A Herbicide Resistant *Euglena* Mutant Carrying a Ser to Thr Substitution at Position 265 in the D 1 Protein of Photosystem II

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Z. Naturforsch. **47 c**, 245–248 (1992); received October 31, 1991

Herbicides, D 1-Protein, Point Mutation, Resistance, *Euglena*

A herbicide resistant *Euglena* mutant (MSI) has been obtained by adapting wild type cells to increasing concentrations of DCMU (3-(3′,4′-dichlorophenyl)-1,1-dimethylurea). Lower resistance levels towards DCMU and metribuzin were observed in MSI when compared with *Euglena* or *Chlamydomonas* mutants with Ser 264 to Ala substitutions. RNA-sequence analysis identified a Ser to Thr change at position 265 (equivalent to position 264 in other organisms), thus making it possible to compare the influence of amino acids Ser, Ala and Thr at identical positions on the inhibitory effect of structurally different herbicides in the same species.

Introduction

The D 1 subunit of the photosystem II reaction center is the target for a variety of inhibitors some of which are potent herbicides. These inhibitors displace the loosely bound plastoquinone molecule Q_B and thus can serve as probes for scrutinizing the Q_B-binding niche located in a specific region of the D 1 protein. A detailed structural model describing the role of individual amino acid residues within this binding niche is gradually evolving [1–6]. The model is based on specific homologies to the bacterial reaction center of purple bacteria [7], on photoaffinity labeling studies [8, 9], and on the analysis of point mutations in the D 1 protein of herbicide resistant organisms [3, 4, 10].

Eight different, single amino acid substitutions have been reported in higher plants, algae and cyanobacteria. Mutations have been identified by sequencing the *psb*A gene, which codes for D 1 and is located on the chloroplast genome. All mutations are clustered between amino acids 211 and 275. Depending on the position of the mutation and on the amino acid substitution, different cross resistancies towards different inhibitor classes are observed. The most frequently observed amino acid substitution occurs at position 264 in higher plants and *Chlamydomonas reinhardtii,* where a Ser is replaced by a Gly or an Ala, respectively. In herbicide resistant *Euglena,* a Ser to Ala change has been detected at position 265, which corresponds to codon 264 in all other organisms due to an additional amino acid at the N-terminus [11, 12].

According to a recent model elaborated by molecular graphics methods [3], Ser 264 is in contact with the Q_B-binding niche and provides hydrogen bonds to various inhibitors. In addition, this amino acid stabilizes the conformation of the binding niche by forming a hydrogen bond to His 252. The identification of a Ser to Thr change at position 265 in an *Euglena* mutant described here offers a unique possibility to independently compare the influence of the amino acids Ser, Ala and Thr at identical positions on the effect of structurally different inhibitors in the same organism.

Materials and Methods

The *Euglena* mutant MSI was obtained by adapting cells from *Euglena gracilis* Z (Sammlung von Algenkulturen, Göttingen) to increasing DCMU concentrations [13]. Thylakoids were isolated by a modification of the procedure described

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Verlag der Zeitschrift für Naturforschung, D-W-7400 Tübingen
0939–5075/92/0300–0245 $ 01.30/0

by Janatkova and Wildner [14]. Cells harvested in the logarithmic growth phase were suspended in 20 mM HEPES buffer, pH 7.5, 0.3 M sorbitol, 5 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM KCl at a concentration of 0.3 mg chlorophyll per ml, and broken by two passages through the Yeda press at 4000 kPa. Thylakoids were sedimented at 8000 × *g* for 5 min, resuspended in the above mentioned buffer and the chlorophyll concentration adjusted to 1 mg/ml. For determination of pI_{50}-values inhibitors were preincubated for 2 min in the dark before measuring uncoupled electron transport from water to 2,6-dichlorophenol-indophenol (DCPIP). Control rates varied between 60 and 70 µmol DCPIP/mg/h in assays containing 3 µg chlorophyll/ml.

RNA from *Euglena* cells was isolated according the method of Weeks *et al.* [15]. For direct sequencing of RNA, a 5′-end-labeled, synthetic oligonucleotide (5′-GCTGTAAACCAAATACC) was annealed to 70 µg total RNA. Labeled cDNA fragments were produced by reverse transcriptase in the presence of dideoxynucleotides (ddNTPs), separated on 8% polyacrylamideurea gels and visualized by autoradiography [16].

Results

The *Euglena* mutant MSI has been obtained by adapting cultures of *Euglena gracilis* Z to increasing concentrations of DCMU. The mutant has been extensively characterized [13]. It proved to be stable under non-selective conditions and its growth rate did not differ from that of the wild type under photoautotrophic or photoheterotrophic conditions.

Initial experiments showed that photosynthetic electron transport in the MSI mutant is resistant to various inhibitors in whole cells as well as in isolated thylakoids. Especially intriguing was the observation, that the MSI mutant was significantly less resistant to DCMU or metribuzin as compared with the earlier characterized *Chlamydomonas reinhardtii* mutant MZ 1 with an amino acid substitution from Ser to Ala at position 264 [17]. This prompted us to analyze that part of the *Euglena* MSI *psb*A mRNA which codes for amino acids in and between helices IV and V of the D 1 protein, *i.e.* the region in which all point mutations responsible for herbicide resistance have been located to date [4].

As has been described earlier [16], total RNA isolated from wild type and mutant strain was directly sequenced using a synthetic, end-labeled oligonucleotide and reverse transcriptase. The autoradiogram of a sequencing gel shown in Fig. 1 unambigously identifies a transversion from T to A in the mutant. The base change results in an amino acid substitution from Ser to Thr at position 265. The absence of any signal in the T lane at codon 265 indicates, that most if not all mutant *psb*A copies carry this modification.

This mutant and the previously characterized mutant ZR with a Ser to Ala change at position 265 [12, 18] were analyzed by determining the sensitivity of photosynthetic electron transport rates from water to DCPIP towards various classes of inhibitors (Table I). R/S values express the ratio of I_{50} values from mutant and wild type *Euglena* thylakoids. The most remarkable difference is observed for the classical urea-type inhibitor DCMU. The ZR mutant is about 16 times more resistant to DCMU than the MSI mutant. Benzthiazuron, a substituted urea derivative, and the triazinone metribuzin are also more effective inhibitors in MSI cells as compared with ZR cells. Inhibitory potencies of cyanoacrylate and the triazine compound atrazine are slightly reduced in the MSI mutant when compared with ZR cells. Phenolic compounds like ioxynil, ketonitrile and BNT become better inhibitors in both mutants.

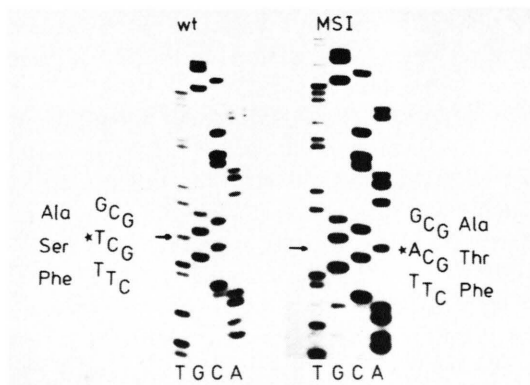

Fig. 1. Autoradiogram of RNA-sequencing gels identifying the transversion from a T in wild type *Euglena* (wt) to an A in *Euglena* mutant MSI (arrows, stars). T, G, C and A indicate the nucleotides complementary to the ddNTP added to the reverse transcriptase reaction.

Table I. R/S- and pI_{50}-values (negative log of herbicide concentration giving 50% inhibition) of various herbicides for inhibition of uncoupled electron flow from water to DCPIP in thylakoid membranes isolated from wild type (wt) and mutant (ZR, MSI) *Euglena* cells.

Inhibitor	Wild type Ser 265	*Euglena* mutants ZR Ala 265		MSI Thr 265	
	pI_{50}	pI_{50}	R/S	pI_{50}	R/S
DCMU[a]	7.0	4.5	316	5.7	20
Benzthiazuron[b]	6.3	4.4	79	5.0	20
Metribuzin[c]	6.3	>4	>200	4.5	63
Atrazine[d]	5.9	4.3	40	4.0	80
Cyanoacrylate[e]	7.1	5.7	25	5.4	50
Ioxynil[f]	6.1	6.5	0.4	6.9	0.2
Ketonitrile[g]	7.6	7.9	0.5	7.7	0.8
BNT[h]	7.2	8.0	0.2	7.7	0.3

[a] 3-(3,4-dichlorophenyl)-1,1-dimethylurea
[b] 1-(benzothiazol-2-yl)-3-methylurea
[c] 4-amino-6-(t-butyl)-4-methylthio-1,2,4-triazine-5-one
[d] 2-chloro-4-(ethylamino)-6-(iso-propylamino)-s-triazine
[e] 2-cyano-3-ethyl-3-(4-chlorbenzyl)-aminoacrylate ethoxyethylester
[f] 4-hydroxy-3,5-diiodobenzonitrile
[g] 2-phenylthiazolyl-3-hydroxy-4-phenyl-butenonitrile
[h] bromo-nitrothymol

Discussion

In eukaryotic organisms several mutations in the D1 protein have been described. Most of them were detected in the green alga *Chlamydomonas reinhardtii,* which has either been chemically mutagenized or, recently, created by site-directed mutagenesis [19]. For understanding the interaction of individual amino acids with inhibitors as well as with Q_B by molecular modelling methods it is of special importance to collect and analyze a great variety of mutants.

The most profound effect on herbicide binding is observed when amino acid 264 has been changed. A single substitution at this position in D1 was first found in herbicide resistant *A. hybridus* changing Ser 264 to Gly 264 [20]. Subsequently, the same substitution was reported in other herbicide-resistant higher plant species like *Solanum nigrum* [21], *Brassica napus* [22], *Chenopodium album* [23, 24], *Poa annua* [25], *Phalaris paradoxa* [26] and *Amaranthus retroflexus* [27]. In herbicide resistant cyanobacteria and algae Ser 264 is replaced by Ala 264 [12, 17, 28–31]. A Ser 264 to Asn 264 has been reported for triazine-resistant *Nicotiana plumbaginifolia* growing as a photomixotrophic cell culture [32, 33].

In atrazine-resistant cell cultures of *Nicotiana tabacum* codon 264 was changed from Ser to Thr [34]. This mutant cell line with the same amino acid substitution as the *Euglena* mutant described here has been compared in cross resistance studies with the *Amaranthus retroflexus* biotype with a Gly at position 264 [27, 34]. In a study with cyanobacteria Ohad and Hirschberg [35] compared the cross resistance of a Ser to Ala with a Ser to Gly mutant of *Synechococcus* PCC 7942.

When the cross resistance pattern of the two *Euglena* mutants carrying a Ser to Ala and a Ser to Thr mutation is compared (Table I), the most dramatic differences are observed with metribuzin and DCMU. Metribuzin is supposed to form a hydrogen bond to the side chain hydroxyl of Ser 264 [3]. Substitution by an Ala strongly weakens binding, but introduction of an OH-group through Thr in the MSI mutant might restore this interaction to some extent.

The hydroxyl group of Ser 264 does not seem to be especially relevant for binding of DCMU, because Ser to Gly [20, 35] or Asn [33] mutations in higher plants do not confer significant DCMU resistance. Replacement of Ser by Thr increases DCMU resistance by a factor of 20 (Table I) or 40 in *Nicotiana tabacum* [27]. Introduction of an Ala enhances resistance up to a factor of 316 (Table I) or 200 in *Chlamydomonas* [6]. These results suggest that, depending on the nature of the substituted amino acid, different conformational changes occur, which allow to accomodate the urea compound to a different extent. It is interesting to note, that in the bacterial quinone binding site a specific mutation confers major structural changes, which lead to DCMU sensitivity to otherwise DCMU insensitive reaction centers [36, 37].

Resistance to atrazine appears to be moderately high irrespective of the mutation. The herbicide supposedly shares a hydrogen bond with the OH-group of Ser [3], which is lost by substitution with Gly, Asn or Ala. A Thr is obviously not capable of restoring this bond, since a slight decrease rather than an increase in sensitivity can be observed. Alternatively, this bond might be of minor significance for atrazine binding. The increased sensitivity (supersensitivity) to phenolic com-

pounds observed here is seen in all Ser264 mutants.

Minor changes in the herbicide binding niche can have drastic effects on the sensitivity of electron flow towards different classes of inhibitors. Since no X-ray data of photosystem II are available right now, the combination of mutant analysis and computer modeling studies will help to identify amino acids crucial for Q_B- and inhibitor-binding and to assign functions to individual amino acids in the herbicide binding niche.

Acknowledgements

We thank Prof. A. Trebst for valuable discussions and Ursula Altenfeld and Ursula Hilp for excellent technical assistance. Automated oligonucleotide synthesis was supported by a grant from the "Minister für Wissenschaft und Forschung des Landes Nordrhein-Westfalen". This work was supported by the "Bundesministerium für Forschung und Technologie" (project no. 319308A).

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