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Resistance to nitrophenolic herbicides and metronidazole in the cyanobacterium *Synechocystis* sp. PCC 6803 as a result of the inactivation of a nitroreductase-like protein encoded by *drgA* gene

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Abstract Dinoseb is a herbicide known to inhibit photosystem II electron transfer like DCMU, triazine and phenolic-type herbicides. The mutant Din7 of the cyanobacterium *Synechocystis* sp. PCC 6803, selected for resistance to dinoseb, and the mutant Ins2, constructed by the insertion of the kanamycin resistance cassette into the *drgA* gene, were cross-resistant to other nitrophenolic herbicides (DNOC, 2,4-dinitrophenol) and to the cell inhibitor metronidazole but not to the photosystem II inhibitors DCMU or ioxynil. The Din7 mutant had the same characteristics of photosystem II inhibition by dinoseb as the wild type. This result suggested the existence of another site for dinoseb inhibition. The wild type cells modified dinoseb to a non-toxic product that gave an absorption spectrum similar to that of dithionite treated dinoseb containing reduced nitro groups. In contrast, the Din7 mutant did not modify dinoseb. These phenomena were controlled by the *drgA* gene encoding a protein which showed similarity to several enzymes having nitroreductase activity. The addition of superoxide dismutase to the medium relieved the toxic effect of dinoseb in wild type cells but not in Din7. It is proposed that in wild type cells of *Synechocystis* sp. PCC 6803 the DrgA protein is involved in detoxification of dinoseb via the reduction of the nitro group(s) and this process is accompanied by the formation of toxic superoxide anions. Mutations blocking the activity of the DrgA protein lead to the development of resistance to nitrophenolic herbicides and metronidazole.

Key words: Nitrophenolic herbicide; Metronidazole; Photosystem II inhibition; Dinoseb modification; *drgA* gene; *Synechocystis* sp. PCC 6803

1. Introduction

Phototrophic cyanobacteria are an excellent model system to study genetic control and mechanisms of resistance to herbicides that inhibit photosynthesis. The herbicidal activity of 'classic' herbicides (triazines, urea, some phenolic-type herbicides) is due to inhibition of photosystem II (PS II). They block electron transfer on the acceptor side of PS II by competing with Q_B, the secondary quinone acceptor of PS II, for binding to the Q_B pocket of the D1 protein. The resistance to these herbicides is determined by mutations in the *psbA* gene (encoding the D1 protein) which lead to a decreasing affinity of D1 protein to herbicides [1,2]. The phenolic-type herbicides generally produce additional effects on photosynthesis, such as inhibition of the PS II electron donor side and uncoupling of photophosphorylation at concentrations several times higher than required for PS II inhibition [3]. The same mechanisms of action have been proposed for nitrophenolic herbicides.

It has been shown that most nitroaromatic compounds such as nitrotoluenes, nitrofurans, and nitroimidazoles require metabolic activation in bacterial cells in order to exert their toxic effects. A key step in such activation processes is the reduction of the nitro functional group of the molecule [4–6]. However, little is known about the genetic control of nitroaromatic compound activation and the mechanisms of resistance related to conversion of the nitro group in phototrophic organisms.

Using the cyanobacterium *Synechocystis* sp. PCC 6803 mutants, resistant to the nitrophenolic herbicide dinoseb, a novel gene *drgA* responsible for resistance has been isolated [7]. This gene encoded a protein of deduced molecular mass 23.7 kDa. The insertional inactivation of *drgA* led to the development of resistance to dinoseb and metronidazole in *Synechocystis* sp. PCC 6803 cells. It was suggested that resistance to both inhibitors was due to the absence of the active product of the *drgA* gene.

In the present work, we analyzed the molecular nature of the protein encoded by the *drgA* gene and the different effects of dinoseb on the wild type and dinoseb resistant mutants of *Synechocystis* sp. PCC 6803.

2. Materials and methods

2.1. Strains and growth conditions

Synechocystis sp. PCC 6803 wild type and dinoseb resistant mutants Din7 and Ins2 were from the collection of the Department of Genetics of Moscow State University. The mutation in the *drgA* gene of the mutant Din7 leads to the replacement of Leu-45 by His. The mutant Ins2 was constructed by the insertion of the kanamycin resistance cassette into the *Bam*HI site of the *drgA* gene [7]. *Synechocystis* sp. PCC 6803 cells were grown at 30–32°C in BG-11 liquid medium or on plates containing 1.2% agar at a light intensity of 40 mE/m^2/s [8]. If added, the superoxide dismutase (from bovine erythrocytes, Sigma) concentration in the medium was 400 U/ml.

2.2. Oxygen measurements

Oxygen evolution by whole cells (20 mg chlorophyll/ml) was measured by polarography using a Clark-type oxygen electrode at 20°C in growth medium buffered at pH 7 with 50 mM HEPES.

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Abbreviations: PS II, photosystem II; dinoseb, 2-*sec*-butyl-4,6-dinitrophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNOC, 4,6-dinitro-*o*-cresol; 2,4-DNP, 2,4-dinitrophenol; ioxynil, 4-hydroxy-3,5-di-iodobenzonitrile; metronidazole, 1-(2-hydroxyethyl) 2-methyl-5-nitroimidazole

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Table 1
Cross-resistance of wild type and dinoseb resistant mutants of *Synechocystis* sp. PCC 6803 to different inhibitors

Strain	Inhibiting concentration of agent (μM)					
	Dinoseb	DNOC	2,4-DNP	Metronidazole	Ioxynil	DCMU
WT	7	80	5	2	400	5
Din7	100	250	80	20	400	5
Ins2	100	250	80	20	400	5

Inhibiting concentrations indicated are the minimal concentrations needed to inhibit cell growth on agar.

2.3. Spectroscopy measurements

Room temperature chlorophyll fluorescence induction was performed as in [9]. To determine inhibition of electron transfer on the acceptor and the donor side of PS II, the same protocol as described in [3] was used. Cell concentrations were equivalent to 1 mg chlorophyll/ml in growth medium buffered at pH 7 with 50 mM HEPES.

2.4. Nucleotide sequence accession number

The nucleotide sequence of the *drgA* gene described in this communication has been deposited in the EMBL Data Library under accession number L29426.

3. Results

3.1. Cross-resistance of Synechocystis sp. PCC 6803 wild type and mutants to inhibitors

Table 1 presents the levels of resistance of the wild type and of the Din7 and Ins2 mutants to different inhibitors during cell growth on a solid medium. Besides dinoseb these mutants were cross-resistant to the other nitrophenolic-type herbicides DNOC and 2,4-DNP, and to cell inhibitor metronidazole [10]. Both mutants had the same level of resistance as wild type to DCMU and to the phenolic-type herbicide ioxynil that block the photosynthetic electron transport at the level of PS II. Thus the inactivation of the *drgA* gene by the point mutation in the Din7 mutant or insertion of the kanamycin cassette in the Ins2 mutant resulted in the development of resistance to dinoseb, DNOC, 2,4-DNP and metronidazole. These data indicate that the mechanisms of resistance of *Synechocystis* sp. PCC 6803 cells to nitrophenolic-type herbicides and metronidazole differ from the mechanism of resistance to DCMU and ioxynil that results from mutations in the *psbA* gene.

3.2. Effect of dinoseb on PS II activity

Phenol-type inhibitors produce inhibition of electron transfer on both sides of the PS II. To measure each type of inhibition the same protocol as in [3] was used (Fig. 1). The electron transfer inhibition curves were similar for both *Synechocystis* sp. PCC 6803 wild type and Din7 (Fig. 2). The I_{50}s, i.e. concentrations of dinoseb producing 50% inhibition of electron transfer on the acceptor and on the donor sides, were 2×10^{-4} M and 2×10^{-3} M respectively. These high values indicate that dinoseb does not show high affinity to PS II as compared to other PS II herbicides. For comparison, the I_{50} of the inhibition for the acceptor side by ioxynil was very close for both cultures and equal to 5×10^{-7} M. Oxygen evolution of *Synechocystis* sp. PCC 6803 wild type and Din7 was also measured in the presence of various concentrations of dinoseb (not shown), and the I_{50} was determined to be 2×10^{-4} M for both strains.

Fig. 2 also shows the effects of increasing concentrations of dinoseb on the growth rates of wild type and Din7 cells under photosynthetic conditions in a liquid medium. For Din7 the growth rate inhibition curve was similar to that of PS II electron transfer on the acceptor side giving a I_{50} around 2×10^{-4} M. In contrast, the inhibition of wild type growth occurred at a much lower concentration (5×10^{-5} M). These results suggest that inhibition of photosynthesis cannot be a primary mode of dinoseb action and there is a target for dinoseb action other than PS II in *Synechocystis* sp. PCC 6803 wild type cells.

3.3. Comparison of the deduced DrgA protein with other proteins

The gene *drgA*, responsible for resistance to dinoseb and metronidazole, was cloned earlier from the genomic DNA libraries of several dinoseb resistant mutants [7]. The molecular nature of the product of *drgA* gene was not clear at that time.

Comparison of the DrgA deduced amino acid sequence using the translated GenBank database (Fig. 3) revealed similarity to that of oxygen insensitive NAD(P)H nitroreductases of *Salmonella typhimurium*, *Enterobacter cloacae* and *Mycoplasma*-like organism as well as with NAD(P)H-flavin oxidoreductases of *Vibrio fisheri*, *Escherichia coli* and H_2O_2-forming NADH oxidase from *Thermus thermophilus* HB8 [11–14]. The latter three enzymes also have nitroreductase activity. Thus the *drgA* gene may encode a nitroreductase-like protein. Two highly conserved regions (at positions 1–48 and 134–176) have been identified in the amino acid sequences (Fig. 2). It should be noted that mutations resulting in dinoseb and metronidazole resistance in the ealier described [7] mutants Din7, Din9 and Md31 are localized in these regions which

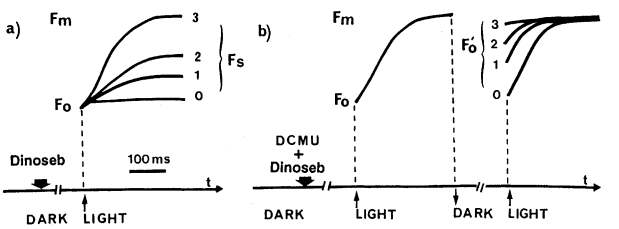

Fig. 1. Schematic representations of fluorescence measurements to estimate the inhibition by dinoseb on the acceptor (a) and donor (b) sides of photosystem II. In (a) no addition (0) or various concentrations of dinoseb (1, 2) or 10^{-5} M DCMU (3) were added in dark adapted samples 30 s before fluorescence recording. Inhibition is given by:

$$\frac{F_{s(\text{dinoseb})}-F_{s(o)}}{F_{s(\text{DCMU})}-F_{s(o)}}$$

In (b) 10^{-5} M DCMU was added in all the samples and at the same time either nothing (0) or various concentrations of dinoseb (1, 2) or 2 mM NH_2OH (3) were added. The two illuminations were separated by 20 s darkness. Inhibition is calculated as:

$$\frac{F'_{o(\text{dinoseb})}-F'_{o(o)}}{F'_{o(NH_2OH)}-F'_{o(o)}}.$$

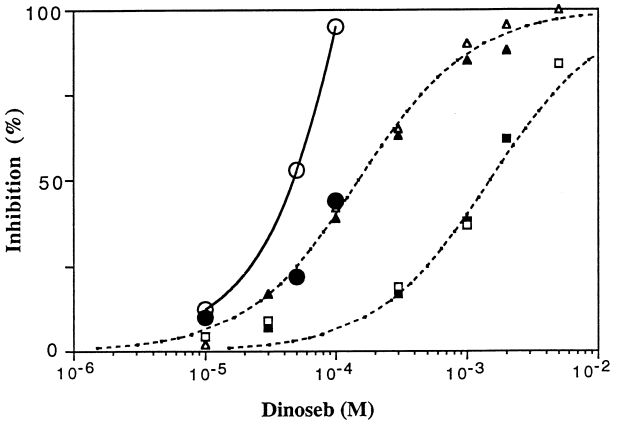

Fig. 2. Inhibition by dinoseb of *Synechocystis* sp. PCC 6803 wild type (empty symbols) and Din7 mutant (filled symbols) on the electron acceptor side of PS II (△, ▲) on the electron donor side of PS II (□, ■), and on the growth rate under photosynthetic conditions (○, ●). Inhibition of PS II activity was measured by fluorescence as described in Fig. 1. Growth rates were calculated from the slopes of growth curves.

might represent the functionally important domains of the DrgA protein.

It was proposed that the reduction of nitro functional group(s) of dinoseb, DNOC, 2,4-DNP and metronidazole might be the key step in the toxic effect of these inhibitors in wild type cells of *Synechocystis* sp. PCC 6803.

3.4. Dinoseb modification by wild type cells

During continuous growth of wild type cells in the presence of dinoseb the medium changed color from yellow (due to the presence of dinoseb) to reddish. This effect was not observed when dinoseb resistant mutants were grown in the presence of dinoseb.

Fig. 4A,B shows the absorption spectra of supernatants from wild type and Din7 cultures grown in the presence of dinoseb. At the beginning of growth the absorption spectra for wild type and Din7 supernatants were the same having a peak at 375 nm and a shoulder near 410 nm characteristic of the absorption spectrum of dinoseb. After 24 h of growth these peaks were still present in the Din7 supernatant but were completely absent from the supernatant of wild type cells. Instead the supernatant from wild type cells showed a broad peak with a maximum at 470 nm. These data indicate that wild type cells modify dinoseb, whereas such a modification cannot be realized by the *drgA* mutant Din7. As this gene encodes a protein showing similarity to known nitroreductases, the modification of dinoseb by wild type cells might be a consequence of the reduction of the nitro group(s) of dinoseb.

To test this hypothesis sodium dithionite was added under anaerobiosis to a dinoseb solution buffered at pH 8 by 20 mM

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SyDrgA   1  MD--TFDAIYQRRSVKHFDPDHRLTAEEERKLHEAAIQAPTSFNIQQLWRF  48
SaNR     1  MD--IVSVALQRYSTKAFDPSKKLTAEEADKIKTLLQYSPSSTNSQPWHF  48
EnNR     1  MD--IISVALKRHSTKAFDASKKLTAEEAEKIKTLLQYSPSSTNSQPWHF  48
EsFR     1  MD--IISVALKRHSTKAFDASKKLTPEQAEQIKTLLQYSPSSTNSQPWHF  48
ViFR     1  MTHPIIHDLENRYTSKKYDPSKKVSQEDLAVLLEALRLSASSINSQPWKF  50
MyNR     1  MN--LLTS---RRAIRTFENNHKIPRNVFKKILKDTSYAPSSFNLQPWHF  45

SyDrgA  49  LIIR--DPQLRQTIREKYG---NQAQMTDASLLILVAADVNAWD------  87
SaNR    49  IVASTEEGKARVAKSAAGNYTFNERKMLDASHVVVFCAKTAMDDAWLERV  98
EnNR    49  IVASTEEGKARVAKSAAGTYVFNERKMLDASHVVVFCAKTAMDDAWLERV  98
EsFR    49  IVASTEEGKARVAKSAAGNYVFNERKMLDASHVVVFCAKTAMDDVWLKLV  98
ViFR    51  IVIESDAAKQGMHDSFANMHQFNQPHIKACSHVILFANKLSYTRDDYDVV  100
MyNR    46  FIIETLENKKKLAICLNNG---NRSQLETSSAMILIFGDINKKE------  86

SyDrgA  88  KDPARYWRNAPREVANYLVGAIASFYGGKPQLQ-RDE---AQRSIGMAMQ  133
SaNR    99  VDQEDADGRFATPEAKAANDKGRRFFADMHRVSLKDDHQWMAKQVYLNVG  148
EnNR    99  VDQEEADGRFNTPEAKAANHKGRTYFADMHRVDLKDDDQWMAKQVYLNVG  148
EsFR    99  VDQEDADGRFATPEAKAANDKGRKFFADMHRKDLHDDAEWMAKQVYLNVG  148
ViFR   101  LSKAVADKRITEEQKEAAFASFK--FVELNCDENGEHKAWTKPQAYLALG  148
MyNR    87  LKTKIY-NDLETSKKEIIFKKINNYYDSMTNEEIKNE---LFLECGIVSL  132

SyDrgA 134  NLMLAAKAMGYDSCPMIGFDLQKVAELVKLPAD-YAIGPMVAIGKRTEDA  182
SaNR   149  NFLLGVAAMGLDAVPIEGFDAEVLDAEFGLKEKGYTSLVVVPVG-H-HSV  196
EnNR   149  NFLLGVGAMGLDAVPIEGFDAAILDEEFGLKEKGFTSLVVVPVG-H-HSV  196
EsFR   149  NFLLGVAALGLDAVPIEGFDAAILDAEFGLKEKGYTSLVVVPVG-H-HSV  196
ViFR   149  NALHTLARLNIDSTTMEGIDPELLSELFADELKGYECHVALAIGYH-HPS  197
MyNR   133  QLMLSAKNYGYETCPIGGFNKKKINELFKIEKK-YLPILIVAIGKKVTKD  181

SyDrgA 183  PGKRRSNSPGRIPLGKLLCLTKVWCLAI                        210
SaNR   197  EDFNAGLPKSRLPLETTLT-------EV                        217
EnNR   197  EDFNATLPKSRLPLSTIVT-------EC                        217
EsFR   197  EDFNATLPKSRLPQNITLT-------EV                        217
ViFR   198  EDYNASLPKSRKAFEAVIT-------IL                        218
MyNR   182  EETKNF----KMETTDF----THW---L                        198
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Fig. 3. Amino acid sequence alignments of the predicted *Synechocystis* sp. PCC 6803 DrgA protein (SyDrgA) with oxygen-insensitive NAD(P)H nitroreductases of *Salmonella typhimurium* (SaNR, accession number X17250) [11], of *Enterobacter cloacae* (EnNR, accession number M6803) [5] and of *Mycoplasma*-like organism (MyNR, accession number L22217), and with NAD(P)H-flavin oxydoreductases of *Vibrio fisheri* (ViFR, accession number 17743) [13] and of *Escherichia coli* (EsFR, accession number D25414) [13]. Identical amino acids are in boxes. Only similarity to DrgA is shown. Bold letters in a circle indicate the amino acids which are substituted in the *Synechocystis* sp. PCC 6803 dinoseb resistant mutants [7].

Tris. The yellow solution turned red. The absorption spectrum of the dithionite reduced dinoseb (Fig. 4C) was remarkably similar to that of the 24 h wild type culture supernatant (Fig. 4A).

To analyze the toxicity of the modified dinoseb for *Synechocystis* sp. PCC 6803 cells the wild type and Din7 supernatants were collected after 24 h growth in the presence of dinoseb and used as media for fresh cultures. In Din7 supernatant, containing unmodified dinoseb, inhibition of growth was stronger for wild type cells than for Din7. In wild type supernatant, containing modified dinoseb, growth curves for both wild type and mutant were the same as for cells growing without dinoseb (data not shown). These data indicate that the product of dinoseb modification present in the wild type culture supernatant is not toxic for *Synechocystis* sp. PCC 6803 cells and represents, probably, a product of dinoseb detoxification.

Thus the inhibiting effect of dinoseb on wild type cells was connected with the presence of active DrgA protein which reduced the dinoseb to non-toxic compound. The dinoseb resistant mutant Din7, lacking active DrgA protein, could not detoxify dinoseb. It was suggested that some biologically

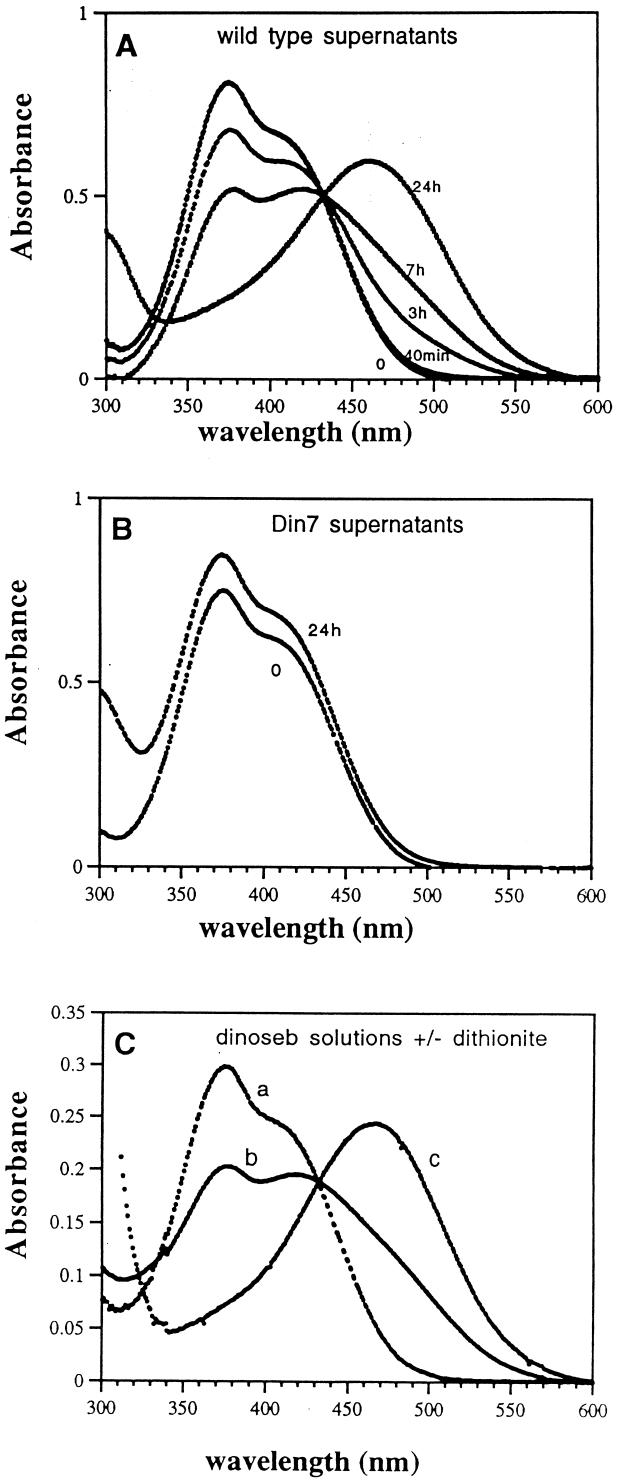

Fig. 4. Absorption spectra of supernatants of the wild type (A) and Din7 (B) cultures after various times of growth in the presence of dinoseb. Cell concentrations at zero point were about 10^8 cells/ml. C: Absorption spectra of dinoseb solutions: a, untreated; b and c, partially and totally reduced by dithionite respectively.

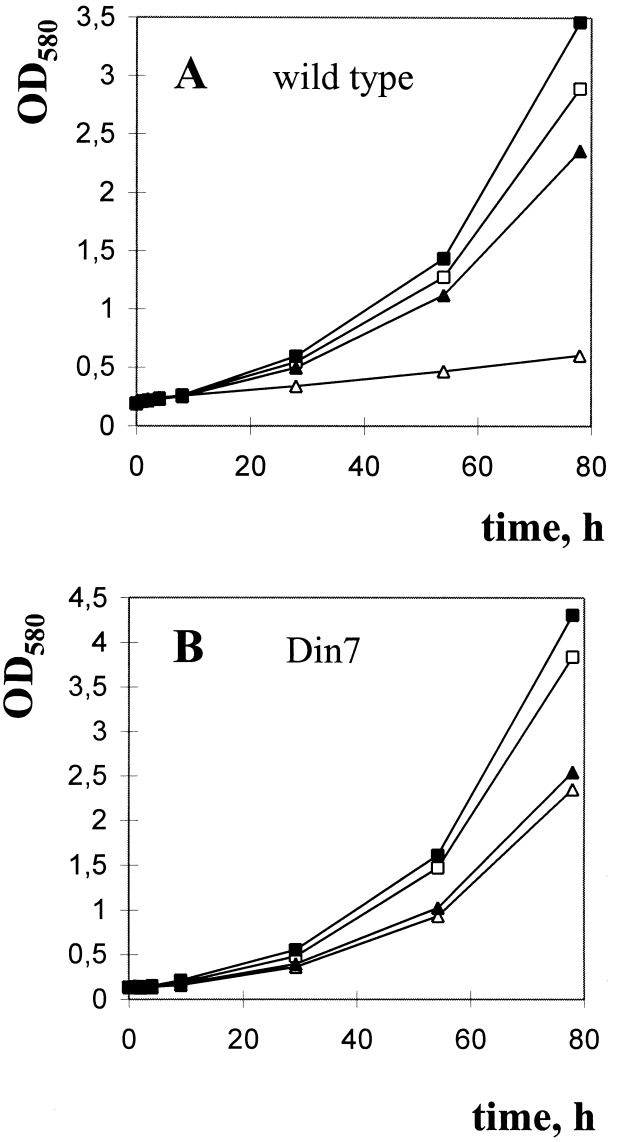

Fig. 5. Effect of superoxide dismutase on the growth of wild type (A) and the Din7 mutant in the presence (△, ▲) and in the absence (□, ■) of dinoseb. Open symbols: without superoxide dismutase. Closed symbols: superoxide dismutase (400 U/ml) was added to the growth medium.

active intermediates could arise during dinoseb modification in wild type cells.

3.5. Effect of superoxide dismutase on wild type and Din7 growth in the presence of dinoseb

To test the possibility that biologically active superoxide anions could be generated during dinoseb modification in *Synechocystis* sp. PCC 6803, the wild type and Din7 cells were incubated in liquid medium with dinoseb in the presence and in the absence of superoxide dismutase. The same slight inhibitory effect of dinoseb was observed for the Din7 (or Ins2) mutant in the presence and in the absence of superoxide dismutase. In contrast, the addition of superoxide dismutase to wild type culture medium relieved the inhibiting effect of dinoseb on cell growth (Fig. 5). The addition of superoxide dismutase did not prevent the wild type cells from modifying dinoseb. These results indicate that superoxide anions arise during dinoseb modification by DrgA protein in wild type cells and these anions may be involved in the toxic mode of dinoseb action.

4. Discussion

It is accepted that most nitroaromatic compounds undergo biotransformation to their toxic products in bacterial cells. The nitroreductases which perform the reduction of the nitro functional group of the molecule play a principal role in such activation processes [4–6]. Several enzymes possessing nitroreductase activity have been found in bacterial cells and genes encoding these enzymes have been cloned and sequenced [5,11–16]. The primary structure of the DrgA protein deduced from the nucleotide sequence was found to be similar to that of several nitroreductase-like proteins of Enterobacteriaceae [11–14]. Thus the *drgA* gene may encode a nitroreductase-like protein and the reduction of nitro functional group(s) may be the key reaction in the toxic effect of dinoseb, DNOC, 2,4-DNP and metronidazole in wild type cells of *Synechocystis* sp. PCC 6803.

Indeed, we have shown that the Din7 mutant had the same characteristics of PS II inhibition by dinoseb as the wild type and that the toxic effect of dinoseb on wild type cells of *Synechocystis* sp. PCC 6803 occurred at a dinoseb concentration lower than that needed to inhibit PS II electron transfer. Therefore dinoseb acts in at least two different ways on *Synechocystis* sp. PCC 6803. The first one, connected with PS II inhibition, can be observed both in wild type and in the Din7 mutant and does not require the DrgA protein activity. This type of inhibition may arise from interaction of the herbicide with the Q_B pocket of D1 protein as previously shown for other phenolic herbicides [3]. The second mode of dinoseb action for which we provide evidence here takes place in wild type cells but not in the Din7 mutant. This type of inhibition depends on the presence of active DrgA protein and on the modification of dinoseb leading to its detoxification. This detoxification product probably corresponds to reduced dinoseb containing amino group(s) because its absorption spectrum was similar to that of a solution of dinoseb reduced by dithionite.

The reduction of nitro groups seems to be the essential part of detoxification and biodegradation of some nitroaromatic compounds including dinoseb and 2,4-DNP [17–19]. A two-electron reduction of nitro group to the fully reduced amino adduct proceeds through the nitroso and hydroxylamine intermediates, whereas one-electron reduction was found to involve the reoxidation of nitro-anion radical to the parent compound with the concomitant formation of superoxide anion [5,20,21]. Our data indicate that the toxic effect of dinoseb is greatly diminished in the presence of superoxide dismutase, i.e. superoxide anions may arise during dinoseb modification by DrgA protein.

The results presented indicate that the resistance to nitrophenolic herbicides and metronidazole in *Synechocystis* sp. PCC 6803 mutants is associated with the absence of active product of the *drgA* gene. DrgA protein may be involved in the reduction of nitroaromatic compounds. It is proposed that the toxic effect of dinoseb is due to the formation of active oxygen radicals during the dinoseb modification by DrgA protein. These data may lead to new insights into the mechanisms of inhibition by nitrophenolic herbicides and metronidazole in photosynthetic organisms.

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