Inhibition of the Acetyl-CoA Carboxylase of Barley Chloroplasts by Cycloxydim and Sethoxydim

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Z. Naturforsch. **42c**, 1361–1363 (1987);
received September 14, 1987

Acetyl-CoA Carboxylase Inhibition, Cyclohexane-1,3-dione Herbicides, Fatty Acid Biosynthesis, Mode of Action of Cycloxydim, Clethodim, Sethoxydim

The effect of the three cyclohexane-1,3-dione derivatives cycloxydim, sethoxydim and clethodim on the incorporation of ^{14}C-labelled acetate, malonate, acetyl-CoA or malonyl-CoA into fatty acids was studied in an enzyme preparation isolated from barley chloroplasts (*Hordeum vulgare* L. var. "Alexis"). The herbicides cycloxydim, clethodim and sethoxydim block the *de novo* fatty acid biosynthesis from [2-^{14}C]acetate and [1-^{14}C]acetyl-CoA, whereas that of [2-^{14}C]malonate and [2-^{14}C]malonyl-CoA is not affected. The data indicate that the mode of action of the cyclohexane-1,3-dione derivatives in the sensitive barley plant consists in the inhibition of *de novo* fatty acid biosynthesis by blocking the acetyl-CoA carboxylase (EC 6.4.1.2.).

Introduction

The cyclohexane-1,3-dione derivatives cycloxydim and sethoxydim are very effective and selective herbicides for postemergence control of annual and perennial grasses in many dicotyledonous crop plants [1–4]. This is also the case for the structurally related active ingredient clethodim (proposed common name) [5]. The three herbicides cause growth inhibition in leaves and meristematic zones of many Poaceae, block chloroplast biogenesis and accumulation of chlorophylls and carotenoids and inhibit the biosynthesis of all cellular membrane-bound phospho- and glycolipids [6–9]. The target of the three cyclohexane-1,3-dione derivatives appears to be the *de novo* fatty acid biosynthesis, which in higher plants proceeds exclusively in the plastids (chloroplasts) [10]. In chloroplasts isolated from the sensitive maize, oat and wheat seedlings they block the *de novo* fatty acid biosynthesis from [2-^{14}C]acetate in a dose-dependent manner [9, 11, 12]. The point of interaction with the different enzyme activities in the biosynthetic sequence of fatty acid formation was not known hitherto. Here we provide evidence that the point of interaction of the cyclohexane-1,3-dione herbicides is the acetyl-CoA carboxylase.

Abbreviations: clethodim, 2-(1-(3-chloro-2-propenyl)-oxymino)-propyl-5-(2-ethylthiopropyl)-2-cyclohexane-1,3-dione; cycloxydim, 2-(1-ethoxyimino)-butyl-5-(thian-3-yl)-2-cyclohexane-1,3-dione; sethoxydim, 2-(1-ethoxyimino)-butyl-5-(2-ethylthiopropyl)-2-cyclohexane-1,3-dione.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/87/1100–1361 $ 01.30/0

Materials and Methods

Chloroplasts from barley seedlings (*Hordeum vulgare* L. var. "Alexis") in the two-leaf stage were isolated according to the procedure described before [13]. From these by ammonium sulfate fractionation an active enzyme preparation was isolated [13], which was active in the incorporation of labelled acetate and malonate into fatty acids. Applied were [2-^{14}C]acetate, [2-^{14}C]malonate, [1-^{14}C]acetyl-CoA and [2-^{14}C]malonyl-CoA purchased by Amersham.

The incubation medium contained ACP (Calbiochem), NADPH, NADH, ATP, $MgCl_2$ and Na_2HPO_4 as given in [13]. In the experiments with [^{14}C]malonate and [^{14}C]malonyl-CoA cold acetyl-CoA (25 μM) was added. The three herbicides, dissolved in methanol, were applied to the enzyme suspension with a final methanol concentration, in the controls also, of 0.2%. After an incubation time of 15 min in the case of labelled malonyl-CoA and acetyl-CoA and of 30 min after applying labelled acetate and malonate, the reaction was stopped by 40% KOH, to hydrolyze the thioesters. After addition of 12 N H_2SO_4 and 30% trichloroacetic acid the fatty acids, with cold oleic acid as carrier, were extracted with light petrol (b.p. 50–70 °C). After separation by TLC [14] the radioactivity of the fatty acids was determined in a liquid scintillation counter (Packard Tricarb 2000 CA). The protein determination was performed after a modified Lowry method [15].

Results and Discussion

By foliar spray application of cycloxydim and sethoxydim (ca. 125 g·ha^{-1}) to barley seedlings it was checked that the barley variety used was sensitive to the cyclohexane-1,3-dione herbicides. The crude enzyme preparation, isolated from barley chloroplasts is active in the incorporation of [2-^{14}C]-acetate into fatty acids when provided with the appropriate cofactors. That the incorporation rate is

linear to up to 20 min [13] was confirmed by us. This *de novo* fatty acid biosynthesis from [^{14}C]acetate is blocked by cycloxydim and sethoxydim in a dose-dependent manner (Fig. 1). Clethodim given at a 10 μM concentration reduced the [^{14}C]acetate incorporation to a similar degree (59%) as cycloxydim (60%) and sethoxydim (56%). The inhibition of the *de novo* fatty acid biosynthesis of this enzymic system is not complete even at a 100 μM dosis.

In contrast to the results obtained with [^{14}C]acetate, the *de novo* fatty acid biosynthesis of the enzyme preparation from [2-^{14}C]malonate is not affected by the herbicides cycloxydim, sethoxydim and clethodim even at a 100 μM concentration (Fig. 2). Similar results were obtained by studying the incorporation of [^{14}C]malonyl-CoA. All three cyclohexane-1,3-dione derivatives had no effect on the *de novo* fatty acid biosynthesis from the labelled malonyl-CoA (Fig. 3). This indicates that the three cyclohexane-1,3-dione herbicides do not interfere with the activation of malonate to malonyl-CoA or the use of the malonyl-CoA for the formation of new fatty acids. The target of the three active ingredients must therefore be an enzymatic step in the utilization of acetate and its transformation to malonyl-CoA,

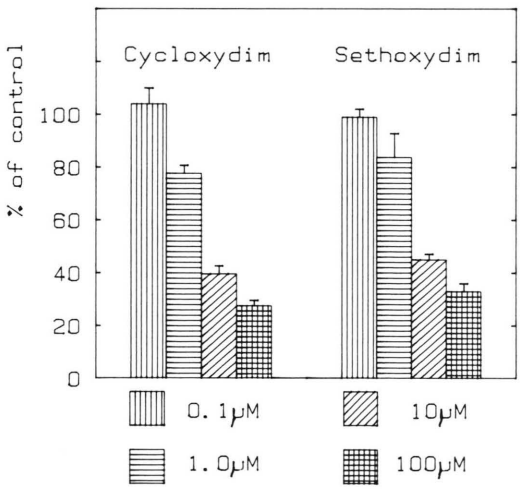

Fig. 1. Reduction by cycloxydim and sethoxydim of [2-^{14}C]acetate incorporation into the total fatty acid fraction by an enzyme preparation isolated from chloroplasts of barley seedlings. Applied were 0.86 μCi (ca. 15 nmol) [2-^{14}C]acetate per condition. The incorporation rate of the control amounted to 2600 Bq per mg protein·h and to an incorporation of 3.4% of the applied radioactivity. Mean of 4 determinations from two separate chloroplast isolations and enzyme preparations with standard deviation.

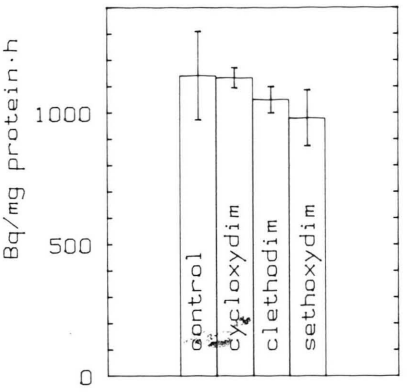

Fig. 2. Incorporation of [2-^{14}C]malonate into the total fatty acid fraction by an enzyme fraction isolated from barley chloroplasts with and without addition (10^{4} M) of the herbicides cycloxydim, clethodim and sethoxydim. About 5% of the applied radioactivity (0.3 μCi [2-^{14}C]malonate ≙ 15 nmol) was incorporated. Mean of 3 determinations with standard deviation.

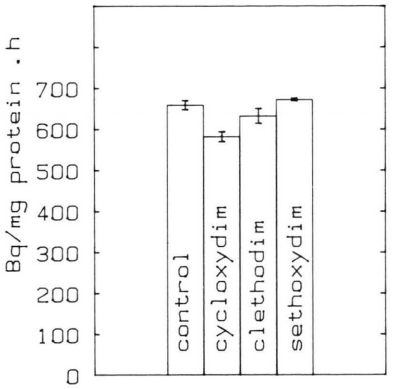

Fig. 3. Incorporation of [2-^{14}C]malonyl-CoA into the fatty acid fraction by an enzyme preparation from barley chloroplasts with and without application (10^{-4} M) of the herbicides cycloxydim, clethodim and sethoxydim. About 14% of the applied radioactivity (0.05 μCi malonyl-CoA ≙ 12 nmol per condition) was incorporated. Mean of 3 determinations with standard deviation.

which could be either the acetyl-CoA synthetase and/or the acetyl-CoA-carboxylase, both of which are known to be localized in the chloroplasts [16, 17].

In order to decide which of the two possible enzymes is the site of interaction, we applied labelled [^{14}C]acetyl-CoA to the isolated enzyme preparation. Our preliminary results indicate that the incorporation of [1-^{14}C]acetyl-CoA can also be blocked by the herbicides cycloxydim, clethodim and sethoxydim

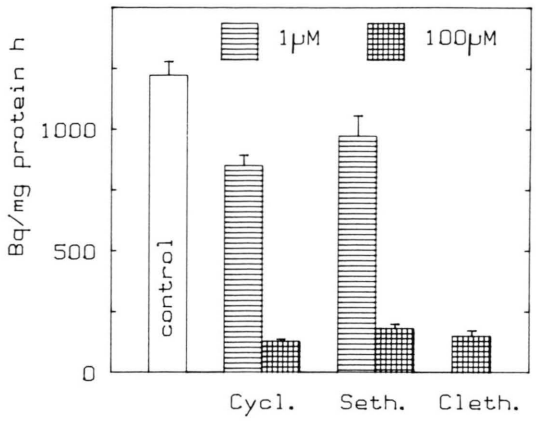

Fig. 4. Incorporation of [1-^{14}C]acetyl-CoA into the fatty acid fraction by a crude enzyme preparation isolated from barley chloroplasts with and without addition of the herbicides cycloxydim, sethoxydim and clethodim. The incorporation rate of the control amounted to 1223 Bq per mg protein·h. About 30% of the applied radioactivity (0.02 μCi ≙ 7 nmol) was incorporated. Mean of 3 determinations with standard deviation.

(Fig. 4). This finding strongly suggests that the acetyl-CoA carboxylase (EC 6.4.1.2.) is the target of the three cyclohexane-1,3-dione herbicides. All other effects of the cyclohexane-1,3-dione herbicides such as inhibition of cell and chloroplast multiplication, biomembrane formation, phospho- and glycolipid biosynthesis as well as *de novo* fatty acid biosynthesis [4–9, 11, 12, 18] can be explained by this mode of action on the activity of the acetyl-CoA carboxylase.

That the inhibition of the [^{14}C]acetate incorporation into fatty acids is not complete even at a 100 μM concentration may be due to the possibility that some cold malonyl-CoA and acyl-ACPs may have been present in the multi-enzyme preparation with fatty acid biosynthesis activity, for which [^{14}C]acetate and [^{14}C]acetyl-CoA can be used as starter molecules. One has also to take into account that the crude enzyme preparation used in this investigation contained many individual enzyme activities and that not all sites of interactions may have been accessible to the herbicides involved. Further investigations for a direct isolation of the acetyl-CoA carboxylase from barley chloroplasts and appropriate inhibitor studies with the three herbicides are in progress.

Acknowledgements

This work was supported by financial aid from the BASF-AG, D-6703 Limburgerhof, and Nippon Soda, Tokyo, which is gratefully acknowledged. We wish to thank Dr. G. Retzlaff, BASF-AG, Agricultural Research Station for providing cycloxydim and sethoxydim and Dr. A. Rendina, Chevron Chemical Company, Richmond, California for a sample of the herbicide clethodim, Dr. T. J. Bach for a helpful discussion and Ms. U. Prenzel and I. Schürer for excellent assistance.

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