Photosynthesis Research **23**: 73–79, 1990.

Regular paper

A similar structure of the herbicide binding site in photosystem II of plants and cyanobacteria is demonstrated by site specific mutagenesis of the *psbA* gene

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Received 8 November 1988; accepted 13 February 1989

Key words: cyanobacteria, D1 protein, herbicide-resistance, Photosystem II, *psbA* gene

Abstract

Many herbicides inhibit the photosynthetic electron transfer in photosystem II by binding to the polypeptide D1. A point mutation in the chloroplast gene *psbA* , which leads to a change of the amino acid residue 264 of D1 from serine to glycine, is responsible for atrazine resistance in higher plants. We have changed serine 264 to glycine in *Synechococcus* PCC7942 and compared its phenotype to a mutant with a serine to alanine shift in the same position. The results show that glycine at position 264 in D1 gives rise to a similar phenotype in cyanobacteria and in higher plants, indicating a similar structure of the binding site for herbicides and for the quinone Q_B in the two systems. A possible mode of binding of phenyl-urea herbicides to D1 is predicted from the difference in herbicidal cross-resistance between glycine and alanine substitutions of serine 264.

Abbreviations; DCPIP – 2,6-dichlorophenolindophenol, I_{50} – concentration of herbicide giving 50% inhibition, K_b – binding constant, kb – kilobase, MES – 2(N-morpholino)ethanesulfonic acid, PS II – photosystem II.

Introduction

The primary photochemical reactions of photosynthesis in green plants, algae and cyanobacteria are carried out by photosystem II (PS II). This protein-pigment complex drives electron transfer from water to plastoquinone. Two plastoquinones serve as intermediate electron acceptors in PS II, the first one is a stably bound quinone – Q_A, which transfers the electron to a second electron acceptor, a weakly bound quinone called Q_B.

Many chemically distinct herbicides block transfer of electrons from Q_A to Q_B by displacing Q_B from its binding site on the 32 kilodalton polypeptide–D1, which is a component of PS II (Tischer and Strotmann 1977, Pfister et al. 1981, Vermass et al. 1983). Since different herbicides compete with each other and with the quinone on binding to D1, a common, though not absolutely identical, binding site for herbicides and Q_B was postulated (Trebst and Draber 1979, Thiel and Böger 1984). D1 is a hydrophobic protein which, before processing has 353 amino acid residues in higher plants and 360 in cyanobacteria (Erickson et al. 1985). A remarkable conservation was found in the deduced amino acid sequence of D1–98% homology between different higher plants and 85–90% between all PS II–containing species.

A single point mutation in the chloroplast gene *psbA*, which codes for D1, was found in an atrazine-resistant mutant of *Amaranthus hybridus* (Hirschberg and McIntosh 1983). This mutation leads to an amino acid substitution of residue 264 in D1 from serine to glycine. Exactly the same mutation in *psbA* has also been observed in several other atrazine–resistant mutants of higher plants (reviewed in Hirschberg et al. 1987a). In all cases, this specific mutation in D1 leads to a dramatic

reduction in the binding of s-triazines, e.g., atrazine (2-cloro-4-ethylamino-6-isopropylamino-*s*-triazine), while binding of phenyl-urea herbicides, e.g., diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea), is almost unaffected (Arntzen et al. 1982, Hirschberg et al. 1984). The same serine residue is changed to alanine in herbicide-resistant mutants of algae (Erickson et al. 1984, Johanningmeier and Hallick 1987) and cyanobacteria (Golden and Haselkorn 1985, Ohad et al. 1987). However, in contrast to plants, these mutants exhibit a totally different phenotype being less resistant to s-triazines but highly resistant to phenyl-urea inhibitors. It has been suggested (Rochaix and Erickson 1988) that the conformation of the binding niche for Q_B and herbicides in D1 of algae and cyanobacteria is different than that of higher plants.

In order to address this question we have compared the effects of glycine and alanine substitutions of serine 264 in the D1 polypeptide of the cyanobacterium *Synechococcus* PCC7942 (*Anacystis nidulans* R2). The structure and function of PS II in this organism are essentially similar to those of higher plants (Bryant 1986), yet their prokaryotic genetic nature facilitates the isolation of a large number of mutants and allows genetic manipulations such as DNA-mediated transformation.

The existance of a *psbA* gene family in cyanobacteria (Mulligan et al. 1984, Curtis and Haselkorn 1984, Jansson et al. 1987) in contrast to a single gene found in plastids of plants, is still and enigma. *Synechococcus* PCC7942 contains three *psbA* genes, two of them: *psbAII* and *psbAIII*, code for an identical polypeptide (Golden et al. 1986). However, *psbAI* is the gene that accounts for most of the *psbA* mRNA found in the cells (Golden et al. 1986, Brusslan and Haselkorn 1987). Moreover, all the mutations leading to herbicide resistance that have been observed so far in this organism, have occured in *psbAI* only (Golden and Haselkorn 1985, Hirschberg et al. 1987b, Ohad et al. 1987). For these reasons we have introduced the mutation into *psbAI*.

Materials and methods

Strains and growth conditions

Cell cultures of *Synechoccocus* PCC7942 (Pasteur culture collection No. 7942) were grown in BG11 medium at 35°C as described by Williams and Szalay (1983). For selection of herbicide-resistant mutants, cells were plated on solid BG11 medium containing 1.5% agar and the appropriate concentration of herbicides. Isolation of the atrazine-resistant mutant Dil has been previously described (Hirschberg et al. 1987b).

Molecular cloning and sequencing

Methods for isolating DNA from *Synechococcus* PCC7942 and for transformation of cyanobacterial cells were according to Williams and Szalay (1983). Restriction enzyme digestion, Southern hybridization and cloning followed conventional protocols (Maniatis et al. 1982). The *E. coli* strain MV1190 was used as host for the plasmid vectors pBR328 and pUC118. DNA sequencing was carried out by the dideoxy nucleotide chain termination method (Sanger et al. 1977).

Site-directed mutagenisis of psbAI

In order to change the serine residue at position 264 of D1 to glycine, a 1.5 kb SalI–BamHI DNA fragment, containing about 85% of the coding region of the *psbAI* gene from mutant Dil, was subcloned in the plasmid pUC118. This gene has been previously cloned and sequenced (Hirschberg et al. 1987b). Single stranded DNA was prepared (Zoller and Smith 1987) and served as template for oligonucleotide-directed mutagenesis that was carried out by the method of Nakamaye and Eckstein (1986) as illustrated in Fig. 1(a). The linear 1.5 kb fragment carrying the new mutation was transfected to wild type cells and herbicide-resistant transformants were selected on plates containing 10.0 μM atrazine. The new mutation was designated G264.

Measurement of PS II dependent electron transfer

Cells from a 0.5 liter suspension culture of *Synechoccoccus* PCC7942 were harvested by centrifugation and washed once in distilled water. The cells were resuspended in 5 ml of MES buffer (50 mM

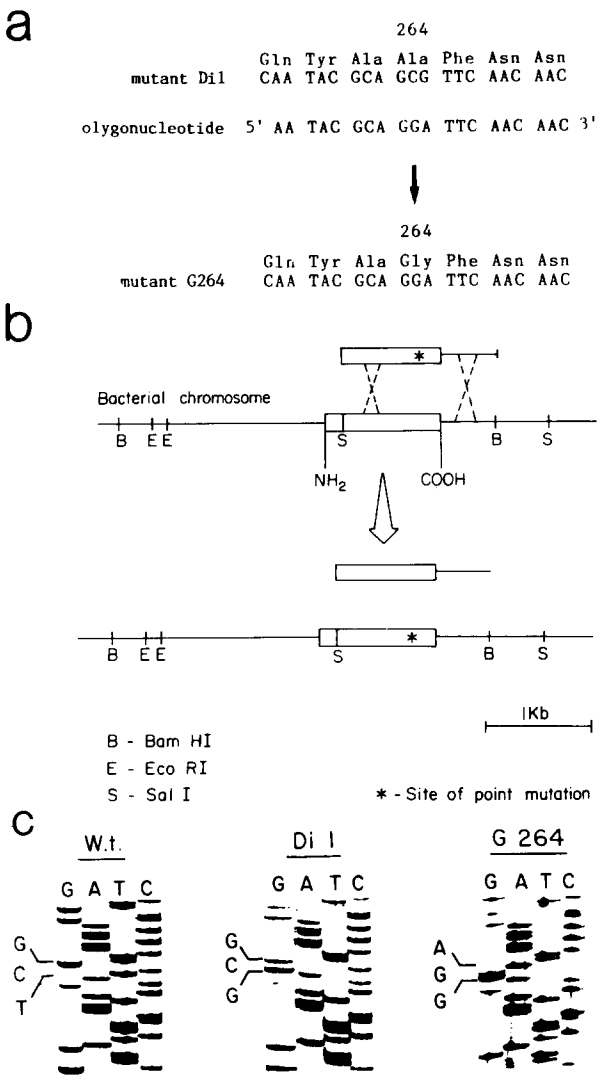

Fig. 1. Site-specific mutagenesis of *psbAI* in *Synechococcus* PCC7942. A 1.5 kb SalI-BamHI DNA fragment, containing 85% of the coding region (boxed) and the 3'-flanking region of the *psbAI* gene from mutant Dil (Ohad et al., 1987b), has been subcloned in the *E. coli* plasmid-pUC118. Single stranded DNA was prepared and two nucleotides were substituted, using an anti-sense oligonucleotide (Nakamaye and Eckstein 1986) as indicated (a). Wild-type cells of *Synechococcus* PCC7942 were transfected with the 1.5 kb linear DNA fragment containing the mutation. Homologous recombination between the transfected DNA and the cyanobacterial chromosome, which involved a double crossover event, introduced the mutation to the wild-type gene by a reciprocal exchange (b). Hericide-resistant transformants were selected on solid BG11 medium containing 10.0 μM atrazine. The *psbAI* genes from the wild-type and the two isogenic mutants were completely sequenced (Sanger et al. 1977). The region in the sequencing gel, showing the changes of serine in the wild type to alanine in mutant Dil and to glycine in G264, is presented (c).

MES pH = 6.5 with 5 mM $CaCl_2$, 100 mM sucrose, 1 mM α-aminocaproic acid (Sigma) and 1 mM benzamidine) at 4°C. The suspension was sonicated three times for 15 s each and centrifuged at 2500 × *g* for 2 min. The supernatant was loaded on top of 2 ml 60% (w/v) sucrose and centrifuged for 15 min at 4°C in a Beckman Ti50 rotor at 40 000 rpm. The green band, that was accumulated on top of the sucrose, was collected, resuspended in 5 ml MES buffer and centrifuged at 40 000 rpm as above. The green pellet was resuspended in 200 μl of MES buffer. These membranes could be stored at −80°C. PS II-dependent electron transfer from H_2O to DCPIP was analyzed in these membranes by measuring the rate of photochemical reduction of DCPIP in a dual wavelength spectrophotometer (Aminco-Chance). I_{50} was determined as the concentration of herbicide that inhibited the rate of DCPIP reduction by 50%. The average experimental error in these measurements was about 15%. All herbicides used in this study were chemically pure and were supplied by various companies which manufacture them or by Chem Service, West Chester, PA, USA. The herbicides were dissolved in methanol in stock solutions of 20 mM. Maximal concentration of methanol in the samples was 3%. This concentration had no effect on the photosynthetic electron transport.

Results and discussion

A point mutation in *psbAI*, leading to an alanine substitution of the serine residue at position 264 of D1, has been previously found in the atrazine-resistant mutant Dil that was isolated following mutagenesis of wild-type cells and selection for atrazine resistance (Hirschberg et al. 1987b, Ohad et al. 1987). The same mutation has been found in *Synechococus* PCC7942 by Golden and Haselkorn (1985). In all cyanobacteria and algae that have been examined the codon used for serine 264 is TCG in contrast to AGT in higher plants. A serine to glycine mutation in these organisms would have required two nucleotide changes. This is probably the reason why this mutation has not been isolated by random mutagenesis and selection. In order to change the serine residue 264 of D1 to glycine by site-specific mutagenesis we have subcloned a DNA fragment containing 85% of the coding region and

Table 1. Properties of wild-type (w.t.) and herbicide resistant mutants of *Synechococcus* PCC9742 and *Phalaris paradoxa*. Photosystem II-dependent electron transfer from H_2O to DCPIP was measured in isolated thylakoid membranes. The concentration of herbicide that inhibits the rate of electron transfer by 50% (I_{50}) was determined for each herbicide. Relative resistance is expressed by the ratio of I_{50} of the mutants to that of the wild-type strain.

Herbecide	Synechococcus PCC7942			P.Paradoxa	
	I_{50} [M] w.t.	Relative resistance		I_{50} [M] w.t.	Relative resistance
		Mutant Dil (264 Ser → Ala)	Mutant G264 (264 Ser → Gly)		mutant (264 Ser → Gly)
Ureas					
fenuron	3.0×10^{-6}	1000	50	5.0×10^{-6}	10
monuron	8.0×10^{-7}	250	40	7.0×10^{-7}	12
diuron	3.0×10^{-8}	100	10	1.5×10^{-7}	2
linuron	1.5×10^{-7}	100	20	9.0×10^{-8}	10
fluomethuron	2.0×10^{-6}	10	4	2.0×10^{-6}	3
tebuthiuron	4.0×10^{-6}	25	7	1.5×10^{-6}	11
ethidimuron	2.0×10^{-6}	25	15	2.5×10^{-6}	10
s-triazines					
simazine	9.0×10^{-8}	60	500 <	1.0×10^{-7}	1000
atrazine	3.0×10^{-7}	33	1000	3.0×10^{-7}	1000
propazaine	6.0×10^{-7}	3.5	160	5.0×10^{-7}	400
ametryne	5.0×10^{-8}	30	1000	4.0×10^{-8}	1000
terbutryne	1.5×10^{-8}	15	45	2.5×10^{-8}	62
prometryne	9.0×10^{-8}	3	450	7.0×10^{-8}	500

(a) *Herbicides*: ametryne, 2-ethylamino-4-(isopropylamino)-6-(methylthio)-s-triazine; atrazine, 2-chloro-4-ethylamino-6-isopropylamino-s-triazine; diuron (DCMU), 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ethidimuron, N-[5-(ethylsulfonyl)-1,3,4-thidiazol-2-yl]-N,N-dimethylurea; fenuron, 1,1-dimethyl-3-phenylurea; fluometuron, 1,1-dimethyl-3-(α,α,α-trifluoro-m-toly)-urea; linuron, 3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea; monuron, 3-(4−chlorophenyl)-1,1-dimethyl-urea; prometryne, 2,4 bis(isopropylamino)-6-(methylthio)-s-triazine; propazine, 2-chloro-4,6-bis(isopropylamino)-s-triazine; simazine, 2-chloro-4,6-bis(ethylamino)-s-triazine; tebuthiuron, N-[5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]-N,N-dimethylurea; terbutryne, 2-(tert-butylamino)-4-(etylamino)-6-(methylthio)-s-triazine;

the 3' flanking region of *psbAI* from mutant Dil. Codon 264 was changed from GCG (Ala) to GGA (Gly) using oligonucleotide-directed mutagenesis (Fig. 1a). The new mutation was designated — G264.

Following DNA transformation of wild-type cells with the linear 1.5 kb fragment containing *psbAI*, either from Dil or from G264, cells were plated on an atrazine-containing solid medium and herbicide resistant colonies were selected. These transformants were generated by a reciprocal exchange between homologous nucleotide sequences in the transfecting DNA and the bacterial chromosome (Fig. 1b). As has been previously shown (Golden and Haselkorn 1985, Ohad et al. 1987), a mutation in *psbAI* only was found in the herbicide-resistant mutants. The possibility of alteration of *psbAII* or *psbAIII* could be ruled out since Southern hybridization analysis of the DNA from the transformed strain, using the oligonucleotide that had been used to create the mutation (Fig. 1a) as a probe, revealed that only *psbAI* was carrying the mutation (data not shown). The transformants of the two mutations are, therefore, completely isogenic with the wild type strain except for the *psbAI* gene that they carry. Hence, it is possible to relate the herbicide-resistance phenotype exclusively to the specific alterations in D1 without interference of genetic polymorphism that might exist in other genes of the different strains. The complete *psbAI* genes of the wild-type strain and mutants Dil and G264 were sequenced. The only difference between these genes was observed in codon 264 (Fig 1c).

In order to compare the precise phenotype of the two mutants, the degree of resistance towards various herbicides was determined *in vitro* by measuring the sensitivity of PS II-dependent electron transfer, from H_2O to an artificial electron acceptor - DCPIP, in thylakoid membranes iso-

lated from the mutants and from the wild type strains. The resistance to each of the inhibitors is expressed by the I_{50} concentration that has been measured (Table 1). These values have been found to be proportional to herbicide binding constants (K_b) (Kuhn et al. 1986). A similar analysis was carried out with atrazine-resistant and atrazine-susceptible biotypes of the grass *Phalaris paradoxa*. A change of serine 264 to glycine has been previously found in this mutant (Schönfeld et al. 1987).

The data presented in Table 1 show that the serine to glycine change in D1 gave rise to a similar pattern of herbicide cross-resistance in higher plants and in cyanobacteria, while the serine to alanine shift had a completely different phenotype. This result clearly demonstrates the similar contribution of amino acid residue 264 to the conformation of the herbicide binding site in D1 from the two phylogenetically distant organisms. It also provides an additional support to the use of cyanobacteria as a model system to investigate the structure and function of PS II, and particularly the molecular mechanisms of herbicide action and herbicide resistance.

Studying the molecular mechanisms or inhibitors of PS II can shed light on its structure and function (Renger 1986). Valuable information about the fine stucture of the overlapping binding sites for herbicides and Q_B can be obtained from analysis of herbicide-resistant mutants (Trebst 1987, Hirschberg et al. 1987a, Michel and Deisenhofer 1988). In this context the difference in the degree of resistance to the herbicides diuron and atrazine between mutants G264 and Dil is very significant and could indicate a different mode of binding of these compounds to the protein D1.

It is widely accepted now that polypeptides D1 and D2 form the core of the PS II reaction center in a similar fashion to subunits L and M of the photosynthetic reaction center in the purple bacteria (Trebst and Depka 1985, Michel and Deisenhofer 1986, Hearst 1986, Nanba and Satoh 1987, Barber 1987). There are numerous structural and functional analogies between PS II and the reaction center in the purple bacteria. Noteworthy in this discussion are: (1) the electron transfer reactions between the components at the acceptor side of PS II are similar to those in purple bacteria (Rutherford 1987); (2) triazines inhibit electron transfer in PS II and in purple bacteria by a similar mechanism which is displacement of the second quinone acceptor-Q_B from its binding site in D1 of PS II, and in subunit L of the bacterial reaction center;(3) there is an amino acid sequence homology between D1 and subunit L; (4) amino acid substitutions in D1 and L in the region of sequence homology, are involved in mutations that confer herbicide resistance. Such is the case with serine 264 of D1. The corresponding serine residue in the L subunit (serine 223) is changed to alanine in *Rps. viridis* (Sinning and Michel 1987) and to proline in *Rb. sphaeroides* (Paddock et al. 1987) in triazine-resistant mutants.

The complete structures of the reaction centers of *Rps. viridis* (Deisenhofer et al. 1985, Michel et al. 1986) and *Rb. sphaeroides* (Allen et al. 1987) have been determined by crystallographic X-ray analysis. It has been established (Sinning and Michel 1987, Michel and Deisenhofer 1988) that terbutryne (a triazine derivative) forms two hydrogen bonds with the L polypeptide of *Rps. viridis*, one of them between the hydroxyl group of serine 223 and a nitrogen of the aminoethyl side chain of terbutryne, and the other between a ring nitrogen of terbutryne and an N–H group of the polypeptide backbone. The elimination of one hydrogen bond due to the replacement of serine 223, leads to a decrease in the binding of triazines in the herbicide–resistant mutants. By analogy, similar mutations in serine 264 of D1 should give rise to the same result. This is in agreement with the triazine resistance exhibited by mutants Dil and G264. However, the difference in the degree of resistance is probably due to additional effects of the mutations. The serine replacement by glycine is expected to cause structural rearrangements in the polypeptide that could affect other interactions between D1 and triazines so that the binding affinity is further reduced.

There is a dramatic difference in diuron resistance between the Dil and G264 mutants. The mode of binding of phenyl-urea compounds to D1 is unknown. However, based on the similarity of their chemical structure to other PS II inhibitors and to the quinone, it is likely that they form a hydrogen bond, possibly via the phenyl-amino nitrogen or the carbonyl oxygen, to the side chain of serine 264. It has been recently suggested (Michel and Deisenhofer 1988) that a hydrogen bond

between the carbonyl oxygen of Q_B and the L subunit of *Rps. viridis* is shared between the O–H of serine 223 and a polypeptide backbone nitrogen. It is possible that a similar situation exists also in the binding of phenyl-urea compounds to D1. We suggest that due to structural rearrangements in D1 caused by glycine, but not by alanine, substitution at position 264, the phenyl-urea inhibitors are able to form a hydrogen bond with a backbone N–H (perhaps residue 264 or 265), and thus their binding to D1 is almost unchanged.

The experiment described above is the first reported site-specific modification of D1 which sets an example for the possibility in the future of genetic engineering of herbicide resistance.

Acknowledgement

This work was supported by a Grant, DISNAT 137, from GSF München, Germany and NCRD, Israel.

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