Volume 246, number 1,2, 207–210 FEB 06974 March 1989

Mutation in phenol-type herbicide resistance maps within the *psb*A gene in *Synechocystis* 6714

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Received 29 December 1988; revised version received 6 February 1989

A *Synechocystis* 6714 mutant resistant to the phenol-type herbicide ioxynil was isolated and characterized. Sensitivity to DCMU and atrazine was measured in whole cells and isolated thylakoids. The mutant presents the same sensitivity to atrazine as the wild type and a slightly increased sensitivity to DCMU. A point mutation has been found at codon 266 in the *psb*AI coding locus (AAC to ACC) resulting in an amino acid change from asparagine to threonine in the D_1 protein.

Herbicide; Ioxynil; Mutant; Photosystem II; (*Synechocystis*)

1. INTRODUCTION

Different classes of herbicides have been known for many years to inhibit photosynthesis at the level of photosynthesis II by blocking electron transfer. Competition between the herbicide molecules and the secondary electron acceptor Q_B prevents electron transfer from Q_A^- (the primary acceptor) to Q_B. D_2 and D_1 polypeptides bind Q_A and Q_B, respectively. These two polypeptides and cytochrome *b*-559 constitute the reaction center of PS II.

Much information on the modification of D_1 protein in mutants resistant to DCMU and/or atrazine is available in the literature [1–9] but nothing concerning phenol-type herbicide-resistant mutants has appeared. These herbicides exhibit several inhibitory effects, including one on the PS II donor side [10,11]. Information on binding sites was obtained by radiolabeling with azido derivatives of the phenolic type herbicides. The first study to identify the polypeptide target of these herbicides demonstrated binding not to D_1, as for azido derivatives of DCMU or atrazine, but to a 41 kDa polypeptide [12]. More recently the D_1 polypeptide was also shown to be labeled [13].

The strong analogy between polypeptides D_1 and D_2 of PS II and the L and M subunits of bacterial reaction centers [14] and the localization of Q_B and herbicides in bacterial reaction centers [15] have led to models being proposed for PS II [16–18]. Trebst put forward the proposal that phenol-type inhibitors would be oriented towards His 215 into the D_1 binding niche. Recently, sequencing of proteolytic fragments of the D_1 protein tagged with ^{125}I-labeled azido-ioxynil (a phenol-type inhibitor) has shown that this component binds to Val 249 of D_1 [19].

In *Synechocystis* 6714 we have been able to isolate mutants resistant to the phenolic inhibitor ioxynil. Here we describe the characterization and molecular analysis of one of the mutants, Iox-I.

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS II, photosystem II

2. MATERIALS AND METHODS

The strain *Synechocystis* 6714, kindly provided by Dr R.Y. Stanier, was no. ATCC 27178 of the American Type Culture Collection.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

2.1. *Growth conditions*

The minimal medium (MM) for growth was that defined by Herdman et al. [20] with twice the concentration of nitrate. For the solid medium, 1.5% agar autoclaved separately was added. Standard photosynthetic growth was achieved by incubation in a Gallenkamp rotary shaker under constant agitation at 34°C and illumination at 4000 lux in a CO_2-enriched atmosphere. The generation time was 6 h.

2.2. *PS II activity assays*

Fluorescence under continuous illumination was measured as described [21]. The fluorescence, excited with a tungsten lamp through Corning 4-96 and 5-59 filters, was detected in the red region through a Corning 2-64 filter and a Wratten 90 filter. Recording was carried out using a multichannel analyzer. The cell suspension contained about 1 μg chlorophyll per ml.

PS II activity of thylakoids, prepared as in [22], was measured with dichlorophenolindophenol (DPIP) as an electron acceptor, at pH 6.8, from the absorption change at 580 nm.

3. RESULTS

3.1. *Isolation of mutants*

Usually, herbicide-resistant mutants are selected with a herbicide concentration which completely inhibits wild-type growth. The maximal concentration of ioxynil which can be used without effect on the donor side of PS II in *Synechocystis* 6714 is 10^{-4} M (Ajlani et al., in preparation). At this concentration, the wild-type strain is not completely inhibited. Because of this residual growth of the wild type at 10^{-4} M ioxynil, specific procedures are required for isolation of resistant mutants. Ioxynil was added to 20 cultures containing 50 ml cell suspension at 10^7 cells/ml. After 10 days, five cultures had survived. Two of them were diluted in a series of 20 subcultures, always in the presence of 10^{-4} M ioxynil. Cultures which exhibited significant growth were kept and subcultured twice. Subsequently, samples of these cell suspensions were plated in selective solid medium. Colonies obtained from different cultures were tested for their stability by being grown in the absence then in the presence of 10^{-4} M ioxynil. One of them, Iox-I, was used for further studies.

3.2. *Characterization of the mutant*

3.2.1. Whole-cell sensitivity to ioxynil and cross-resistance to other types of herbicides

The mutant Iox-I was grown in the presence of various ioxynil concentrations as described in [23]. Its growth inhibition I_{50} was determined to be 1.5×10^{-4} M ioxynil compared to 5×10^{-5} M for the wild type. The growth medium was not buffered and the pH during growth varied between 8.6 and 7.7. Ioxynil has a pK of 3.96, and very probably, only the undissociated (protonated) form is able to penetrate into the cells. For a more accurate determination of I_{50}, we measured at pH 7.0 the efficiency of various concentrations of ioxynil and other herbicides by measuring variable fluorescence in whole cells (table 1).

Cells of Iox-I are 10-times more resistant to ioxynil than wild-type cells. They also show resistance to bromoxynil, another phenol-type herbicide. They did not develop resistance to DCMU or atrazine and even slightly increased sensitivity was observed for DCMU in the mutant.

3.2.2. Thylakoid sensitivity to ioxynil and other herbicides

To ensure that the resistances to herbicides were retained in isolated thylakoids, i.e. that the mutant we obtained was not a detoxification or permeation mutant, inhibition of the Hill reaction by ioxynil was measured in thylakoids isolated from wild-type and mutant cells. Table 1 shows that higher concentrations of ioxynil are needed to inhibit the mutant. The sensitivity of thylakoids from the mutant to the herbicides bromoxynil, DCMU and atrazine presented the same characteristics as those of whole cells (table 1).

3.3. psb*AI* *cloning and nucleotide sequence determination*

A genomic DNA library of the Iox-I mutant was constructed in the vector λEMBL$_3$. This library was probed with the *psb*AI gene of the wild-type

Table 1

Herbicide sensitivity of wild-type and Iox-I mutant

Inhibitor	Whole cells		Thylakoids	
	WT	Iox-I	WT	Iox-I
Ioxynil	8×10^{-6}	8×10^{-5}	4×10^{-7}	3.6×10^{-6}
Bromoxynil	3×10^{-4}	$>10^{-3}$	1×10^{-5}	1.5×10^{-4}
DCMU	1.3×10^{-7}	8×10^{-8}	1.2×10^{-7}	8×10^{-8}
Atrazine	3×10^{-6}	2.5×10^{-6}	7×10^{-7}	7×10^{-7}

(Whole cells) I_{50} (M) is the concentration needed to block half of the variable fluorescence; (thylakoids) I_{50} represents the concentration which blocks half of the Hill activity

Synechocystis 6714 strain which we had previously cloned [9]. A positive recombinant phage was isolated. This phage contained a 15 kb *Bam*HI fragment from which a 2 kb *Hin*dIII-*Eco*RI fragment containing *psb*AI was subcloned into Bluescript plasmid. A 0.7 kb *Kpn*I fragment containing 50% of the 3'-end of the *psb*AI gene was subcloned again into Bluescript plasmid to be sequenced. Comparison with the wild-type sequence [9] showed a point mutation at codon 266 in the *psb*AI coding locus (AAC to ACC) resulting in an amino acid change from asparagine to threonine in the Q_B-binding domain of the D_1 protein.

4. DISCUSSION AND CONCLUSIONS

We have developed a method for selection of mutants modified only on the acceptor side of PS II. The Iox-I mutant was found to be 10-times more resistant to ioxynil than the wild-type strain without modification of the sensitivity of its donor side. This strain was also resistant to bromoxynil, another phenol-type herbicide, but still sensitive to DCMU and atrazine. These characteristics were retained in the isolated thylakoids.

Molecular analysis of other herbicide-resistant mutants of this strain has clearly shown that it was the copy *psb*AI which carried the different mutations responsible for DCMU and/or atrazine resistance [9]. In the Iox-I mutant, it was also in this copy that we found a point mutation at codon 266 resulting in a change from asparagine to threonine in the D_1 polypeptide.

On the scheme (fig.1) from the model developed by Trebst and Draber [17] we have indicated the mutation of the Iox-I mutant. We have also denoted the amino acid tagged by ^{125}I-labeled azido-ioxynil at position 249 [19]. These two amino acids (266, 249) are located on either side of the cytoplasmic helix, which is localised between the two transmembrane helices IV and V. This may help in specifying the conformation of this part of D_1.

In fig.1 we have also represented Ser 264. A change in this amino acid to either alanine or glycine, depending on the organism, was correlated with the resistance to urea-triazine-type herbicides. Some of these mutants show increased sensitivity to ioxynil [24]. This is not the case for the *Synechocystis* mutant modified at this position

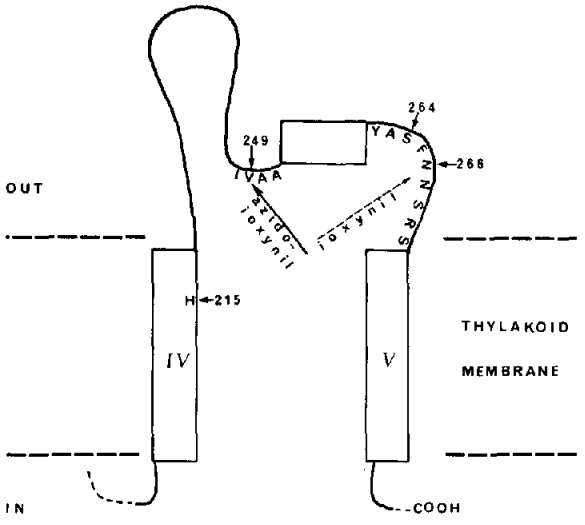

Fig.1. Scheme based upon the model of Trebst and Draber [17] for the herbicide-binding environment in the D_1 protein. The presumed helices are represented by boxes. Only that region of D_1 is shown which covers the segment from the beginning of the 4th membrane-spanning helix to the COOH end of the polypeptide. Arrows denote amino acid residues involved in fixation of azido-ioxynil (249) [18], ioxynil resistance (266) and urea-triazine-type herbicide resistance (264).

(Ser 264/Ala) [9]. From thermoluminescence and fluorescence analysis after a saturating flash, we estimated the affinity constant of Q_B in its site: whereas it decreased slightly in the DCMU-resistant mutant (Ser 264/Ala) [25], it remained unchanged in the ioxynil-resistant mutant (not shown).

From this mutant strain, it will be possible to select new mutants resistant to higher concentration of ioxynil. These mutants might have modifications on the donor side and thus provide information on the function of the oxidizing side of PS II.

Acknowledgement: We thank Dr A.-L. Etienne for stimulating discussions.

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