinuous sucrose gradient centrifugation (210 000 × g, 1 h). The green thylakoid band was collected, diluted with the same buffer, pelleted and finally resuspended in 60 mM Na_2CO_3; 60 mM DTT and solubilized in 2% SDS. The thylakoid membrane proteins were fractionated by SDS-polyacrylamide gel electrophoresis according to the method of Chua [8]. The gels were stained with Coomassie brillant blue R, destained, dried and autoradiographed using Kodak X-Omat AR X-ray film. The radioactive bands were excised and the radioactivity determined by liquid scintillation counting.

Under our experimental conditions the uptake of $^{32}P_i$ into the cells was 99%, and in the incorporation into thylakoids was about 15%. The major thylakoid proteins, which were labeled by ^{32}P can be assigned to a molecular weight range of 10–14 kDa, 26–29 kDa, 32–35 kDa, and 46–48 kDa. The results of the incorporation experiments with different metribuzin resistant mutants are summarized in Table II. The pattern of the stained proteins and of the autoradiography was identical in all four mutants and the wild-type thylakoids. The quantitative determination of the radioactivity reveals that the ratio of ^{32}P incorporated into the protein of the 32–35 kD range and the light harvesting chlorophyll *a*/*b* protein complex (LHCP) was also constant (Table II). It can be concluded that the mutation of the Q_B-protein and the concomitant resistance against herbicides of the "DCMU-type" do not interfere with the phosphorylation of the protein. This observation could be explained either by assuming that the Q_B-protein and the phosphorylated protein are not identical or that the mutated site on the Q_B-protein and the phosphorylation site do not interfere.

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