Bleaching Herbicide Norflurazon Inhibits Phytoene Desaturase by Competition with the Cofactors

Jürgen Breitenbach, Changfu Zhu, and Gerhard Sandmann*

Botanisches Institut 213, J.W. Goethe Universität, P.O. Box 111932, D-60054 Frankfurt, Germany

Cofactor requirement was determined for the heterologous expressed phytoene desaturases from the cyanobacterium *Synechococcus* and the higher plant *Gentiana lutea*. The cyanobacterial enzyme is dependent on either NAD(P) or plastoquinone, whereas only quinones such as plastoquinone can function as a cofactor for the phytoene desaturase from *G. lutea*. Enzyme kinetic studies were carried out to determine a possible competition between the cofactors and the bleaching herbicide norflurazon. For the *Synechococcus* enzyme, competition between norflurazon and NADP, as well as plastoquinone, could be demonstrated. The K_m values for these cofactors were 6.6 mM and 0.23 μM, respectively. Inhibition of the phytoene desaturase from *G. lutea* by norflurazon was also competitive with respect to plastoquinone. The K_m values of both enzymes for plastoquinone were very close.

Keywords: *Bleaching herbicides; competitive inhibitor; enzyme kinetics; norflurazon; phytoene desaturase*

INTRODUCTION

Bleaching herbicides such as norflurazon, fluridone, and diflufenican interfere with the carotenoid biosynthetic pathway. They block phytoene desaturation, accumulating phytoene at the expense of colored cyclic carotenoids (*1*). Thus, bleaching herbicides prevent the formation of enough carotenoids to ensure efficient photoprotection of the photosynthetic apparatus. As a consequence, chlorophyll is degraded, depending on the intensity of illumination, which leads to the typical bleaching symptoms. The herbicides mentioned above directly interact with the enzyme phytoene desaturase (*2*). In vitro enzyme kinetic studies with cyanobacterial enzymes have shown that the type of inhibition is noncompetitive with respect to the substrate phytoene (*3*, *4*). Cofactors for the cyanobacterial phytoene desaturase are NAD and NADP (*5*). Recently, it was shown for the homologous ζ-carotene desaturase from the higher plant *Capsicum annuum* that plastoquinone is the hydrogen acceptor (*6*). Participation of quinones in the desaturation reactions of *Narcissus pseudonarcissus* has been proposed (*7*).

Our knowledge on the inhibition mechanism of bleaching herbicides at the phytoene desaturase target is far from complete. Especially for higher plants, the nature of the cofactor and its interaction with bleaching herbicides at the enzyme is still open but can be answered with enzyme kinetic studies. A better understanding of cofactor−herbicide interaction should be helpful for the design and development of new phytoene desaturase inhibitors. Therefore, in the present study, cofactor requirements of the heterologous expressed and isolated phytoene desaturases from the higher plant *Gentiana lutea* and from the cyanobacterium *Synechococcus were* compared. In addition, enzyme kinetic studies in the presence of norflurazon were carried out.

* To whom correspondence should be addressed: fax +49 69 798 24822; e-mail sandmann@em.uni-frankfurt.de.

MATERIALS AND METHODS

Transformation and Growth of *Escherichia coli*. *Escherichia coli* strain JM101 was transformed with individual plasmids for expression of Pds from *Synechococcus* and *G. lutea* or for production of phytoene as substrate in the enzyme reaction. Growth was in LB medium supplied with appropriate antibiotics in concentrations of 100 μg/mL ampicillin and 34 μg/mL chloramphenicol (*8*). To the enzyme expressing strains, isopropylthiogalactose (0.1 mM) was added to the culture after an optical cell density (OD_{600}, 1 cm light path) of 0.1 was reached. Growth was for 24 h at a temperature of 25 °C.

Plasmid pUC8-pds_{Gl}, which mediates the overexpression of Pds from *G. lutea*, was constructed by pcr amplification of the corresponding cDNA using the primers 5′-TTGAATTCC ATG TCT CAA TTG GGA CAC ATA-3′ (start) together with 5′-TTGGATCC T GTG CAT GTC AGG AGA TGT C-3′ (end) and cloning in frame into the *Eco*RI/*Bam*HI sites of the expression vector pUC8. Plasmids pPDSdel35 for overexpression of the *Synechococcus* Pds (*9*) and pACCRT-EB (*10*) for production of phytoene were described previously.

Preparation of Substrate and Enzyme; Phytoene Desaturase Assay. Phytoene was extracted fom *E. coli* JM101/pACCRT-EB freeze-dried cells by heating with methanol containing 6% KOH (20 min, 60 °C), partitioning into diethyl ether/petroleum ether (bp. 35−60 °C) (1:9, v/v), and evaporation of the solvent in a stream of nitrogen. After resuspension in acetone, phytoene was applied into the assay.

For the preparation of the enzyme source, JM101/pPDSdel35 or JM101/pUC8-pds_{Gl} cells were disrupted by passing twice through a French pressure cell at a pressure of 50 MPa. After addition of DNAse (10 mg/mL), a soluble supernatant fraction was obtained by centrifugation at 40000*g* for 20 min.

The assay mixture contained the following in a final volume of 1 mL: 5 μL of l-α-phosphatidylcholine suspension, 0.7 mL of enzyme extract, and 5 μg of the substrate phytoene in 200 mM phosphate buffer, pH 7.2. The cofactors NAD and decyl plastoquinone (purchased from Sigma, Deisenhofen, Germany) were added in concentrations of 0.3−10 μM and 0−0.06 μM, respectively. Decyl ubiquinone was from the same supplier. Norflurazon was added from a 10 mM stock solution in methanol as indicated. Incubations of Pds from *Synechococcus* were carried out under anaerobic conditions which were established by addition of glucose (2 mM), glucose oxidase (20

10.1021/jf0106751 CCC: $20.00
Published on Web 10/19/2001

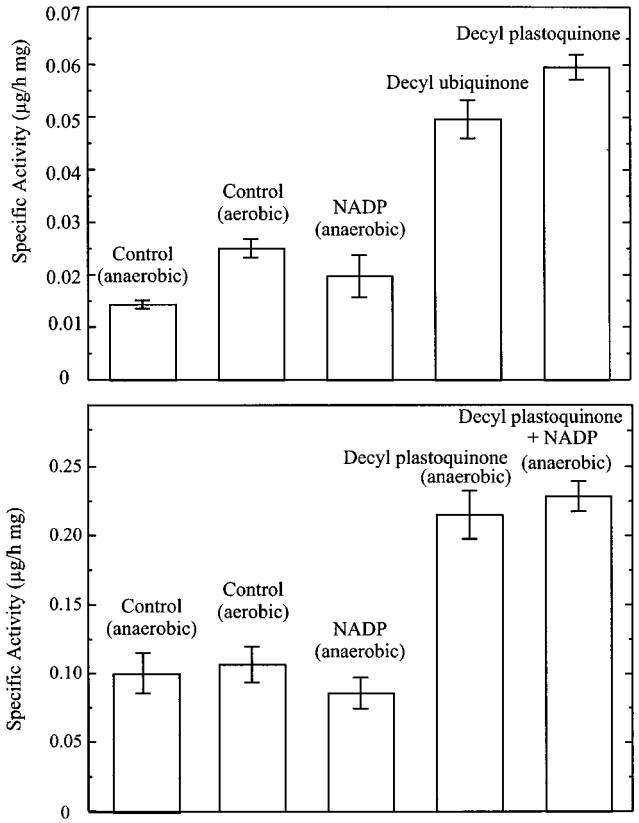

Figure 1. Cofactor requirements of phytoene desaturases from the cyanobacterium *Synechococcus* (top) and the plant *Gentiana lutea* (bottom). Cofactor concentrations were 100 μM in each case.

U/mL), and catalase (20000 U/mL) in a tightly sealed vessel. The l-α-phosphatidylcholine suspension was prepared by dissolving 0.1 g in 2 mL of chloroform and drying under a nitrogen stream. After addition of 1 mL of the buffer mentioned above, the lipid was dispersed by sonication. The assays were terminated after incubation for 2 h at 28 °C by addition of methanol (4 mL). The residual phytoene and enzymatically formed ζ-carotene were extracted from the incubation mixture with diethyl ether/petroleum ether (bp 35−60 °C) (1:9, v/v), evaporated to dryness in a stream of N_2, and resuspended in acetone. The amount of ζ-carotene was quantitated from the recorded absorption spectra using the peak at 425 nm with an extinction coefficient $E_{1\%/1\ cm}$ of 2555 as previously described (*11*). The values in Figure 1 are the means of three determinations and are given with their standard errors. Figures 2 and 3 show typical sets of experiments which were repeated three times; the probabilities of linearity are indicated. Protein was determined with the Biorad protein assay.

RESULTS AND DISCUSSION

The introduction of double bonds into the phytoene molecule involves proton and electron transfer to a cofactor. Depending on the type of phytoene desaturase which catalyzes this reaction, the cofactor will be FAD (*12*) or NAD (*13*). Either NAD or NADP were identified as cofactors of a purified phytoene desaturase from *Synechococcus* (*9*). Figure 1A shows the effect of 100 μM NADP on the *Synechococcus* phytoene desaturase which stimulates desaturation only under anaerobic conditions. Oxygen alone is able to mediate the reaction to the same extent as demonstrated previously (*5*). Because phytoene desaturation by daffodil chromoplast preparations (*7*) and the isolated pepper ζ-carotene desaturase are quinone-dependent (*6*), the effect of decyl

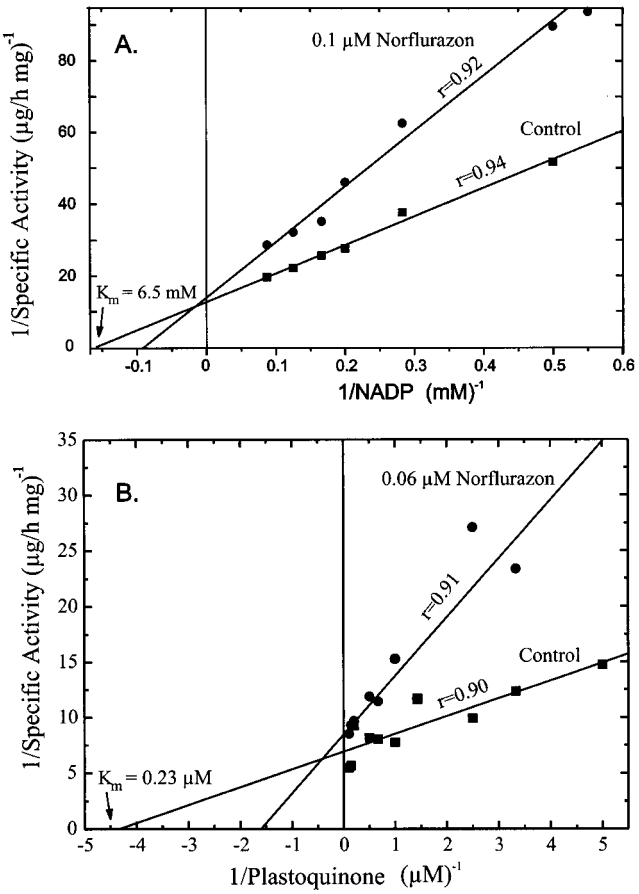

Figure 2. Double-reciprocal plot of cofactor concentrations, NADP (A) and decyl plastoquinone (B), versus the specific activity of the *Synechococcus* phytoene desaturase. One set of experiments was carried out in the absence of norflurazon and another was carried out in its presence.

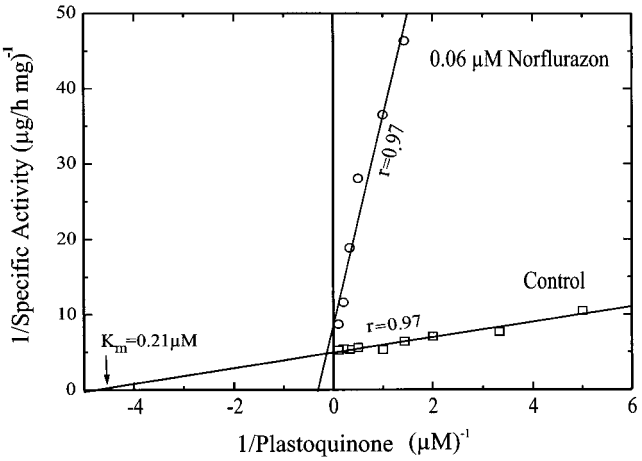

Figure 3. Double-reciprocal plot of decyl plastoquinone concentrations versus the specific activity of the phytoene desaturase from *Gentiana lutea*. One set of experiments was carried out in the absence of norflurazon and another was carried out in its presence.

plastoquinone and decyl ubiquinone on the *Synechococcus* phytoene desaturase was investigated. These quinone derivatives were used in this study because the natural C_{45}-plastoquinone and C_{45}-ubiquinone are not available commercially. A concentration of 100 μM resulted in very high specific activities of the enzyme.

The cofactor studies were extended to a phytoene desaturase from a higher plant. The enzyme from *G.*

lutea exhibited a low residual activity with or without oxygen (Figure 1B). In contrast to the *Synechococcus* enzyme, the phytoene desaturase from *G. lutea* did not respond to NADP. Decyl plastoquinone was very effective as a cofactor of desaturation. The simultanous presence of NADP had no influence on the activity. With respect to the indifference toward NADP and the affinity for plastoquinone, the *G. lutea* phytoene desaturase closely resembles the ζ-carotene desaturase from *C. annuum* (*6*). The genes encoding both enzymes are closely related, as they belong to the same gene family (*14*).

Because bleaching herbicides, including norflurazon, do not compete with the substrate phytoene for the same binding site at phytoene desaturase (*3*, *4*), the interactions with the cofactors NADP or decyl plastoquinone were analyzed using the *Synechococcus* enzyme (Figure 2). Depending on their affinities for phytoene desaturase, the concentrations of both cofactors were varied, and the resulting phytoene desaturase activity was determined in the presence or absence of norflurazon. The double-reciprocal plots for the NADP-dependent reactions without or with 0.1 μM norflurazon gave straight lines which both had intercepts close to the *y*-axis (Figure 2A). This result indicates that the V_{max} values are very little influenced by norflurazon, which reveals a competition between NADP and norflurazon for the cofactor binding site of phytoene desaturase. Very recently, a competition with NADP was also shown for a bleaching diphenylpyrrolidinone derivative (*15*).

Very similar plots were obtained when decyl plastoquinone was the cofactor. Again, the V_{max} values were rather close (Figure 2B) suggesting a competitive interaction between norflurazon and plastoquinone. The K_m values for decyl plastoquinone and NADP, which are obtained from the intercept with the *x*-axis in the control experiments, differ by several orders of magnitude reflecting the superior affinity of *Synechococcus* phytoene desaturase for plastoquinone.

Competition experiments between norflurazon and decyl plastoquinone were also performed with the phytoene desaturase from *G. lutea*. For the plant enzyme, the same competitive interaction between cofactor and inhibitor were found (Figure 3). The lines from the set of experiments in the presence or in the absence of norflurazon intercepted close to the *y*-axis. The K_m values for decyl plastoquinone were almost identical for the plant and the cyanobacterial phytoene desaturase (Figure 2B, Figure 3).

CONCLUSION

The phytoene desaturases from *G. lutea* and *Synechococcus* are homologous proteins. They exhibit the same high affinity for the cofactor plastoquinone. For each enzyme, inhibition by norflurazon proceeds by competition for the cofactor binding site. Thus, the phytoene desaturase from *Synechococcus* represents a good model for the plant enzyme in inhibitor studies. The finding of a competitive behavior of a bleaching herbicide with plastoquinone should have an impact on the modeling and prediction of herbicidal activity of new phytoene desaturase inhibitors.

LITERATURE CITED

(1) Böger, P.; Sandmann, G. Carotenoid biosynthesis inhibitor herbicides − mode of action and resistance mechanisms. *Pestic. Outlook* **1998**, *9*, 29−35.

(2) Sandmann, G.; Böger, P. Phytoene desaturase as target for bleaching herbicides. In *Herbicide Activity: Toxicology, Biochemistry and Molecular Biology*; Roe, R. M., Burton, J. D., Kuhr, R. J., Eds.; IOS Press: Amsterdam, 1997; pp 1−10.

(3) Sandmann, G.; Linden, H.; Böger, P. Enzyme-kinetic studies on the interaction of norflurazon with phytoene desaturase. *Z. Naturforsch.* **1989**, *44C*, 787−790.

(4) Kowalczyk-Schröder, S.; Sandmann, G. Interference of fluridone with the desaturation of phytoene in membranes of the cyanobacterium *Aphanocapsa*. *Pestic. Biochem. Physiol.* **1992**, *42*, 7−12.

(5) Schneider, C.; Böger, P.; Sandmann G. Phytoene desaturase: Heterologous expression in an active state, purification, and biochemical properties. *Protein Expression Purif.* **1997**, *10*, 175−179.

(6) Breitenbach, J.; Fernandez-Gonzales, B.; Vioque, A.; Sandmann, G. A higher-plant type ζ-carotene desaturase in the cyanobacterium *Synechocystis* PCC6803. *Plant Mol. Biol.* **1998**, *36*, 725−732.

(7) Mayer, M. P.; Beyer, P.; Kleinig, H. Quinone compounds are able to replace molecular oxygen as terminal electron acceptors in phytoene desaturation in chromoplasts of *Narcissus pseudonarcissus* L. *Eur. J. Biochem.* **1990**, *191*, 359−363.

(8) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: a Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.

(9) Fraser, P. D.; Linden, H.; Sandmann, G. Purification and reactivation of recombinant *Synechococcus* phytoene desaturase from an overexpressing strain of *E. coli*. *Biochem. J.* **1993**, *291*, 687−692.

(10) Sandmann, G.; Misawa, N. New functional assignment of the carotenogenic genes *crtB* and *crtE* with constructs of these genes from *Erwinia* species. *FEMS Microbiol. Lett.* **1992**, *90*, 253−258.

(11) Sandmann, G.; Schneider, C.; Böger, P. A new nonradioactive assay of phytoene desaturase to evaluate bleaching herbicides. *Z. Naturforsch.* **1996**, *51C*, 534−538.

(12) Fraser, P. D.; Misawa, N.; Linden, H.; Yamano, S.; Kobayashi, K.; Sandmann, G. Expression in *Escherichia coli*, purification and reactivation of the recombinant *Erwinia uredovora* phytoene desaturase. *J. Biol. Chem.* **1992**, *267*, 19891−19895.

(13) Hausmann, A.; Sandmann G. A single 5-step desaturase is involved in the carotenoid biosynthesis pathway to β-carotene and torulene in *Neurospora crassa*. *Fungal Genet. Biol.* **2000**, *30*, 147−153.

(14) Breitenbach, J.; Kuntz, M.; Takaichi, S.; Sandmann, G. Catalytic properties of an expressed and purified higher plant type ζ-carotene desaturase from *Capsicum annuum*. *Eur. J. Biochem.* **1999**, *265*, 376−383.

(15) Ogawa, H.; Yamada, I.; Arai, K.; Hirase, K.; Moriyasu, K.; Schneider, C.; Sandmann, G.; Böger, P.; Wakabayashi, K. Mode of bleaching phytotoxicity of herbicidal diphenylpyrrolidinones. *Pestic. Manage. Sci.* **2001**, *57*, 33−40.

Received for review May 22, 2001. Revised manuscript received August 12, 2001. Accepted August 14, 2001.

JF0106751