Enzyme-Kinetic Studies on the Interaction of Norflurazon with Phytoene Desaturase

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Bleaching herbicides inhibit carotene biosynthesis in photosynthetic organisms. The interaction of norflurazon [4-chloro-5-methylamino-2-(3-trifluoromethylphenyl)-pyridazin-3(2H)one] with its target enzyme phytoene desaturase, has been characterized by enzyme-kinetic studies. A Lineweaver-Burk plot showed a non-competitive manner for norflurazon inhibition. Binding of norflurazon to phytoene desaturase was reversible as demonstrated by complete replacement of bound ^{14}C-labeled herbicide by unlabeled norflurazon. In a linear Dixon plot the k_i value for norflurazon was determined as 0.09 μM. With the *in vitro* system from *Anacystis* used in this study it was possible to perform structure-activity studies with selected *m*-phenyl-substituted pyridazinones. A linear relationship between inhibitory properties of these compounds and their lipophilicity could be established.

Introduction

Norflurazon belongs to the phenylpyridazinones which are the oldest group of bleaching herbicides. They decrease the carotenoid and chlorophyll content of treated plants and concurrently accumulate phytoene, an intermediate in the pathway leading to colored carotenes and xanthophylls [1]. After the development of *in vitro* systems for carotenogenesis from daffodil chromoplasts [2] and from photosynthetic membranes of the blue-green alga *Aphanocapsa* [3], it could be shown that inhibition of the carotenogenic pathway as well as phytoene accumulation originated from a specific interference of norflurazon with phytoene desaturase. Furthermore, we have characterized norflurazon-resistant *Anacystis* mutants with resistant phytoene desaturases (unpublished results).

The availability of *in vitro* assays makes it possible to perform enzyme kinetics with the phytoene-desaturase reaction in the presence of norflurazon. For this purpose, we have used the cell-free system from *Anacystis*, which has the advantage of high phytoene conversion rates and efficient metabolism into β-carotene with little detectable intermediates [4]. From these kinetic studies information can be obtained on the type of interaction of norflurazon with phytoene desaturase. Either direct interaction of phytoene-desaturase inhibitors with the active site or allosteric modification of enzyme activity as observed for several carotenes have been discussed [5]. This paper reports on Michaelis-Menten kinetics together with binding studies using ^{14}C-labeled norflurazon to give an answer whether inhibition is competitive or non-competitive and whether norflurazon-binding is reversible or irreversible.

Materials and Methods

Anacystis R2 (= *Synechococcus* PCC 7942) was cultivated as described for other unicellular cyanobacteria [3]. The carotene-deficient mutant *Phycomyces blakesleeanus* C5 was grown for four days according to Ref. [6] and the carotene-deficient *Fusarium moniliforme* SG4 in 2.4% potato-dextrose broth (w/v; Difco Laboratories, Detroit, U.S.A.) for 5 days. *In vitro* carotenogenesis was carried out in a coupled system with *Phycomyces* C5 extracts as source of phytoene from R-[2-^{14}C]mevalonic acid (Amersham-Buchler, Braunschweig, Germany) and *Anacystis* membranes performing the subsequent pathway to β-carotene [7, 8]. In the experiment of Fig. 3 *Phycomyces* extracts were replaced by geranylgeranyl pyrophosphate-accumulating extracts from *Fusarium* SG4. Fungal extracts were prepared by rubbing freeze-dried mycelium through a sieve with a mesh size of 0.4 mm. Then 8 times (v/w) the amount of 0.4 M Tris-HCl buffer, pH 8.0, was added containing 5 mM dithiothreitol. The paste was stirred with a spatula for 1 min and centrifuged for 10 min at $10{,}000 \times g$.

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Anacystis membranes were obtained by French-press treatment (500 bar) of cells resuspended in 0.1 M Tris-HCl buffer, pH 8.0 containing 5 mM dithiothreitol. After centrifugation ($12{,}000 \times g$, 15 min) the pellet was resuspended in the same buffer and the membranes were ready for the assay. Incubations were carried out in 0.5 ml reaction mixture, containing mevalonic acid 0.5 μCi, NAD 0.75 μmol, ATP 10 μmol, $MnCl_2$ 3 μmol, $MgCl_2$ 2 μmol, fungal extracts containing 0.15 mg protein and *Anacystis* thylakoids equivalent to 100 μg of chlorophyll in a final Tris-HCl concentration of 0.1 M at pH 8.0.

After incubation for 2 h in the light at 30 °C and saponification of chlorophyll with 6% KOH for 15 min at 60 °C, the carotenes were partitioned into petrol (b.p. 40–60 °C) and separated by HPLC on a 25 cm Spherisorb ODS-1 5 μ column with acetonitrile/methanol/2-propanol 85:10:5 (v/v/v) as eluent [9]. Radioactivity was continuously recorded by a radioactivity flow detector (Ramona LS, Ray Test, Straubenhardt, Germany).

For the Lineweaver-Burk plot the substrate concentration was calculated as radioactivity in phytoene + β-carotene. The latter can be regarded as the part of the substrate phytoene which has been metabolized. The inhibition ratio used in Fig. 4 is defined as the ratio of radioactivity incorporated from [^{14}C]geranylgeranyl pyrophosphate into phytoene *versus* ^{14}C-incorporation into β-carotene at a defined inhibitor concentration (1 μM) divided by the same ratio of the untreated control.

In the binding studies, *Anacystis* membranes equivalent to 100 μg of chlorophyll were suspended in 1 ml of 0.1 M Tris-HCl buffer, pH 8.0, and [^{14}C]norflurazon (3.24 mCi/mmol) was added. After 1 h the mixture was centrifuged (10 min, $15{,}000 \times g$) and the radioactivity in the supernatant determined in a liquid-scintillation counter. For replacement of bound ^{14}C-labeled by non-radioactive norflurazon, the pellet was resuspended in norflurazon-containing buffer. After 20 min and subsequent centrifugation radioactivity was determined in the supernatant as above.

Chlorophyll [10] and protein [11] was determined as described. The fungal mutants were from Prof. E. Cerdá-Olmedo, Departamento de Genética, Universidad de Sevilla, Spain. ^{14}C-Labeled and unlabeled norflurazon was a gift from Sandoz AG, Basel, Switzerland, the other phenylpyridazinones were supplied by BASF AG, Limburgerhof, Germany.

Results and Discussion

The *in vitro* experiments for the characterization of norflurazon/phytoene-desaturase interaction were performed with a coupled system [7] in which the extracts from the *Phycomyces* mutant C5 synthesized [^{14}C]phytoene which was subsequently converted by the *Anacystis* membranes. As both biosynthetic sequences from [^{14}C]mevalonic acid to phytoene and further on to [^{14}C]β-carotene were carried out simultaneously, the amount of [^{14}C]phytoene offered to phytoene desaturase had to be averaged over the whole reaction period by summing-up the radioactivity accumulated in phytoene and in β-carotene at the end of the reaction. The details for this procedure are given in Ref. [1]. These values obtained for substrate concentrations were used to perform Michaelis-Menten kinetics as demonstrated in Fig. 1. Double reciprocal plots of substrate values *versus* radioactivity found in β-carotene, the final product of the reaction sequence, yielded straight lines. With increasing norflurazon concentration an increase of the slope is observed. All three lines obtained for a series of determination with no norflurazon or in the presence of 0.5 and 0.75 μM norflurazon intersected at the same position on the abscissa. This result indicates that phytoene desaturase is inhibited either in a reversible or irreversible non-competitive manner [12].

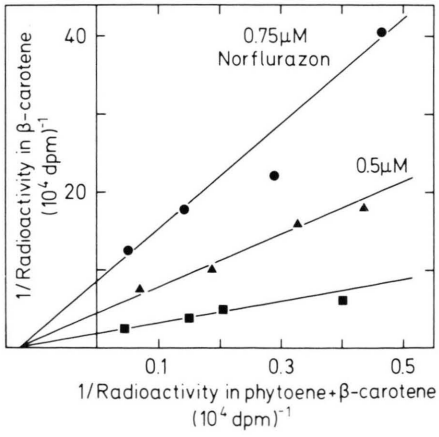

Fig. 1. Lineweaver-Burk plot of substrate dependence of phytoene desaturation in the presence of norflurazon (see text for further explanations).

The binding experiments in Table I were performed to discriminate between both possibilities. First, [^{14}C]norflurazon was bound to the carotenogenic *Anacystis* membranes. Subsequently, the inhibitor was replaced by unlabeled herbicide and washed-off the membranes. Increasing amounts of norflurazon in the washing solution increased the release of membrane-bound norflurazon in a first washing step. After a second step, less than 1% residual norflurazon was still bound to the membranes. From this result we can exclude an irreversible binding of norflurazon to the membrane-associated phytoene desaturase under our assay conditions.

Obviously, the inhibition site of norflurazon is different from the active site. Structural similarities of phytoene-desaturase inhibitors with segments of the phytoene molecule have led to speculations on a possible competition of certain inhibitors with phytoene for the catalytic site at the phytoene-desaturase complex [5, 13]. For norflurazon we assume that it might interact with the allosteric site of phytoene desaturase where intermediates of the carotene biosynthetic pathway exert a negative control on enzyme activity [4, 14].

Comparable studies on inhibitor interaction with enzymes of the carotenogenic pathway are available for the *m*-phenoxybenzamide S 3442 and the dihydropyrone LS 80707 [1, 15]. The first compound gives a non-competitive picture in the Lineweaver-Burk plot for phytoene desaturase and the latter exhibits the same feature for ζ-carotene desaturase. However, no binding experiments were possible since radioactive-labeled inhibitors were not available. Nevertheless, subsequent enzyme kinetics and plots of V_{max} *versus* enzyme concentrations for sets of experiments with and without LS 80707 present indicated an irreversible interaction of this inhibitor with ζ-carotene desaturase [1].

Table I. Binding of [^{14}C]norflurazon to *Anacystis* membranes and replacement by unlabeled norflurazon.

Norflurazon concentration in the washing solution [μM]	Recovery of membrane-bound [^{14}C]norflurazon by washing [%]
0	14
0.5	31
1.0	42

Membranes equivalent to 100 μg of chlorophyll were pretreated with 2700 dpm (= 0.28 μM) norflurazon and 700 dpm were bound corresponding to the 100% value. After a second washing-step the residual bound norflurazon was less than 1%.

Increasing concentrations of norflurazon decrease the *in vitro* conversion of phytoene to β-carotene (Fig. 2). For non-competitive inhibitors k_i values can be determined in a Dixon plot of inhibitor concentration *versus* reciprocal product formation. As shown in the inset of Fig. 2, a straight line was obtained and the k_i values for norflurazon could be determined as 0.09 μM. So far, this k_i value is the lowest one determined *in vitro* as yet for any herbicidal inhibitor of phytoene desaturase [15, 16].

Variations of the substituents of norflurazon modify the herbicidal activity of this inhibitor [17]. Correlation of this activity with physiochemical properties of substituents at the *m*-phenyl position could be obtained with intact cells of the green alga *Scenedesmus* [18]. This investigation showed that lipophilicity π of the ligand exerts the strongest influence together with a contribution of electronic properties. For the experiment in Fig. 3 six phenylpyridazinones were selected with different groups at the *meta* position of the phenyl moiety which all show similar electronic properties. The inhibition ratio as defined in Ref. [5] was determined for all these compounds at a fixed concentration of 1 μM, and the logarithmic ratio plotted against (Fig. 3). A good linear relationship could

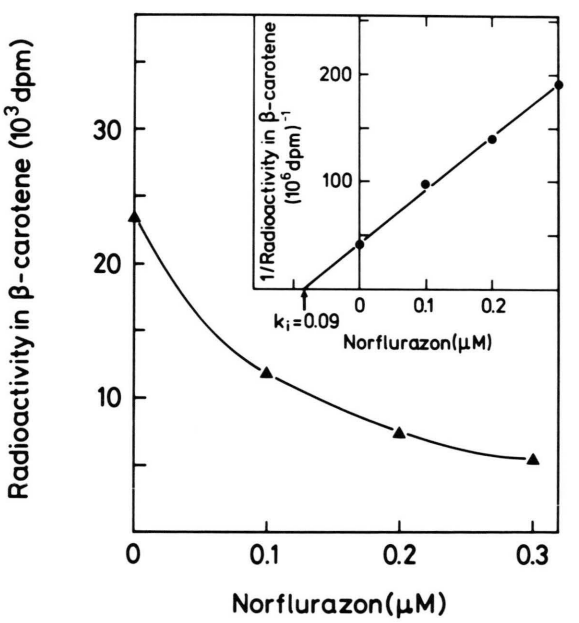

Fig. 2. Inhibition of carotenogenesis by norflurazon and determination of k_i value in a Dixon plot (inset).

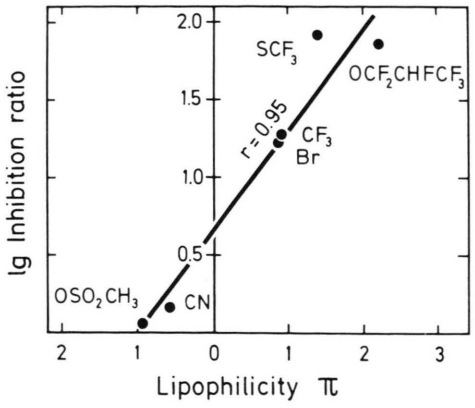

Fig. 3. Relationship between inhibition of phenylpyridazinones and lipophilicity. The pyridazinones were *m*-phenyl-substituted as indicated.

be established. Using the inhibition ratio as a parameter, the cell-free system makes it possible to perform a structure-activity correlation for phenylpyridazinones which reflects direct interaction of these inhibitors with phytoene desaturase. Apparently, this approach may be advantageous to probe the inhibition site of phytoene desaturase.

Acknowledgements

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