Differential Inhibition of Phytoene Desaturases from Diverse Origins and Analysis of Resistant Cyanobacterial Mutants

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Recent work on the molecular biology of carotenogenic genes and enzymes showed that there are two different and unrelated types of phytoene desaturase. One can be found in bacteria and fungi whereas the other is present in cyanobacteria, algae, and higher plants. Studies with herbicidal inhibitors showed that only the latter type of enzyme is affected by the bleaching herbicides norflurazon, fluridone, and flurtamone. I_{50} values have been determined for inhibition of phytoene desaturase in either solubilized membranes of the cyanobacterium *Synechococcus*, membrane preparations of *E. coli* in which the corresponding gene from *Synechococcus* was expressed or purified and reactivated phytoene desaturase from this *E. coli* transformant. The values differ by two orders of magnitude. The importance of the lipid environment of the integral membrane protein phytoene desaturase for its interaction with herbicides has been discussed. With the purified phytoene desaturase it could also be shown that the pyrimidine derivative J852 which is an inhibitor of ζ-carotene desaturase also inhibits phytoene desaturase with an I_{50} value which is about 2700 times higher than for inhibition of phytoene desaturation.

Several mutants of *Synechococcus* resistant against fluridone and flurtamone have been selected, which exhibited different cross resistances to other bleaching herbicides. With one exception, *in vitro* results indicate that the resistances are due to different amino acid changes in the enzyme polypeptide chains. Previous work with norflurazon-resistant mutants showed that parallel to the degree of resistance the phytoene desaturase lost its catalytic activity. In the mutants selected here this loss of active was much more moderate.

Commercial bleaching herbicides, like norflurazon and fluridone interfere with the desaturation of phytoene, the first carotene in the corresponding biosynthetic pathway [1]. This has been demonstrated in higher plants as well as pro- and eucaryotic algae. All herbicidal inhibitors of phytoene desaturase investigated so far, have exhibited a reversible non-competitive type of inhibition upon the enzyme found [2, 3].

Recently, genes for phytoene desaturase have been cloned from bacteria [4–6], cyanobacteria [7, 8] and higher plants [9, 10]. Comparison between their amino acid homology and characterization of their reaction products has shown the existence of two different types of phytoene desaturase. They are represented either by the enzyme from *Erwinia* or from *Synechococcus*.

The availability of both genes made it possible to purify both enzymes after overexpression in *Escherichia coli* and to reconstitute their activities [11, 12]. Consequently, we have carried out inhibition experiments with both phytoene desaturases using the herbicides norflurazon (NFZ), fluridone (FRD) and flurtamone (FTM) as well as the inhibitor of certain phytoene desaturases, diphenylamine (DPA). Furthermore, the availability of the purified enzyme made it possible to look for additional inhibitory potential of the well-characterized ζ-carotene desaturase inhibitor J 852 [1].

The only resistant mutants against any herbicidal phytoene desaturase inhibitor are from the cyanobacteria *Synechocystis* and *Synechococcus* which were selected against NFZ [8, 13]. One tendency observed was a loss of catalytic activity of the phytoene desaturase with increasing resistance against NFZ accompanied by decreased synthesis of colored carotenoids and accumulation of phytoene [13]. In this work we present inhibition data

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on several mutants of *Synechococcus* selected against FRD and FTM showing different patterns of cross-resistance to other bleaching herbicides. In addition, there are examples indicating that the degree of resistance is not closely correlated with loss of biosynthetic activity for formation of colored carotenoids.

Materials and Methods

Cress seedlings, *Synechococcus* (PCC 7942) cells and the *Phycomyces blakesleeanus* mutant C 5 were grown as previously described [14]. Carotenoids were isolated for quantitation or analysis from the seedlings or *Synechococcus* by extraction with 6% KOH/methanol (w/v) for 15 min at 60 °C and partitioned into 10% diethylether/petrol (v/v). Total colored carotenoids were determined directly from this extract by optical absorbance at 445 nm [13]. After concentration of this extract HPLC analysis was carried out on a 25 cm Spherisorb ODS-1, 5 μm column with acetonitrile/methanol/2-propanol (85:10:5, v/v/v) as eluent.

The *in vitro* assays were performed by coupling the [^{14}C]phytoene-generating capability of *Phycomyces* C 5 to the phytoene-consuming desaturase reaction catalyzed by the different enzyme preparations [11–13]. The reaction mixture in 0.5 ml Tris-HCl buffer, pH 8.0, containing 5 mM DTT consisted of 3 μmol ATP, 1 μmol of NAD which was replaced by FAD in the assay with the *Erwinia* enzyme, 4 μmol Mn^{2+}, and 6 μmol of Mg^{2+}, 0.25 μCi of 3 *R* [2-^{14}C]mevalonic acid (specific radioactivity 53.4 mCi/mmol) converted to its Na^+ salt prior to addition. Preparation of C 5 extracts and membranes from *E. coli* carrying the phytoene desaturase genes either from *Erwinia* or *Synechococcus* were as described [11, 12]. Thylakoid membranes from *Synechococcus* were obtained by French press treatment (500 bar), centrifugation for 15 min at 10,000 × *g* and resuspension in 0.1 M Tris-HCl buffer, pH 8.0, containing 5 mM DTT. Then Tween 40 was added to a final concentration of 1%, incubated for 15 min on ice and the supernatant from centrifugation for 15 min at 20,000 × *g* was collected. Details on isolation, purification and reactivation of phytoene desaturases from *Erwinia uredovora* and *Synechococcus* expressed in *E. coli* are given in references 11 and 12. The amount of reactivated homogeneous phytoene desaturase used in the assays was 30–40 μg. Experiments to determine inhibitory properties of norflurazon on *Synechococcus* phytoene desaturase were carried out with membranes from *E. coli* transformed wih the pds gene which was recloned from a library by hybridization of the originally isolated pds gene [7].

Mutagenesis of *Synechococcus* was carried out with UV irradiation by placing a suspension at a distance of 75 cm from an Osram HNS 30 Watt lamp for 30 min or by EMS treatment as described [13]. The resulting mutants were selected either on plates with fluridone or flurtamone. The first 3 letters of the mutant assignation stands for the herbicide which was used as the selecting agent. The letter U at the end indicates generation by UV mutagenesis and E indicates generation by EMS. Mutants FTM 1 X and FRD 1 X were further selected from FTM 1 E and FRD 1 U by continuous cultivation in increasing herbicide concentration over 6 months.

Results and Discussion

Our understanding of the mode of action of bleaching herbicides has been greatly aided by the development of active *in vitro* carotenogenic systems [15, 16]. Subsequently, the recent progression in the molecular genetics of carotenoid biosynthesis has made it possible to overexpress and purify different phytoene desaturases [11, 12]. With respect to the number of double-bonds introduced into phytoene, three different types of enzymes represented by *Erwinia, Rhodobacter* and *Synechococcus* may be discriminated [17]. Based on amino acid homology they can be grouped into a bacterial/fungal on one hand and a cyanobacteria/algal/higher-plant type on the other.

In Table I inhibitory properties of phytoene desaturases originating from a representative species of each of the two non-homologous types were compared. I_{50} values for inhibition by fluridone, flurtamone, and norflurazon were determined with detergent-solubilized thylakoids of *Synechococcus* membranes, from *E. coli* transformed with the *Synechococcus* phytoene desaturase gene, and the purified *Synechococcus* phytoene desaturase. The I_{50} values in the range of 10^{-8} to 10^{-7} M for all three herbicides obtained with thylakoid solubilisates resemble very well the values obtained with

Table I. *In vitro* I_{50} values for inhibition of phytoene desaturase.

Herbicide	Enzyme preparation originating from *Synechococcus*			Enzyme preparation originating from *Erwinia*
	Solubilized thylakoids	*E. coli* membranes*	Purified enzyme	Purified enzyme
FRD	13 nM	0.3 μM	3.5 μM	⩾250 μM
FTM	18 nM	0.3 μM	1.3 μM	⩾250 μM
NFZ	95 nM	1.1 μM	–**	
J852***	–	–	4.8 μM	–
DPA	–	–	⩾250 μM	64 μM

* Membrane preparation of *E. coli* transformed with the *pds* gene from *Synechococcus*.
** Indicates that no values were determined.
*** I_{50} value for inhibition of ζ-carotene desaturase with *Synechococcus* thylakoids = 1.8 nM.

whole thylakoids [13]. However, the same enzyme synthesized in *E. coli* when assayed integrated into *E. coli* membranes showed I_{50} values for all three herbicides which were about 10 to 20 times higher. Furthermore, sensitivity was decreased by another factor of about 4 to 11 when the purified *Synechococcus* phytoene desaturase was used to determine I_{50} values. Obviously, the environment of the phytoene desaturase molecule plays an important role for its sensitivity against bleaching herbicides. For example, *Synechococcus* lipids contain mono-desaturated fatty acids only whereas *Synechocystis* synthesizes also dienic and trienic fatty acids [18]. The I_{50} (= k_i) value for fluridone has been determined previously using *Synechocystis* membranes as the *in vitro* system [3] which was about twice that found with the *Synechococcus* enzyme preparation in Table I. Higher plants are comparably rich in poly-unsaturated fatty acids. Therefore, we also determined I_{50} values for inhibition of formation of colored carotenoids with cress seedlings (Table II). Although the experimental conditions are different, the I_{50} values determined for norflurazon, fluridone, and flurtamone vary from the *in vitro* I_{50} value obtained with solubilized *Synechococcus* membranes only by a factor of 3 for fluridone and even less for the other herbicides.

Table II. I_{50} values for inhibition of the formation of colored carotenoids in cress seedlings germinated for 3 days in darkness.

Herbicide	I_{50} [nM]
Norflurazon	45
Flurtamone	69
Fluridone	24

Inhibitory action of fluridone, flurtamone, and norflurazon was also assayed with the purified phytoene desaturase from *Erwinia* (Table I). Due to restricted solubility of these herbicides only concentrations of up to 250 μM were employed which in all cases showed almost no inhibition. If an I_{50} value for inhibition with *Erwinia* phytoene desaturase is anticipated extrapolation would yield a value high in the mM range. This result demonstrates that phytoene desaturase from *Erwinia* is insensitive to different herbicidal phytoene desaturase inhibitors. However, this enzyme is inhibited by diphenylamine as indicated by an *in vitro* I_{50} value of 64 μM. For intact *E. coli* cells transformants carrying all genes from *Erwinia* necessary for the synthesis of lycopene much higher concentrations of diphenylamine were necessary for half maximum inhibition [17].

Pyrimidine derivatives like J852 inhibit the desaturation of ζ-carotene to lycopene [19]. When intact plants were treated, accumulation of some phytoene besides of ζ-carotene was observed. As the purified phytoene desaturase is now available [12], we looked for its inhibition by J852 (Table I). The I_{50} value for phytoene desaturase inhibition of the purified *Synechococcus* enzyme was 4.8 μM. In contrast, an I_{50} value of 1.8 nM was obtained with *Synechococcus* membranes for inhibition of ζ-carotene desaturation. Obviously, J852 is predominantly an inhibitor of ζ-carotene desaturase but has also the potential to inhibit the cyanobacterial/plant type phytoene desaturase at higher concentrations.

In the last years, mutants from unicellular cyanobacteria exhibiting resistance against norflura-

zon have been selected and the mutations were located as single amino acid changes by sequencing of the gene [7, 8]. The targeted regions of the enzyme polypeptide chain and pattern of cross resistance to other bleaching herbicides of different chemical character have given the first hints as to the herbicide-binding site. In Table III seven new and basically different *Synechococcus* mutants with resistance against either flurtamone or fluridone are presented. *In vivo* as well as *in vitro* I_{50} values for the selecting herbicide either fluridone (FRD) or flurtamone (FTM) were determined. With the exception of FRD3U, the values obtained by determination of carotenoid formation in intact cells or with enzymatic phytoene desaturation were very similar. This is an indication that in these mutants resistance is due to an alteration at the enzyme level and that restricted uptake or metabolism can be excluded. However, the latter possibilities or alternatively higher expression of the phytoene desaturase protein could be the case instead for FRD3U as concluded from the differences between *in vivo* and *in vitro* inhibitor sensitivity.

Mutants FTM1E, FTM2U, and FTM4E were different in their cross-sensitivity against bleaching herbicides others than the selecting agent flurtamone. In addition to this, FTM2U is resistant to fluridone and fluorochloridone, FTM4E to fluridone and norflurazon, and FTM1E to fluridone. Mutant FRD1U shows a pattern of resistance similar to FTM4E whereas FRD3U is resistant (factor of resistance >3) to the selecting herbicide fluridone only. Judging from the cross resistance of all the mutants of Table III and by comparing them with the NFZ mutants known so far from which the resistance genes have been sequenced [7, 8], there may now be at least three completely different new amino acids involved in herbicide-binding after gene sequencing.

FTM1X and FRD1X were selected from the mutants FTM1E and FRD1U, respectively, by continuous cultivation for over 6 months with increasing concentrations of flurtamone for the first and fluridone for the second mutant. This treatment resulted in cultures with much higher degrees of resistance indicated by I_{50} values which were 8- to 13-fold higher. It should be pointed out that *in vivo* as well as *in vitro* values increased accordingly. In FTM1E cross resistance against other bleaching herbicides was not improved. However, in FRD1X cross resistance against flurtamone showed a higher increase than against the selecting herbicide fluridone. Based on the stepwise proce-

Table III. Resistance against herbicidal inhibitors of *Synechococcus* mutants selected against flurtamone (FTM) or fluridone (FRD) indicated as I_{50} values (μM).

Herbicide		FTM Mutants 1E	1X	2U	4E	FRD Mutants 1U	1X	3U
Flurtamone								
in vivo	I_{50} =	0.24	3.22	0.22	0.17	0.13	0.16	0.04
	FR =	8.0	107	7.6	5.9	4.3	51.2	1.4
in vitro	I_{50} =	0.31	4.33	0.19	0.29	n.d.	n.d.	n.d.
	FR =	11	153	6.8	8.6			
Fluridone								
in vivo	I_{50} =	0.24	0.22	0.14	0.15	0.20	1.64	0.13
	FR =	9.9	9.1	5.8	12	8.3	68.1	5.4
in vitro	I_{50} =	n.d.	n.d.	n.d.	n.d.	0.19	1.33	0.02
	FR =					8.3	58.1	1.1
Norflurazon	I_{50} =	0.21		0.25	0.72	0.51	0.15	0.25
	FR =	1.9		2.3	6.6	4.7	1.4	2.3
Fluorochloridone	I_{50} =	0.24		0.91	0.41	0.05	0.08	0.05
	FR =	2.2		8.2	3.7	0.5	0.8	0.5

n.d. is not determined. FR are factors of resistance calculated for the I_{50} values of the mutants divided by the corresponding value of the wild type strain.

Table IV. Carotenoid content (μg/ml pcv) of *Synechococcus* mutants resistant against bleaching herbicides.

Carotenoid	WT	FRD1X*	FTM1X**
Zeaxanthin	1589	1538	1236
β-Cryptoxanthin	44	71	41
β-Carotene	789	601	563
Total colored carot.	2421	2210 (91%)	1840 (76%)
Phytoene	0	39	95

* FR against FRD = 68 and FTM = 51.
** FR against FTM = 107.

dure of selection, we can assume that in case of FTM1X and FRD1X at least double mutants were generated.

Previous results with a range of norflurzon-resistant *Synechococcus* mutants implied that the degree of resistance is negatively related to the activity of the mutated phytoene desaturase and to the amount of colored carotenoids produced in the cell [13]. For example, NFZ49 with a factor of resistance (FR) of 70 for norflurazon inhibition exhibited a 78% decrease of colored carotenoids. In Table IV carotenoid analysis of two mutants, one FRD1X with a comparable FR to NFZ49 and another FTM1X with an FR about 2-fold higher is presented. For FRD1X the concentrations of carotenoids are almost as high as in the wild type. Furthermore, only a small amount of phytoene was detectable. In FTM1X inhibition of carotenoid synthesis (by 26%) as well as accumulation of phytoene was much more affected but not nearly so strong as in NFZ49 [13]. Obviously, different amino acids are exchanged in different mutants which result in more or less severe conformational changes as the reason for impaired catalytic activity.

In conclusion, the work presented here gives basic information of inhibitory properties of different naturally occurring or mutated phytoene desaturases. In nature, two types of phytoene desaturase can be found. Among them phytoene desaturase from *Erwinia uredovora* belonging to the bacterial/fungal type has been found to be naturally resistant against bleaching herbicides. Furthermore, several herbicide-resistant strains were obtained after mutagenesis of *Synechococcus*, a cyanobacterium from which the phytoene desaturase gene has already been cloned [7]. The biochemical results with these mutants point out that different amino acids of the phytoene desaturase polypeptide are modified. As the genes of naturally resistant and mutated desaturases are available now, they can be employed for transformation of higher plants in order to acquire resistance against bleaching herbicides. Work in this direction is in progress.

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