Inhibition of ACCase220 and ACCase240 Isozymes from Sethoxydim-Resistant and -Susceptible Maize Hybrids†

Bev J. Incledon and J. Christopher Hall*

Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Acetyl-coenzyme A carboxylase (ACCase) isozymes were separated from cyclohexanedione-resistant and -susceptible maize. ACCase240 from resistant maize was 3.7-, >77-, and 12.8-fold more resistant to inhibition by clethodim, sethoxydim, and tralkoxydim, respectively, than ACCase240 from susceptible maize. The resistant ACCase240 preparation had 3.0-fold more protein and 14.5-fold lower specific activity than susceptible ACCase240. Resistant ACCase240 has a V_{max} 5.5-fold lower than that of susceptible ACCase240, whereas apparent K_m values were similar. ACCase220 from resistant maize was >25- and 7.2-fold more resistant to inhibition by sethoxydim and tralkoxydim, respectively, than susceptible ACCase220 but was inhibited to the same extent by clethodim. In summary, sethoxydim-resistant corn has an altered herbicide-resistant ACCase220 isozyme and increased expression of a less efficient, herbicide-resistant ACCase240 isozyme. However, to what extent alteration of both isozymes contributes to sethoxydim resistance is not clear.

Keywords: *Sethoxydim; resistance; ACCase; cyclohexanedione herbicides*

INTRODUCTION

Acetyl-coenzyme A carboxylase (EC 6.4.1.2) (ACCase) is the target site for the aryloxyphenoxypropionate (AOPP) and cyclohexanedione (CHD) classes of herbicides and the rate-limiting enzyme in the de novo fatty acid biosynthetic pathway (Secor and Cseke, 1988; Burton et al., 1987; Lichtenthaler et al., 1987). ACCase catalyzes the ATP-dependent conversion of acetyl-coenzyme A to malonyl-coenzyme A by the transfer of a carboxyl group (Finlayson and Dennis, 1983a,b). The carboxylation of acetyl-CoA requires three enzymatic reactions. Multifunctional ACCase enzymes are believed to catalyze all three reactions with one constituent protein. Conversely, multisubunit ACCase enzymes require distinct polypeptides to catalyze the separate enzymatic reactions.

Most plant species belonging to the Graminaceae family are naturally susceptible to the AOPP and CHD herbicides in contrast to dicotyledonous plant species, which are resistant (Burton et al., 1987, 1991; Rendina et al., 1988; Catanzaro et al., 1993; Ashton et al., 1994). Consequently, these herbicides are commonly used for the control of grass weeds in dicotyledonous crops (Ishikawa et al., 1985).

Resistance to the AOPP and CHD classes of herbicides has developed in several grass species following repeated use of these two classes of herbicides (Rendina et al., 1990; Holt et al., 1993; Maneechote et al., 1994; Tardif and Powles, 1994). Resistance to these herbicides is usually due to the presence of an altered ACCase (Evenson et al., 1994, 1997; Herbert et al., 1996a; Burton, 1997; Incledon et al., 1997a). ACCase in many higher plants, excluding the grasses, exists as two distinct forms, a multisubunit-resistant form and a multifunctional-susceptible form (Alban et al., 1994; Dehaye et al., 1994; Konishi and Sasaki, 1994; Konishi et al., 1996). The multisubunit form is located in the chloroplast, and the multifunctional form is extrachloroplastic (Burton, 1997; Ohlrogge and Jaworski, 1997). Until recently it has been reported that there is no multisubunit form of ACCase in the grasses (Konishi et al., 1996; Ohlrogge and Jaworski, 1997). However, Incledon and Hall (1997b) showed that maize has a multisubunit ACCase.

ACCase of maize exists as at least two isozymes (Egli et al., 1993; Somers et al., 1993; Ashton et al., 1994; Herbert et al., 1996a; Burton, 1997; Incledon and Hall, 1997b). These distinct isozymes have been reported to be the products of separate genes and have different sensitivities to the CHD and AOPP classes of herbicides (Marshall et al., 1992; Somers et al., 1993; Ashton et al., 1994). The exact quaternary structure of maize ACCase has not been elucidated. There are reports of maize ACCase isozymes existing solely in a homodimeric form (Nikolau and Hawke, 1984; Egli et al., 1993; Ashton et al., 1994; Dehaye et al., 1994; Herbert et al., 1996a) and as a combination of homodimeric and multimeric forms (Incledon and Hall, 1997a,b). ACCase containing the high molecular mass biotinylated protein (~240 kDa) constituent is localized in the chloroplast, whereas the lower molecular mass biotinlyated protein (~220 kDa) constituent containing ACCase is extrachloroplastic (Egli et al., 1993; Ashton et al., 1994; Herbert et al., 1996b). These two isozymes of maize ACCase can be separated by either anion exchange chromatography on a variety of resins or by dye affinity chromatography on a Dye Matrex Orange A resin (Egli et al., 1993; Ashton et al., 1994; Incledon and Hall, 1997b). Although there have been many reports of multiple isozymes of ACCase in monocotyledonous

* Author to whom correspondence should be addressed [telephone (519) 824-4120, ext. 2740; fax (519) 837-0442; e-mail jchall@evbhort.uoguelph.ca].

† This work was supported by a research grant from Agriculture Canada/NSERC and DowElanco provided to J.C.H. B.J.I. is a recipient of an NSERC postgraduate scholarship (PGS-B).

10.1021/jf980124j CCC: $18.00
Published on Web 12/05/1998

plants, there have been relatively few reports on the inhibition of the isolated isozymes from resistant plants.

Sethoxydim [(±)−(*E*,*Z*)-2-(1-(ethoxyiminobutyl)-5-[2-ethylthio)propyl]-3-hydroxy cyclohex-2-enone]-resistant maize (*Zea mays* L.) hybrids have been selected in cell culture by Parker et al. (1990a). These authors isolated ACCase from sethoxydim-resistant maize and showed that the sethoxydim and haloxyfop IC_{50} values for the resistant hybrids were similar to the IC_{50} for the wild-type maize hybrid. In addition, they demonstrated that the quantity of a biotinylated polypeptide (∼220 kDa) was elevated in the resistant maize hybrids. These investigators proposed that resistance was due to overproduction of ACCase in the resistant line. Subsequently, Parker et al. (1990b) found an altered ACCase in other sethoxydim-resistant maize hybrids that was >100-fold more resistant to inhibition by sethoxydim.

In this study, ACCase isozymes from the sethoxydim-resistant maize hybrid DK404SR were separated and their respective IC_{50} values for sethoxydim, clethodim [(±)-2-[(*E*)-1-[(*E*)-3-chloroallyoxyimino]propyl]-5-[2-(ethylthio)propyl]-3-hydroxycyclohex-2-enone], and tralkoxydim [2-[1-(ethoxyimino)propyl]-3-hydroxy-5-mesitylcyclohex-2-enone] were determined. These IC_{50} values were compared to those of two susceptible hybrids (Pioneer 3902 and DK381). The resistant DK404SR maize hybrid was derived from the susceptible DK381 hybrid. The objective of this study was to extract and separate the ACCase isozymes from resistant and susceptible maize hybrids and compare their cyclohexanedione inhibitions. The constituent biotinylated polypeptides of ACCase isolated from each maize hybrid were compared. On the basis of our results, we hypothesize that CHD herbicide resistance in the maize hybrid DK404SR is conferred both by expression of an altered ACCase220 (extrachloroplastic) and by increased expression of a less efficient, herbicide-resistant isozyme (chloroplastic). Our hypothesis is supported by the results of Parker et al. (1990a,b).

MATERIALS AND METHODS

Plant Material. Susceptible maize hybrids used for this study were Pioneer 3902 and DK381. Resistant maize hybrid was DK404SR and was derived from the maize cell lines selected for sethoxydim resistance by Parker et al. (1990a,b). The DK maize hybrids were a gift from BASF Corp. ACCase isozymes were extracted from etiolated seedlings. Seedlings were grown in vermiculite for 7 days in the dark at 25 °C.

Enzyme Extraction. ACCase isozymes were extracted and partially purified as previously described (Incledon and Hall, 1997b). All reagents were purchased from Sigma unless otherwise noted. Spectra/Por 7 dialysis membrane was obtained from Spectrum Medical Laboratory Products. ACCase was isolated from either resistant (DK404SR) or susceptible (Pioneer 3902, DK381) 7-day-old etiolated maize coleoptiles. Coleoptiles were harvested and homogenized in a volume of buffer A [50 mM Tricine/KOH, 1.0 mM EDTA, 10% (v/v) glycerol, 5 mM DTT, 15 mM $NaHCO_3$, pH 8.3] equivalent to the wet tissue mass. Homogenization was performed using a Waring Blendor. Homogenate was filtered through a combination of four layers of cheesecloth and one layer of Miracloth (Calbiochem). Crude filtrate was centrifuged at 10000*g* for 30 min at 4 °C. After centrifugation, the supernatant was removed and crystalline ammonium sulfate was slowly added (over a 20-min period) to 30% saturation with gentle stirring at 4 °C. Following a 1-h incubation period, the solution was centrifuged as described above, the pellet was discarded, and crystalline ammonium sulfate was added (as described previously), bringing the supernatant to 50% saturation. Proteins were pelleted as described earlier, and the pellet was resuspended in ∼15 mL of buffer A. The resuspended pellet was dialyzed (Spectra/Por 7 50 000 MWCO) against 3 × 1 L of buffer A to remove ammonium sulfate prior to dye ligand affinity chromatography.

Affinity Purification. Following dialysis, the solution containing the resuspended 50% ammonium sulfate pellet was applied to a 2.6 × 25 cm Reactive Blue 4 agarose dye affinity column (Sigma) that had been previously equilibrated with buffer A. The column was washed with buffer A until all unbound protein was eluted, as indicated by absorbance at 280 nm. Bound protein was eluted using a 0−1 M linear KCl gradient over 400 mL at a constant flow rate of 1.0 mL/min. Eluted protein was detected by absorbance at 280 nm using a Bio-Rad Econo system flow-through UV detector. Eluted fractions were collected and assayed for ACCase activity as described in the following section. Active ACCase fractions were pooled, concentrated, and desalted using an Amicon positive pressure ultrafiltration apparatus equipped with a YM100 (100 000 MWCO) membrane. Desalted, concentrated protein was applied to a novel cyclohexanedione affinity column (Incledon and Hall, 1997b). The inhibitor affinity column was made by covalently linking the CHD herbicide 2-[1-[[(3-carboxyphenyl-2-propenyl)oxy)] imino]propyl]-5-phenyl-3-hydroxy-2-cyclohexen-1-one to ω-aminohexyl-Sepharose using 1-ethyl-3-(dimethylaminopropyl)carbodiimide according to the manufacturer's instructions. Inhibitor affinity purification was achieved by applying the desalted, concentrated ACCase active fraction onto a 1.5 × 5 cm CHD affinity column at a constant flow rate of 0.5 mL/min in buffer A. Unbound protein was removed with 4 column volumes of buffer A as indicated by the baseline absorbance at 280 nm. Bound protein was eluted with 1 column volume of buffer B (buffer A + 1.0 M KCl). Eluted protein was collected and assayed for ACCase activity. Active ACCase fractions were pooled, concentrated, and desalted by positive pressure ultrafiltration (YM100). Concentrated, desalted CHD affinity column eluant was applied to a 2.6 × 20 cm Dye Matrex Gel Orange A column (Amicon) equilibrated with buffer A. The column was washed with 5 column volumes of buffer A to remove unbound protein. Unbound protein was collected and assayed for ACCase activity. ACCase-active protein was pooled and concentrated by YM100 positive pressure ultrafiltration. Bound protein was eluted with a 400-mL linear 0−1 M KCl gradient (Figure 1). Fractions were assayed for ACCase activity, and active fractions were pooled, concentrated, and desalted by positive pressure ultrafiltration (YM100). Protein concentrations were estimated using a Coomassie protein assay reagent (Pierce) in the microplate format using a bovine serum albumin standard curve. Final ACCase preparations were stored at −20 °C.

ACCase Activity and Inhibition Assays. ACCase activity was measured by monitoring the production of the heat- and acid-stable product, [^{14}C]malonyl coenzyme-A, from $NaH^{14}CO_3$ according to previously described methods (Burton et al., 1987, 1991; Alban et al., 1994; Evenson et al., 1994; Incledon and Hall, 1997b). Samples (100 μL) were mixed with 100 μL of reaction solution [50 mM Tricine, 5.0 mM $MgCl_2$, 20 mM DTT, 2.0 mM ATP, 14.97 mM $NaHCO_3$, 0.03 mM $NaH^{14}CO_3$ (3.7×10^7 Bq ^{14}C), pH 8.3] and preincubated for 15 min at 30 °C. Reactions were initiated by addition of 50 μL of 1.5 mM acetyl-coenzyme A to give a final concentration of 0.3 mM. Following a 20-min incubation, the reaction was quenched with 100 μL of 6 M HCl. The mixtures in the reaction tubes were evaporated to dryness at 100 °C and reconstituted with 100 μL of distilled deionized water. Reconstituted samples were added to 5.0 mL of EcoLite scintillation cocktail, and the concentration of heat- and acid-stable ^{14}C was determined by liquid scintillation counting. Fixed ^{14}C was identified as malonyl-CoA by comigration of radioactive species with a nonlabeled malonyl-CoA standard on reverse phase $KC_{18}F$ thin-layer chromatography plates (Whatman) using a 50% (v/v) CH_3CN mobile phase. For determination of acetyl-CoA K_m a substrate concentration range of 0.03−1.5 mM was used. For

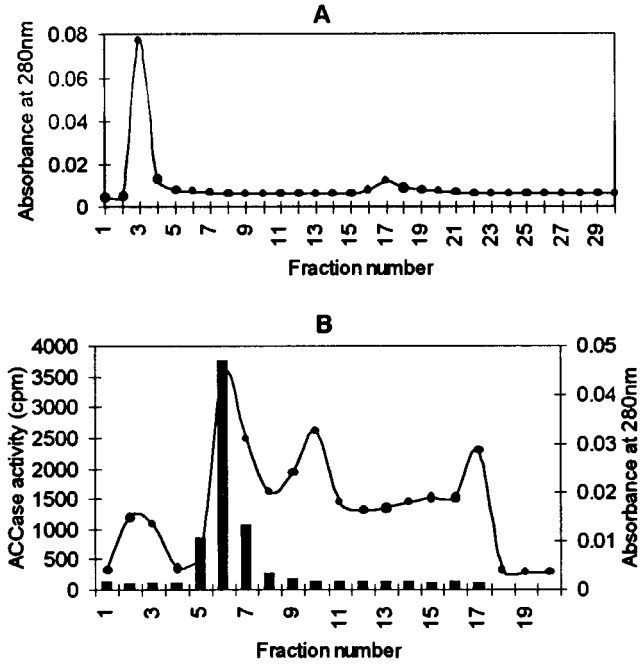

Figure 1. Typical elution profiles for the purification of ACCase220 and ACCase240 by Dye Matrex Gel Orange A and MacroPrep Q anion exchange chromatography from susceptible maize hybrid Pioneer 3902: (A) plot of absorbance at 280 nm versus fraction number for the elution of ACCase240 from the Dye Matrex Gel Orange A dye ligand affinity column; (B) plot of absorbance at 280 nm (—) versus fraction number for the purification of ACCase220 by MacroPrep Q anion exchange chromatography. ACCase activity (■) is also shown for each fraction.

inhibition assays the methods followed were as described above with the exception that the enzyme was added in a 50-μL aliquot and the inhibitor was added in a 50-μL aliquot. Sethoxydim, clethodim, and tralkoxydim (Figure 2) were dissolved in methanol and diluted to final assay concentrations such that the final concentration of methanol was 0.4% (v/v). To facilitate the solubilization of sethoxydim and tralkoxydim, these compounds were diluted in H_2O adjusted to pH 8.5. IC_{50} values for sethoxydim, clethodim, and tralkoxydim were estimated using four-parameter logistic analysis with simple weighting using GraFit (Erithacus Software Ltd.; Leatherbarrow, 1992).

Detection of Biotinylated Polypeptides. Biotinylated proteins were transferred to poly(vinylidene difluoride) (PVDF) membranes (0.02-μm, Bio-Rad) and subsequently detected by probing with avidin−HRP (Pierce). Transfer was performed in cooled transfer buffer [15.6 mM Tris, 120 mM glycine, 10% (v/v) CH_3OH, pH 8.3] using the Protean II minigel apparatus (Bio-Rad) equipped with an electrotransfer apparatus. Proteins were electroblotted to PVDF membranes at 100 V (constant voltage) for 1 h at 4 °C. PVDF membranes were blocked using 3% (w/v) bovine serum albumin in phosphate-buffered saline [10 mM phosphate, 15 mM NaCl, 10% (v/v) glycerol, pH 7.5] (PBS) for 30 min with shaking. Avidin−HRP was diluted 1:1000 in PBS and incubated with the membrane for 2 h. Membranes were washed three times with PBS, containing 0.05% (v/v) Tween 20. Biotinylated bands were visualized using the substrate 4-chloro-1-naphthol (30 μg/mL, Pierce). Substrate was prepared by dissolving premeasured tablets in 10 mL of methanol, which was added to 90 mL of PBS. Immediately prior to development of the blot, 19.6 μL of 50% (v/v) H_2O_2 was added to the 4-chloro-1-naphthol solution. The reaction was quenched by washing the membrane in distilled deionized water. Results were recorded by photographing the dried PVDF membranes.

RESULTS AND DISCUSSION

ACCase Extraction. ACCase220 (extrachloroplastic) and ACCase240 (chloroplastic) isozymes could be separated on a Gel Matrex Orange A column (Figure 1A) because the chloroplastic ACCase240 (purified 68-fold) bound to the Gel Matrex Orange A column and was eluted with NaCl. In contrast, extrachloroplastic ACCase220 (purified 79-fold) did not bind to the Orange A column and was present in the void volume (Figure 1A). SDS−PAGE followed by electrotransfer and detection of biotinylated bands using avidin−HRP blotting indicated that ACCase240 preparations from both resistant and susceptible maize hybrids contained biotinylated proteins with molecular masses of approximately 85 and 240 kDa (Figure 3). ACCase220 preparations from resistant (**R**) and susceptible (**S**) hybrids contained a biotinylated contaminant protein in addition to the 85- and 220-kDa proteins (Figure 3). ACCase220 was not further purified by anion exchange chromatography as in previous studies (Incledon and Hall, 1997b) because the contaminating proteins showed no ACCase activity (Figure 1B). Furthermore, cyclohexanedione inhibition of anion exchange purified ACCase220 yielded results similar to those obtained with the ACCase220 preparations for which purification was stopped prior to anion exchange chromatography (results not shown).

ACCase240 (chloroplastic) preparations from susceptible Pioneer 3902 and DK381 contained 3.0- and 4.0-fold less protein, respectively, than did the **R** hybrid (DK404SR; Table 1). ACCase isolation from both DK hybrids was conducted only once because this was sufficient to provide ample enzyme for inhibition studies; therefore, an estimate of error is not provided (Table 1). However, ACCase isolation from Pioneer 3902 was conducted several times ($n = 10$), and the enzyme yield was consistent (Table 1). Our results agree with those of Parker et al. (1990a), who reported that the level of expression of chloroplastic ACCase from sethoxydim-resistant corn was higher than in the susceptible wild-type hybrid. Furthermore, we agree with their hypothesis that increased expression of ACCase is responsible for resistance.

ACCase240 from both DK381 and DK404SR contained similar biotinylated polypeptides as determined by avidin−HRP blotting (Figure 3). However, the specific activity of ACCase240 isolated from susceptible DK381 was 14.5-fold higher than that from resistant DK404SR (Table 2). Furthermore, the V_{max} of ACCase240 from DK381 [$(1.2 \pm 0.08) \times 10^{-8}$ μmol min^{-1}] was 5.5-fold greater than that of DK404SR [$(2.2 \pm 0.03) \times 10^{-9}$ μmol min^{-1}]. The lower specific activity of DK404SR ACCase240 may result from both decreased expression and a lower V_{max} of the isozyme. In contrast, the quantity and specific activity of ACCase220 from both **R** and **S** DK hybrids were similar (data not shown).

Acetyl-coenzyme A Binding Kinetics. K_m values using acetyl-CoA as a substrate were determined for each of the ACCase isozymes isolated from the three maize hybrids (Table 3). Only acetyl-CoA binding kinetics will be discussed because the CHD family of herbicides is known to interact with the binding site of this substrate within the ACCase enzyme (Burton, 1997; Incledon and Hall, 1997a). Furthermore, there were no differences among the binding kinetics of the three hybrids when the other two substrates (0.20−25 mM ATP and 5.0−50 mM HCO_3^-) were used. Apparent K_m values were estimated from a plot of rate versus rate/

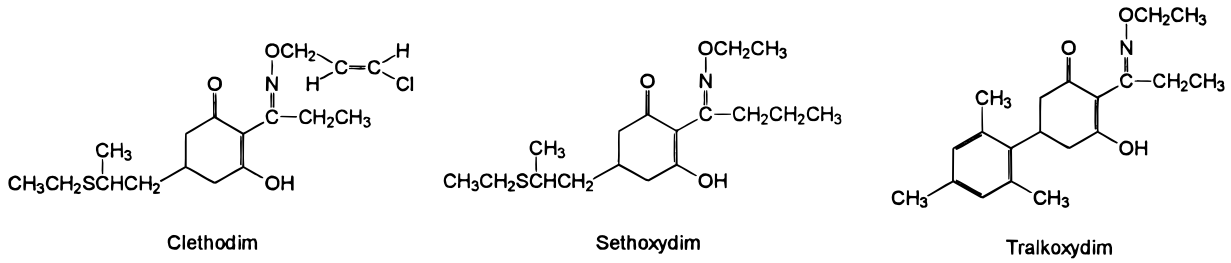

Figure 2. Structures of the CHD herbicides used for the inhibition studies.

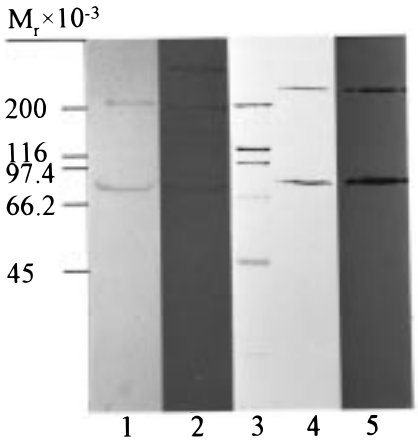

Figure 3. Avidin−HRP blot of ACCase showing three biotinylated polypeptides corresponding to apparent molecular masses of 85, 220, and 240 kDa: (lane 1) resistant ACCase220; (lane 2) susceptible ACCase240; (lane 3) biotinylated molecular mass markers; (lane 4) susceptible ACCase240; (lane 5) resistant ACCase240. Biotinylated polypeptides were detected with avidin−HRP using 4-chloro-1-naphthol chromogenic substrate; molecular masses of markers are indicated adjacent to lane 1.

Table 1. Quantity of ACCase240 Isozyme Partially Purified from Pioneer 3902, DK381, and DK404SR Maize Hybrids

maize hybrid	amount of ACCase240 (μg/g of wet tissue)
Pioneer 3902 (susceptible)	0.290 ± 0.039[a]
DK381 (susceptible)	0.390[b]
DK404SR (resistant)	1.15

[a] Standard error, $n = 10$. [b] Error not included; enzyme preparations performed only once.

Table 2. Comparison of Specific Activities for ACCase240 from Susceptible and Resistant Maize Hybrids

maize hybrid	specific activity[a]
susceptible	578390 ± 46384[b]
resistant	39889 ± 5272

[a] Specific activity is measured as disintegrations per min (mg of protein)$^{-1}$ 15 min^{-1}. Identical lots of radioactive substrate were used to ensure specific activities of the product were identical. [b] Standard error, $n = 3$.

acetyl-CoA concentration. This plot was used to determine K_m because this type of analysis also provides an indication of whether the ACCase enzyme preparation deviates from typical Michaelis−Menton single active site kinetics. A nonlinear plot of rate versus rate/[acetyl-CoA] may indicate multiple acetyl-coenzyme A binding sites with different binding affinities (Engel, 1996). In the case of a nonlinear plot, apparent K_m values were estimated from a plot of rate versus [acetyl-CoA] and are consequently an estimate of the combined K_m values of the competing activities. Due to the non-Michaelis−Menton kinetic behavior of the susceptible isozyme, it was not possible to make a direct comparison of true K_m values for the ACCase220 isozymes from **R** and **S** hybrids. However, there was a significant difference in acetyl-CoA binding kinetics among the hybrids (Table 3). The K_m of **S** ACCase220 was 4-fold higher than that of the **R** (Table 3), indicating that there may be an alteration in the **R** isozyme that results in a higher binding affinity for acetyl-CoA. In contrast, the K_m values for **R** and **S** ACCase240 were the same (Table 3). **S** but not **R** ACCase220 has a curved plot of rate versus rate/[acetyl-CoA], indicating a deviation from Michaelis−Menton kinetics. The nature or implication of this deviation is not clear and requires further study.

Table 3. Comparison of the Apparent K_m Values Using Acetyl-coenzyme A as a Substrate

	K_m (μM)	
maize hybrid	ACCase240	ACCase220
DK381 (susceptible)	13 ± 2[a]	260 ± 39
DK404SR (resistant)	10 ± 0.9	66 ± 5

[a] $n = 3$.

Table 4. IC_{50} Values for Inhibition of Resistant and Susceptible ACCase Isozymes by Clethodim, Sethoxydim, and Tralkoxydim

		IC_{50} (μM)	
maize hybrid	inhibitor	ACCase240	ACCase220
Pioneer 3902[a]	clethodim	12.7 ± 0.8	19.5 ± 1.3
DK381	clethodim	48 ± 8	157 ± 74
DK404SR	clethodim	176 ± 254	178 ± 46
Pioneer 3902	sethoxydim	2.2 ± 0.2	1.8 ± 0.9
DK381	sethoxydim	1.3 ± 0.3	3.9 ± 0.6
DK404SR	sethoxydim	>100	>100
Pioneer 3902	tralkoxydim	1.7 ± 0.8	0.9 ± 0.09
DK381	tralkoxydim	1.25 ± 0.07	2.2 ± 0.06
DK404SR	tralkoxydim	16 ± 3	16 ± 2

[a] Inhibition assays for Pioneer 3902 hybrid were performed only once, and the error indicated represents the standard error associated with the nonlinear regression.

Inhibition of ACCase. ACCase240 and ACCase220 isolated from Pioneer 3902, DK381, and DK404SR maize hybrids were inhibited by clethodim, sethoxydim, and tralkoxydim. IC_{50} values were calculated using four-parameter logistic curve fitting (Table 4). ACCase240 from the **S** hybrids (Pioneer 3902 and DK381) was more sensitive to all three herbicides than was ACCase from the **R** (DK404SR). Inhibition by sethoxydim of ACCase240 from DK404SR did not reach 50%; the IC_{50} was estimated at >100 μM. ACCase240 from the **R** hybrid has reduced affinity for the CHD herbicides and a lower maximum velocity than that from the

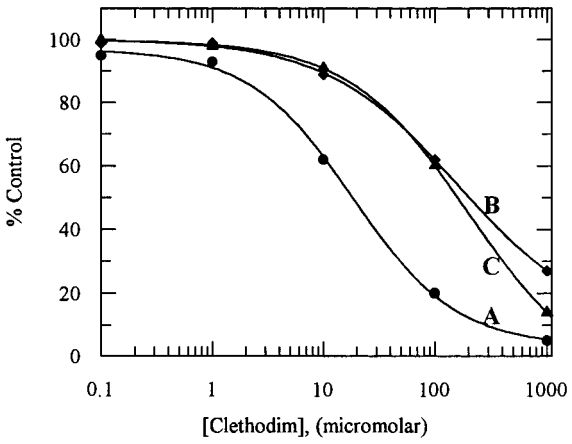

Figure 4. Representative dose−response curves for the inhibition of ACCase220 by clethodim. Pioneer 3902 ACCase220 (A) has an IC_{50} of 19.5 ± 1.3 μM. Resistant DK404SR ACCase220 (B) has an IC_{50} of 178 ± 46 μM, and susceptible DK381 ACCase220 (C) has an IC_{50} of 157 ± 74 μM.

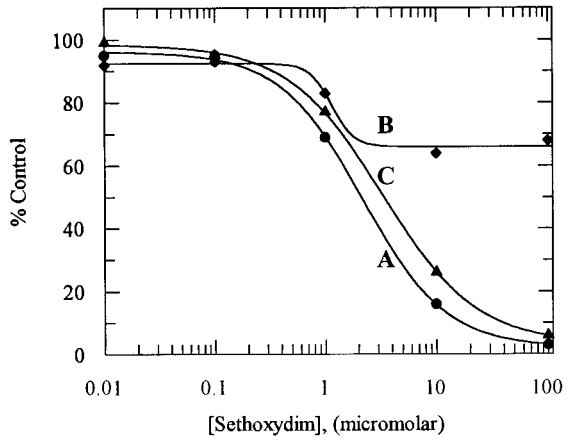

Figure 5. Representative dose−response curves for the inhibition of ACCase220 by sethoxydim. ACCase isolated from susceptible Pioneer 3902 maize (A) has an IC_{50} of 1.8 ± 0.9 μM and a maximum of 100% inhibition. ACCase from resistant DK404SR maize (B) has an IC_{50} > 100 μM and a maximum inhibition of 30% of control. ACCase220 from susceptible DK381 maize (C) has an IC_{50} of 3.9 ± 0.6 μM and a maximum inhibition of 100% of control.

S hybrids, indicating that there may be an alteration of the herbicide-binding site.

ACCase220 isozymes from both the **R** and **S** DK hybrids had similar IC_{50} values when inhibited by clethodim (DK404SR = DK381 > Pioneer 3902; Figure 4). In a field study, Vangessel et al. (1997) showed that DK404SR was resistant to sethoxydim but susceptible to clethodim. Similarly, we have shown that in vitro DK404SR ACCase220 is >25- and 7-fold more resistant to sethoxydim and tralkoxydim inhibition than DK381; however, the IC_{50} values for clethodim were the same for DK404SR (**R**) and DK381 (**S**) (Table 4). A maximum inhibition of only 30% was achieved for the DK404SR ACCase220 at the highest possible concentration of sethoxydim (100 μM). Therefore, the IC_{50} was estimated to be >100 μM (Figure 5). In a subsequent experiment using an in situ assay, we determined that extrachloroplastic ACCase (ACCase220) from Pioneer 3902 was susceptible to sethoxydim inhibition, whereas the chloroplastic ACCase (ACCase240) was not (Incledon et al., unpublished results). Furthermore, the extrachloroplastic isozyme DK404SR was resistant to sethoxydim inhibition at 100 μM. Therefore, on the basis of our results and those of Vangessel et al. (1997), ACCase220 of the **R** hybrid may be the primary isozyme responsible for conferring sethoxydim resistance.

In conclusion, similar to the results of Parker et al. (1990a,b), DK404SR resistance to sethoxydim can be attributed to two major mechanisms, overexpression and altered herbicide binding. First, we found DK404SR had increased expression (4-fold) of a less efficient (14.5-fold lower specific activity) chloroplastic (ACCase240) isozyme that had a binding affinity for acetyl-CoA similar to that of the isozyme from the **S** hybrids. This ACCase240 isozyme is less sensitive to inhibition by clethodim, sethoxydim, and tralkoxydim. Furthermore, mutation of the extrachloroplastic (ACCase220) isozyme in DK404SR results in a lower K_m and resistance to sethoxydim and tralkoxydim but not to clethodim (Table 4). The lack of in vitro inhibition of sethoxydim-resistant ACCase220 by clethodim may explain why Vangessel et al. (1997) observed injury to field-grown DK404SR by clethodim but not by sethoxydim. It remains uncertain, however, to what extent alteration of each isozyme contributes to the resistance of DK404SR to sethoxydim.

ACKNOWLEDGMENT

We thank BASF Corp. for the kind gift of the resistant (DK404SR) and susceptible (DK381) maize hybrids.

LITERATURE CITED

Alban, C.; Baldet, P.; Douce, R. Localisation and characterisation of two structurally different forms of acetyl-CoA carboxylase in young pea leaves, of which one is sensitive to aryloxyphenoxypropionate herbicides. *Biochem. J.* **1994**, *300*, 557.

Ashton, A. R.; Jenkins, C. L. D.; Whitfeld, P. R. Molecular cloning of two different cDNAs for maize acetyl CoA carboxylase. *Plant Mol. Biol.* **1994**, *24*, 35.

Burton, J. D. Acetyl-coenzyme A carboxylase inhibitors. In *Herbicide Activity: Toxicology, Biochemistry and Molecular Biology*; Roe, R. M., et al., Eds.; IOS Press: Bruke, VA, 1997; pp 187−205.

Burton, J. D.; Gronwald, J. W.; Somers, D. A.; Connelly, J. A.; Gengenbach, B. G.; Wyse, D. L. Inhibition of plant acetyl-coenzyme A carboxylase by the herbicides sethoxydim and haloxyfop. *Biochem. Biophys. Res. Commun.* **1987**, *148*, 1039.

Burton, J. D.; Gronwald, J. W.; Keith, R. A.; Somers, D. A.; Gengenbach, B. G.; Wyse, D. L. Kinetics of inhibition of acetyl-coenzyme A carboxylase by sethoxydim and haloxyfop. *Pestic. Biochem. Physiol.* **1991**, *39*, 100.

Catanzaro, C. J.; Burton, J. D.; Skroch, W. A. Graminicide resistance of acetyl-CoA carboxylase from ornamental grasses. *Pestic. Biochem. Physiol.* **1993**, *45*, 147.

Dehaye, L.; Alban, C.; Job, C.; Douce, R.; Job, D. Kinetics of the two forms of acetyl-CoA carboxylase from *Pisum sativum*: correlation of the substrate specificity of the enzymes and sensitivity towards aryloxyphenoxypropionate herbicides. *Eur. J. Biochem.* **1994**, *225*, 1113.

Egli, M. A.; Gengenbach, B. G.; Gronwald, J. W.; Somers, D. A.; Wyse, D. L. Characterisation of maize acetyl-coenzyme A carboxylase. *Plant Physiol.* **1993**, *101*, 499.

Engel, P. C. Enzyme kinetics. In *Enzymology LabFax*; Engel, P. C., Ed.; Bios Scientific Publishers: Oxford, U.K., 1996.

Evenson, K. J.; Gronwald, J. W.; Wyse, D. L. Purification and characterisation of acetyl-coenzyme A carboxylase from diclofop-resistant and -susceptible *Lolium multiflorum*. *Plant Physiol.* **1994**, *105*, 671.

Evenson, K. J.; Gronwald, J. W.; Wyse, D. L. Isoforms of acetyl-coenzyme A carboxylase in *Lolium multiflorum*. *Plant Physiol. Biochem*. **1997**, *35* (4), 265.

Finlayson, S. A.; Dennis, D. T. Acetyl-coenzyme A carboxylase from the developing endosperm of *Ricinus communis* i. isolation and characterisation. *Arch. Biochem. Biophys.* **1983a**, *225*, 576.

Finlayson, S. A.; Dennis, D. T. Acetyl-coenzyme A carboxylase from the developing endosperm of *Ricinus communis* ii. a two site kinetic mechanism. *Arch. Biochem. Biophys.* **1983b**, *225*, 586.

Herbert, D.; Price, L. J.; Alban, C.; Dehaye, L.; Job, D.; Cole, D. J.; Pallett, K. E.; Harwood, J. L. Kinetic studies on two isoforms of acetyl-CoA carboxylase from maize leaves. *Biochem J.* **1996a**, *318*, 997.

Herbert, D.; Cole, D. J.; Pallett, K. E.; Harwood, J. L. Susceptibilities of different test systems from maize (*Zea mays*), *Poa annua*, and *Festuca rubra* to herbicides that inhibit the enzyme acetyl-coenzyme A carboxylase. *Pestic. Biochem. Physiol.* **1996b**, *55*, 129.

Holt, J. S.; Holtum, J. A. M.; Powles, S. B. Mechanisms and agronomic aspects of herbicide resistance. *Annu Rev. Plant Physiol. Plant Mol. Biol.* **1993**, *44*, 203.

Incledon, B. J.; Hall, J. C. A Review of Acetyl-Coenzyme A Carboxylase: Quaternary Structure and Inhibition by Graminicidal Herbicides. *Pestic. Biochem. Physiol.* **1997a**, *57*, 255.

Incledon, B. J.; Hall, J. C. Evidence that maize acetyl-coenzyme A carboxylase does not function solely as a homodimer. *J. Agric. Food Chem*. **1997b**, *45* (12), 4838−4844.

Ishikawa, H.; Yamada, S.; Hosaka, H.; Kawana, T.; Okunuki, S.; Kohara, K. Herbicidal properties of sethoxydim for the control of graminaceous weeds. *J. Pestic. Sci.* **1985**, *10*, 187.

Konishi, T.; Sasaki, Y. Compartmentalisation of the two forms of acetyl-CoA carboxylase in plants and the origin of their resistance toward herbicides. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 3598.

Konishi, T.; Shinohara, K.; Yamada, K.; Sasaki, Y. Acetyl-CoA carboxylase in higher plants: most plants other than gramineae have both the prokaryotic and the eukaryotic forms of this enzyme. *Plant Cell Physiol.* **1996**, *37*, 117.

Leatherbarrow, R. J. *GraFit*, version 3.0; Erithacus Software: Staines, U.K., 1992.

Lichthenthaler, H. K.; Kobek, K.; Ishii, K. Inhibition by sethoxydim of pigment accumulation and fatty acid biosynthesis in chloroplasts of Avena seedlings. *Z. Naturforsch.* **1987**, *42C*, 11361.

Maneechote, C.; Holtum, J. A. M.; Preston, C.; Powles, S. B. Resistant acetyl-coenzyme A carboxylase is a mechanism of herbicide resistance in a biotype of *Avena sterilis* ssp. *ludoviciana*. *Plant Cell Physiol.* **1994**, *35*, 627.

Marshall, L. C.; Somers, D. A.; Dotray, P. D.; Gengenbach, B. G.; Wyse, D. L.; Gronwald, J. W. Allelic mutations in acetyl-coenzyme A carboxylase confer herbicide resistance in maize. *Theor. Appl. Genet.* **1992**, *83*, 435.

Nikolau, B. J.; Hawke, J. C. Purification and characterisation of maize leaf acetyl-coenzyme A carboxylase. *Arch. Biochem. Biophys.* **1984**, *228*, 86.

Ohlrogge, J. B.; Jaworski, J. G. Regulation of fatty acid synthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1997**, *48*, 109.

Parker, W. B.; Somers, D. A.; Wyse, D. L.; Keith, R. A.; Burton, J. D.; Gronwald, J. W.; Gengenbach, B. G. Selection and characterisation of sethoxydim-resistant maize tissue cultures. *Plant Physiol.* **1990a**, *92*, 1220.

Parker, W. B.; Marshall, L. C.; Burton, J. D.; Somers, D. A.; Wyse, D. L.; Gronwald, J. W.; Gengenbach, B. G. Dominant mutations causing alterations in acetyl-coenzyme A carboxylase confer resistance to cyclohexanedione and aryloxyphenoxypropionate herbicides in maize. *Proc. Natl. Acad. Sci. U.S.A.* **1990b**, *87*, 7175.

Rendina, A. R.; Felts, J. M.; Beaudoin, J. D.; Craig-Kennard, A. C.; Look, L. L.; Paraskos, S. L.; Hagenah, J. A. Kinetic characterisation, stereoselectivity, and species selectivity of the inhibition of plant acetyl-CoA carboxylase by the aryloxyphenoxypropionic acid grass herbicides. *Arch. Biochem. Biophys.* **1988**, *265*, 219.

Rendina, A. R.; Craig-Kennard, A. C.; Beaudoin, J. D.; Breen, M. K. Inhibition of acetyl-coenzyme A carboxylase by two classes of grass-selective herbicides. *J. Agric. Food Chem.* **1990**, *38*, 1282.

Secor, J.; Cseke, C. Inhibition of acetyl-CoA carboxylase activity by haloxyfop and tralkoxydim. *Plant Physiol.* **1988**, *86*, 10.

Somers, D. A.; Keith, R. A.; Egli, M. A.; Marshall, L. C.; Gengenbach, B. G.; Gronwald, J. W.; Wyse, D. L. Expression of Acc1 gene-encoded acetyl-coenzyme A carboxylase in developing maize (*Zea mays* L.) kernels. *Plant Physiol.* **1993**, *101*, 1097.

Tardif, F. J.; Powles, S. B. Herbicide multiple-resistance in a *Lolium rigidum* biotype is endowed by multiple mechanisms: isolation of a subset with resistant acetyl-CoA carboxylase. *Physiol. Planta.* **1994**, *91*, 488.

Vangessel, M. J.; Johnson, Q.; Isaacs, M. Response of sethoxydim-resistant corn (*Zea mays*) hybrids to postemergence graminicides. *Weed Technol.* **1997**, *11* (3), 598.

Received for review February 10, 1998. Revised manuscript received September 22, 1998. Accepted September 22, 1998. J.C.H. acknowledges Agriculture Canada/NSERC, Ontario Ministry of Agriculture, Food and Rural Affairs, and Dow-Elanco for financial support for this research. B.J.I. is a recipient of a NSERC PGS-B scholarship.

JF980124J