Interactions of plant acetohydroxy acid isomeroreductase with reaction intermediate analogues: correlation of the slow, competitive, inhibition kinetics of enzyme activity and herbicidal effects

Renaud DUMAS,*‡ Claire CORNILLON-BERTRAND,* Patricia GUIGUE-TALET,† Pierre GENIX,† Roland DOUCE* and Dominique JOB*

*Unité Mixte de Recherche CNRS/Rhône-Poulenc (Unité associée au Centre National de la Recherche Scientifique, UM 41)
and †Service de Biochimie Moléculaire et de Biologie Cellulaire, Rhône-Poulenc Agrochimie, 14–20 rue Pierre Baizet, 69253 Lyon Cédex 09, France

N-Hydroxy-*N*-isopropyloxamate (IpOHA) is known to inhibit extremely tightly (K_i of 22 pM) the bacterial acetohydroxy acid isomeroreductase (EC 1.1.1.86) [Aulabaugh and Schloss (1990) Biochemistry **29**, 2824–2830], the second enzyme of the branched-chain-amino-acid-biosynthetic pathway. Yet, although the same pathway exists in plant cells, this compound presents only very poor herbicidal action. Towards the goal of gaining a better understanding of this behaviour, we have studied the mechanism of interaction of this compound with a highly purified acetohydroxy acid isomeroreductase of plant origin, i.e. the spinach (*Spinacia oleracea*) chloroplast enzyme. IpOHA behaved as a nearly irreversible inhibitor of the enzyme. Encounter complex formation was very slow (association rate constant 1.9×10^3 $M^{-1} \cdot s^{-1}$) and involved a single bimolecular step. Since inhibition was competitive with respect to acetohydroxy acid substrates, the time needed to achieve substantial (90%) inhibition *in vitro* of enzyme activity in the simultaneous presence of substrates and inhibitors was extremely long (for example of the order of hours at 1 μM IpOHA and 100 μM acetohydroxy acid substrates). Thus, under *in vivo* conditions, binding of the inhibitor may be so slow that it may delay considerably the time required for inhibition of the target enzyme. Similar kinetic behaviour was observed with another reaction intermediate analogue described by Schulz, Spönemann, Köcher and Wengenmayer [(1988) FEBS Lett. **238**, 375–378], 2-dimethylphosphinoyl-2-hydroxyacetic acid (Hoe 704), which displays a higher herbicide activity than IpOHA. The herbicidal potency of these two compounds appeared to be correlated with their rates of association with the plant acetohydroxy acid isomeroreductase, since the bimolecular rate constant for Hoe 704 (2.2×10^4 $M^{-1} \cdot s^{-1}$) was higher than that for IpOHA.

INTRODUCTION

The biosynthetic pathways leading to isoleucine, leucine and valine in micro-organisms and plants have three enzymes in common: acetohydroxy acid synthase (EC 4.1.3.18), acetohydroxy acid isomeroreductase (EC 1.1.1.86) and dihydroxy acid dehydratase (EC 4.2.1.9) [1]. Acetohydroxy acid isomeroreductase, the second common enzyme in these biosynthetic pathways, catalyses a two-step reaction in which the substrate, either 2-acetolactate (ultimate products valine and leucine) or 2-aceto-2-hydroxybutyrate (ultimate product isoleucine) is converted via an alkyl migration and an NADPH-dependent reduction into 2,3-dihydroxy-3-isovalerate or 2,3-dihydroxy-3-methylvalerate respectively. The enzyme activity requires the presence of the bivalent ion Mg^{2+} [2]. Because micro-organisms and plants can synthesize essential amino acids, but animals lack this biosynthetic capability, this pathway has raised strong interest for herbicide development. Experimental compounds such as 2-dimethylphosphinoyl-2-hydroxyacetic acid (Hoe 704) [3] and *N*-hydroxy-*N*-isopropyloxamate (IpOHA) [4] are very potent and selective inhibitors of acetohydroxy acid isomeroreductase purified from *Escherichia coli*, behaving as reaction-intermediate analogues of the enzyme-catalysed reaction. Surprisingly, although IpOHA binds very tightly (K_d 22 pM) to the bacterial enzyme [4], this compound shows only very poor herbicidal action; herbicidal effects are only observed at an application rate of 2 kg/ha. This behaviour does not result from a failure of the inhibitor compound to reach its target site *in vivo*, since Wittenbach et al. [5] showed that IpOHA translocates within plants and penetrates plastids, where acetohydroxy acid isomeroreductase is localized [6,7]. This contrasts with the situation observed with acetohydroxy acid synthase, for which several classes of structurally unrelated potent inhibitors have been discovered. In this case, the inhibitors behave as very efficient herbicides, acting at application rates several orders of magnitude lower than those for acetohydroxy acid isomeroreductase [8–11]. The reasons for this dramatic difference in herbicidal potency are not known.

To characterize better the true herbicide target, that is, the acetohydroxy acid isomeroreductase from a higher plant, we have purified this enzyme from the stroma of spinach (*Spinacia oleracea*) chloroplasts, isolated spinach cDNA encoding this protein, and overexpressed the enzyme in *E. coli* [6,7,12–14]. These studies showed that the biochemical and structural properties of the plant enzyme differ markedly from those determined for its bacterial counterpart [15–18]. The question that arises is whether plant acetohydroxy acid isomeroreductase is inhibited by the same mechanism and to the same extent by IpOHA and Hoe 704 as the *E. coli* enzyme. In the present paper we examine binding of these compounds by spinach chloroplast acetohydroxy acid isomeroreductase and its Mg^{2+} and NADPH complexes.

Abbreviations used: AHB, 2-aceto-2-hydroxybutyrate; AL, 2-acetolactate; Hoe 704, 2-dimethylphosphinoyl-2-hydroxyacetic acid; IpOHA, *N*-hydroxy-*N*-isopropyloxamate.

‡ To whom correspondence should be sent.

EXPERIMENTAL

Plants

Solanum nigrum (black nightshade) and *Ipomoea purpurea* (morning glory) were grown in glass tubes containing 20 ml of nutrient gelose and various amounts of IpOHA or Hoe 704 (0.2–60 mg/l). Plants were also grown in the absence of these compounds (control plants) or in the presence of sulfometuron methyl (0.001–2 mg/l), an inhibitor of acetohydroxy acid synthase [19].

Substrates and inhibitors

Racemic 2-acetolactate and 2-aceto-2-hydroxybutyrate were prepared by the method of Krampitz [20]. Hoe 704 was synthesized as described by Schulz et al. [3], and IpOHA as indicated by Aulabaugh and Schloss [4].

Enzyme

Acetohydroxy acid isomeroreductase was purified from an *E. coli* strain JM105 transformed with pKK-AHRI plasmid encoding the mature acetohydroxy acid isomeroreductase from spinach chloroplasts, as described previously [12]. On SDS/PAGE this enzyme preparation exhibited a single protein band of M_r 57000. The specific activity was of the order of 1.7 units/mg and 8.5 units/mg using 2-acetolactate and 2-aceto-2-hydroxybutyrate as substrate respectively. Protein was determined by absorbance measurements at 205 nm [21].

Activity measurements

Assay mixtures contained 50 mM Tris/HCl, pH 8.2, 3 mM $MgCl_2$ and 0.25 mM NADPH, in a final volume of 1 ml. Reactions were usually initiated by adding either 2-aceto-2-hydroxybutyrate or 2-acetolactate, and the progress of the reaction was monitored by the decrease in absorbance of NADPH at 340 nm (Uvikon 860 spectrophotometer; Kontron). Except where otherwise noted, all reactions were carried out at 25 °C. For all reactions carried out in the absence of inhibitors, there was a linear dependence of enzyme activity with enzyme concentration in the assays, testifying adherence to steady-state conditions. All enzyme concentrations and amounts are given on a per-enzyme-subunit basis.

Stoichiometry of inhibitor binding

IpOHA or Hoe 704 (0.0879 nmol) was incubated with various amounts of enzyme (from 0 to 0.4 nmol) for 20 min with 25 nmol of NADPH and 0.25 μmol of Mg^{2+} in a volume of 10 μl. Reactions were initiated by adding 0.6 mM of 2-aceto-2-hydroxybutyrate in 1 ml of 50 mM Tris/HCl buffer, pH 8.2, containing 3 mM $MgCl_2$ and 0.25 mM NADPH.

Kinetic studies of inhibition

The 1-cm-optical-pathlength cuvette contained 1 ml of 50 mM Tris/HCl, pH 8.2, 3 mM $MgCl_2$, 0.25 mM NADPH, and 61–347 nM enzyme. Reactions were initiated by adding simultaneously the acetohydroxy acid substrate and the inhibitor [IpOHA (0–1 mM) or Hoe 704 (0–0.1 mM)] in a few microlitres of solution on the end of a Teflon agitator. Mixing time was of the order of 8 s.

Recovery of enzyme activity from preformed enzyme–inhibitor complexes

Enzyme (0.879 nmol) was first incubated with IpOHA (1.055 nmol) or Hoe 704 (1.55 nmol) in a 100 μl solution containing 0.25 μmol of NADPH and 2.5 μmol Mg^{2+} at different temperatures (4, 25 and 37 °C). After a 20 min incubation, recovery of enzyme activity was measured upon addition of a large excess (1 μmol) of 2-aceto-2-hydroxybutyrate. Aliquots (10 μl) were taken at various time intervals, and transferred to a 1-cm-optical-pathlength cuvette containing 1 ml of 50 mM Tris/HCl, pH 8.2, 3 mM $MgCl_2$, 0.25 mM NADPH and 0.6 mM 2-aceto-2-hydroxybutyrate. Enzyme activity in the various samples was then measured as described above. For steady-state kinetic studies, the restored enzyme was prepared as follows. Enzyme (4.9 nmol) was first incubated for 20 min at 25 °C with Hoe 704 (15 nmol) in a 0.1 ml solution containing 20 nmol of NADPH and 0.3 μmol of Mg^{2+}, then with a large excess (1 μmol) of 2-aceto-2-hydroxybutyrate, and finally purified by gel filtration on a 1 ml PD10 column (Pharmacia).

Fluorescence

Fluorescence measurements were carried out at 460 nm in an SFM 25 (Kontron) fluorimeter using a 1 ml cuvette and an excitation wavelength of 370 nm. Binding of IpOHA or Hoe 704 to the enzyme was measured in 200 μl of solution containing 50 mM Tris/HCl, pH 8.2, 60 μM NADPH and 20.4 μM enzyme, in the absence or presence of Mg^{2+} (3 mM).

Gel filtration

IpOHA or Hoe 704 (0.1 mM) was incubated for 20 min at 25 °C with 3 mM Mg^{2+}, 40 μM NADPH and various concentrations of enzyme (0–60 μM), in a final volume of 100 μl. This medium was then injected on a HiLoad 16/60 Superdex column (Pharmacia) equilibrated in 25 mM potassium phosphate, pH 7.5. Elution was performed at 1.5 ml/min with the same buffer.

Data analysis

Kinetic and binding data were fitted to the appropriate theoretical equations by using the KaleidaGraph program (Abelbeck Software) and a Macintosh IIsi computer.

RESULTS

Herbicidal effects on *S. nigrum* and *I. purpurea*

To compare the herbicidal activity of Hoe 704, IpOHA and sulfometuron methyl, two dicotyledonous plants, *S. nigrum* and *I. purpurea*, were grown under identical conditions on nutrient gelose containing various concentrations of these compounds. Significant growth inhibition of *S. nigrum* occurred at a concentration of 2 mg/l (13 μM) Hoe 704. Under these conditions, root development was strongly impeded. Although development of aerial organs was less severely affected, the sizes of leaves and stems were about 30 % smaller than those of the control plants. Also, leaves from the treated plants were surrounded by a yellow border. Similar symptoms were observed with IpOHA, yet this required a substantially higher concentration of this compound (30 mg/l, i.e. 177 μM) than of Hoe 704. For both compounds, addition to the culture medium of a mixture of leucine, valine and isoleucine at a concentration of 10 mg/l each restored plant growth. Interestingly, neither IpOHA nor Hoe 704 inhibited the germination process, but acted only on seedling growth. With

sulfometuron methyl, similar symptoms were observed, although at a much lower concentration of 0.02 mg/l (55 nM). For *I. purpurea*, growth inhibition also required concentrations of 2–5 mg/l Hoe 704 and 30–60 mg/l IpOHA. These observations essentially confirmed that (i) Hoe 704 and IpOHA can translocate within the whole plants [3,5], (ii) although inhibition of acetohydroxy acid isomeroreductase can be herbicidal, it is not as effective as inhibition of acetohydroxy acid synthase [22], and (iii) herbicides that inhibit acetohydroxy acid isomeroreductase seem to kill plants in the same distinctive manner as the herbicides that inhibit acetohydroxy acid synthase ([3]; reviewed by Hawkes [23]).

Stoichiometry of inhibitor binding

The fact that IpOHA and Hoe 704 impeded plant growth suggested that these compounds inhibited endogenous acetohydroxy acid isomeroreductase. To substantiate this point, various amounts of purified plant enzyme were incubated with a constant amount of IpOHA or Hoe 704, in the presence of excess Mg^{2+} and NADPH. Following a 20 min incubation at 25 °C enzyme activity was assayed from aliquots of the various samples. In the presence of either IpOHA or Hoe 704, enzyme activity was completely abolished at low enzyme concentration and then resumed upon increasing further the enzyme concentration in the assays (Figure 1). This increase in enzyme activity paralleled that for the control reactions. These features are characteristic for tight-binding inhibition [24–26]. The equivalence point was obtained at 0.98 mol and 1.3 mol of inhibitor/mol of monomeric enzyme unit for IpOHA and Hoe 704 respectively, suggesting a stoichiometric binding of these compounds to the plant acetohydroxy acid isomeroreductase (Figure 1). Maximum inhibition of enzyme activity at low enzyme concentration was only observed when the first incubation was carried out in the presence of enzyme, NADPH, Mg^{2+} and inhibitor. Much lower inhibition was obtained in the absence of Mg^{2+} or NADPH (not shown). Similar results have been reported for inhibition of the *E. coli* enzyme by IpOHA [4].

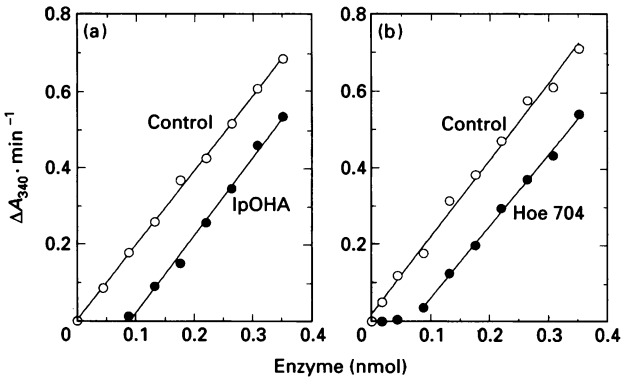


Figure 1 Stoichiometry of inhibitor binding with IpOHA and Hoe 704

IpOHA (**a**) or Hoe 704 (**b**) (0.0879 nmol) was preincubated with various quantities of enzyme (0–0.4 nmol) for 20 min at 25 °C with 25 nmol of NADPH and 0.25 μmol of Mg^{2+} in a volume of 10 μl. Reaction was initiated by adding 0.6 mM of 2-aceto-2-hydroxybutyrate in 1 ml of 50 mM Tris/HCl, pH 8.2, containing 3 mM $MgCl_2$ and 0.25 mM NADPH. ●, Reactions in the presence of inhibitor; ○, reactions in the absence of inhibitor.

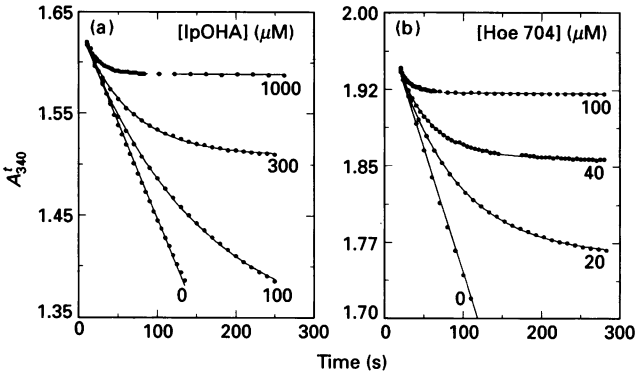


Figure 2 Time course inhibition of spinach acetohydroxy acid isomeroreductase in the presence of IpOHA and Hoe 704

Reactions were initiated by adding simultaneously 2-aceto-2-hydroxybutyrate (0.6 mM) and various concentrations of IpOHA (**a**) or Hoe 704 (**b**) designed in μM in the Figures in 1 ml of 50 mM Tris/HCl, pH 8.2, containing 3 mM $MgCl_2$, 0.25 mM NADPH and 61 nM enzyme. In the absence of inhibitor (0) the data were analysed by linear regression. For the experiments conducted in the presence of inhibitors, the continuous lines were obtained by non-linear least-squares analysis of the data using eqn. (3). This yielded values of the best-fit parameter $k_{obs.}$, the apparent rate constant for inhibition, as a function of inhibitor, acetohydroxyacid substrate, NADPH, or Mg^{2+} concentration (see Tables 1–3).

Inhibition kinetics

The results described above showed that both IpOHA and Hoe 704 were tight-binding inhibitors of plant acetohydroxy acid isomeroreductase. However, this did not help us to understand why these compounds should be such weak herbicides. Inhibition by both IpOHA and Hoe 704 was time-dependent (Figure 2). We therefore characterized the mechanism of inhibition by investigating the effect of substrate and inhibitor concentration on the time-dependence of inhibition [27–29]. When the plant enzyme was assayed in the simultaneous presence of substrate and inhibitor, non-linear time courses of NADPH oxidation were observed, indicating that both IpOHA (Figure 2a) and Hoe 704 (Figure 2b) behaved as slow-binding inhibitors. There was no variation of initial velocities as a function of [IpOHA] or [Hoe 704], contrasting with previous observations on inhibition of the *E. coli* enzyme by IpOHA [4]. In the latter case, biphasic inhibition has been observed, suggesting that the final enzyme–inhibitor complex (K_d of 22 pM) occurred via the isomerization (0.57 min^{-1}) of an initial weaker reversible complex (K_d of 160 nM) [4] (as defined in eqn. 4 below). As Figure 2 shows, at high inhibitor concentrations the inhibition was complete, suggesting that both compounds behaved as nearly irreversible inhibitors of the plant enzyme, in agreement with the data in Figure 1. Furthermore, the time needed to reach the final inhibition state was dependent on the inhibitor concentration (Figure 2). Clearly, at a given inhibitor concentration the inhibition of enzyme activity developed far more slowly (at least 10-fold) in the presence of IpOHA than in that of Hoe 704 (compare the two curves obtained at 100 μM inhibitor in Figure 2).

Kinetic data as shown in Figure 2 were analysed as described by Tian and Tsou [27] and by Liu and Tsou [28]. For the formation of an encounter complex between enzyme and inhibitor:

$$E + I \underset{k_{-0}}{\overset{k_0}{\rightleftarrows}} E\text{–}I \qquad (1)$$

Table 1 Dependence of $k_{obs.}$, the apparent rate constant for inhibition of acetohydroxy acid isomeroreductase by IpOHA and Hoe 704, the concentration of inhibitors

All assays were run at 25 °C in 50 mM Tris/HCl, pH 8.2, containing 61 nM enzyme [2-aceto-2-hydroxybutyrate (AHB) as substrate] or 347 nM enzyme [2-acetolactate (AL) as substrate], 0.6 mM of an acetohydroxy acid substrate, 0.25 mM NADPH, 3 mM Mg^{2+} and variable inhibitor (IpOHA or Hoe 704) in the indicated concentration range. Progress curves as shown in Figure 2 were fitted by non-linear-regression analysis to eqn. (3), allowing the determination of $k_{obs.}$, the apparent inhibition rate constant.

			$k_{obs.}$ versus [inhibitor] plots*		
Acetohydroxy acid substrate	Variable inhibitor	Concentration range (mM)	Slope ($M^{-1} \cdot s^{-1}$)	Origin ordinate (s^{-1})	Correlation coefficient (r)
AHB	IpOHA	0–1	64.1 ± 1.8	0.0003 ± 0.002	0.996
AHB	Hoe 704	0–0.1	593 ± 26	−0.001 ± 0.001	0.996
AL	IpOHA	0–1	57.8 ± 2.0	0.003 ± 0.002	0.996
AL	Hoe 704	0–0.1	663 ± 3.8	−0.003 ± 0.002	0.995

* Plots of $k_{obs.}$ versus the concentration of variable inhibitor were analysed by linear regression.

If the inhibitor binds slowly to the enzyme, and E–I is catalytically inactive, then when the enzyme-catalysed reaction is monitored in the simultaneous presence of the inhibitor and substrate, product formation (eqn. 2) should approach an asymptote [28,29]:

$$[P] = [P]_\infty (1 - e^{-k_{obs.} t}) \quad (2)$$

where [P] and $[P]_\infty$ are respectively the concentrations of product formed at time t and at time approaching infinity, and $k_{obs.}$ is the pseudo-first-order rate constant for inhibition including the excess concentration of inhibitor. Since product formation was measured by monitoring the decrease in absorbance at 340 nm as NADPH is oxidized, eqn. (2) becomes:

$$A^t_{340} = A^\infty_{340} + (A^\infty_{340} - A^0_{340}) e^{-k_{obs.} t} \quad (3)$$

where A^t_{340}, A^0_{340} and A^∞_{340} are respectively the absorbance at 340 nm at time t, at time zero, and at time approaching infinity. As Figure 2 shows, the experimental time courses could be fitted satisfactorily by using eqn. (3) and a nonlinear least-squares programme, allowing the determination of A^0_{340}, A^∞_{340} and $k_{obs.}$ For an irreversible inhibition occurring without prior formation of a reversible intermediate enzyme–inhibitor complex, $k_{obs.}$ is a linear function of [I], the inhibitor concentration [28].

If irreversible inhibition involves the existence of an intermediate reversible complex

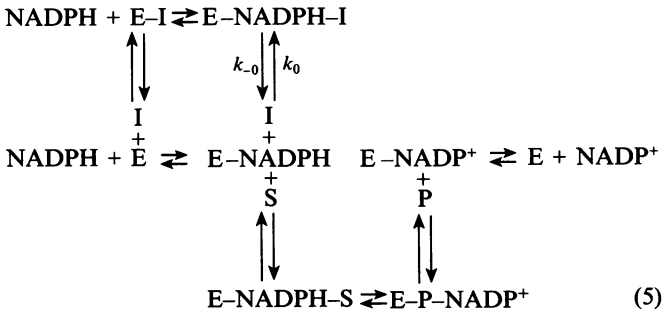
$$E + I \underset{k_{-0}}{\overset{k_0}{\leftrightarrows}} E\text{–}I \xrightarrow{k'} E\text{–}I^* \quad (4)$$

eqns. (2) and (3) describing product formation still apply, but then the representation of $k_{obs.}$ versus [I] is a hyperbola [27,28].

The results summarized in Table 1 show that plots of $k_{obs.}$ against [IpOHA] and [Hoe 704] were linear, suggesting that inhibition of the plant acetohydroxy acid isomeroreductase by these compounds involved a single step, at least in the inhibitor concentration range investigated here (up to 1 mM for IpOHA and 0.1 mM for Hoe 704; above these concentrations inhibition became too fast in these manual mixing experiments to follow the time course of the enzyme-catalysed reaction; see Figure 2). Also, within experimental error, these plots passed through the origin (Table 1), confirming the nearly irreversible character of the binding process. This behaviour also indicates that the rate of inhibitor dissociation, k_{-0}, must be relatively slow, and therefore contributes only slightly to observed rates, even at the lowest concentrations of inhibitor.

The determination of $k_{obs.}$ as a function of the substrate concentration, [S], allows the mechanism of inhibition to be assessed [28]. For competitive and uncompetitive inhibitors plots of $1/k_{obs.}$ are linear with respect to [S] and 1/[S] respectively. For non-competitive inhibitors $k_{obs.}$ is independent of [S]. With both IpOHA and Hoe 704, plots of $1/k_{obs.}$ against acetohydroxy acid substrate concentration were linear (Table 2). In contrast, $k_{obs.}$ was independent of Mg^{2+} and NADPH concentrations. We conclude that both compounds behaved (i) as competitive inhibitors with respect to acetohydroxy acid substrates, as reported for *E. coli* acetohydroxy acid isomeroreductase [3,4], and for inhibition of carrot (*Daucus carota*) [3] and *Saccharomyces cerevisae* [30] acetohydroxy acid isomeroreductases by Hoe 704, and (ii) as non-competitive inhibitors with respect to Mg^{2+} and NADPH.

As for the *E. coli* acetohydroxy acid isomeroreductase [18], a previous kinetic study suggested an ordered sequential mechanism for the plant-enzyme-catalysed reaction, in which NADPH and Mg^{2+} bind first to the enzyme and acetohydroxy acid substrate binds second ([12], eqn. 5). From the data in Table 2, the mechanism of inhibition of spinach chloroplast acetohydroxy acid isomeroreductase by Hoe 704 and IpOHA can be depicted as follows:



(5)

where E stands for the enzyme–Mg^{2+} complex, S for acetohydroxy acid substrate and P for 2,3-dihydroxy-3-isovalerate (substrate 2-acetolactate) or 2,3-dihydroxy-3-methylvalerate (substrate 2-aceto-2-hydroxybutyrate).

For those competitive inhibitors that bind in a single step to the enzyme (eqn. 1), the apparent rate constant $k_{obs.}$ is related by eqn. (6) to the second-order rate constant k_0 for association of inhibitor with enzyme, k_{-0} the dissociation rate constant, the

Table 2 Determination of the type of irreversible inhibition of acetohydroxy acid isomeroreductase by IpOHA and Hoe 704

All assays were run at 25 °C in 50 mM Tris/HCl, pH 8.2, containing 61 nM enzyme (2-aceto-2-hydroxybutyrate as substrate) or 347 nM enzyme (2-acetolactate as substrate), 0.3 mM IpOHA or 40 μM Hoe 704, fixed substrates and metal cofactor as indicated and variable substrates or metal cofactor in the indicated concentration range. Progress curves as shown in Figure 2 were fitted by non-linear-regression analysis to eqn. (3), allowing the determination of $k_{obs.}$, the apparent inhibition rate constant.

Inhibitor	Variable substrate or metal cofactor	Concentration range (mM)	Fixed substrate and metal cofactor	Slope of $1/k_{obs.}$ plots* ($\mu M^{-1} \cdot s$)	Type of Inhibition‖‖
IpOHA	AHB	0–1.8	NADPH† + Mg^{2+}‡	0.104 ± 0.001¶	C
IpOHA	AL	0–2.1	NADPH† + Mg^{2+}‡	0.077 ± 0.007**	C
IpOHA	NADPH	0.05–1.5	AHB§ + Mg^{2+}‡	0††	NC
IpOHA	NADPH	0.05–1.5	AL‖ + Mg^{2+}‡	0††	NC
IpOHA	Mg^{2+}	3–15	AHB§ + NADPH†	0††	NC
IpOHA	Mg^{2+}	3–15	AL‖ + NADPH†	0††	NC
Hoe 704	AHB	0–2.5	NADPH† + Mg^{2+}‡	0.086 ± 0.009‡‡	C
Hoe 704	AL	0–2.5	NADPH† + Mg^{2+}‡	0.059 ± 0.0008§§	C
Hoe 704	NADPH	0.05–1.2	AHB§ + Mg^{2+}‡	0††	NC
Hoe 704	NADPH	0.05–1.2	AL‖ + Mg^{2+}‡	0††	NC
Hoe 704	Mg^{2+}	3–12	AHB§ + NADPH†	0††	NC
Hoe 704	Mg^{2+}	3–12	AL‖ + NADPH†	0††	NC

* Plots of $1/k_{obs.}$ versus the concentration of variable substrate or metal cofactor were analysed by linear regression.
† 0.25 mM NADPH.
‡ 3 mM Mg^{2+}.
§ 0.6 mM 2-aceto-2-hydroxybutyrate (AHB).
‖ 0.6 mM 2-acetolactate (AL).
¶ §§ Origin ordinates: ¶ 2 ± 1 s; ** 10 ± 10 s; ‡‡ −0.3 ± 2.7 s; §§ 1.7 ± 1.2 s.
†† Within standard deviation.
‖‖ C, competitive; NC, non-competitive.

Table 3 Rate constant for binding of IpOHA and Hoe 704 to the acetohydroxy acid isomeroreductase–NADPH–Mg^{2+} complex

All assays were run at 25 °C in 50 mM Tris/HCl, pH 8.2, containing 61–347 nM enzyme, 0.3 mM IpOHA or 40 μM Hoe 704, 3 mM Mg^{2+}, 2-acetolactate (AL; varied in the concentration range 0–2.5 mM) or 2-aceto-2-hydroxybutyrate (AHB; varied in the concentration range 0–1.8 mM). Progress curves as shown in Figure 2 were fitted by non-linear-regression analysis to eqn. (3), allowing the determination of $k_{obs.}$, the apparent inhibition rate constant. Plots of $1/k_{obs.}$ against the concentration of variable acetohydroxy acid substrate were linear, indicating that contribution of k_{-0} to $k_{obs.}$ (eqn. 6) was minor across the range of conditions investigated. The value of the rate constant, k_0, for association of IpOHA or Hoe 704 with the enzyme–NADPH–Mg^{2+} complex was calculated from the slopes of these plots (Table 2), and a K_m value of enzyme for acetohydroxy acid substrate of 20 μM (racemic substrates) [12]. Correlation coefficients: [a]0.999, [b]0.978; [c]0.998; [d]0.996.

IpOHA	Hoe 704	AHB	AL	k_0 ($M^{-1} \cdot s^{-1}$)
+	−	+	−	1940[a]
+	−	−	+	1820[b]
−	+	+	−	22600[c]
−	+	−	+	21300[d]

inhibitor concentration, the substrate concentration, and the apparent K_m of enzyme for substrate [28]

$$k_{obs.} = \frac{k_0[I]}{\left(1 + \frac{[S]}{K_m^S}\right)} + k_{-0} \quad (6)$$

Use of eqn. (6) showed that, at saturating levels of Mg^{2+} and NADPH, the value of k_0 for Hoe 704 was nearly the same by employing either of the two acetohydroxy acid substrates (Table 3). Similar results were obtained for IpOHA (Table 3). However, the value of k_0 for Hoe 704 (2.2×10^4 $M^{-1} \cdot s^{-1}$) was 11-fold higher than that for IpOHA (1.9×10^3 $M^{-1} \cdot s^{-1}$). It is noteworthy that the rate constant for binding of IpOHA to the plant enzyme was 30-fold smaller than that for the *E. coli* enzyme ($k_0 = 5.9 \times 10^4$ $M^{-1} \cdot s^{-1}$; [4]), extending our previous observations that the plant and the bacterial acetohydroxy acid isomeroreductases exhibit variant biochemical properties [7,12]. From eqns. (2) and (6) and the rate constant values listed in Table 3, one can calculate the time needed to reach 90% inhibition of enzyme activity at given substrate and inhibitor concentrations. For example, at 1 μM IpOHA, this time varies from 0.7 to 28 h over the acetohydroxy acid substrate concentration range 10 μM–1 mM. These calculations suggest that, unless a very high inhibitor concentration is used (i.e. in accord with the observation that herbicidal effects are obtained at a high application rate for IpOHA and Hoe 704), a substantial proportion of the acetohydroxy acid isomeroreductase activity would escape *in vivo* to inhibition by IpOHA and Hoe 704.

Recovery of enzyme activity

The above results suggested that both IpOHA and Hoe 704 behaved as irreversible inhibitors of the plant acetohydroxy acid isomeroreductase. It is possible, however, that binding of the inhibitors to the enzyme is reversible to some extent, but that the dissociation rate constants are too low to be accurately determined from the data shown in Figure 2. Since the results in Figure 2 and Table 2 demonstrated that both inhibitors act as competitive inhibitors with respect to acetohydroxy acid substrates, to investigate this point we submitted preformed enzyme–Mg^{2+}–NADPH–inhibitor complexes to a substrate challenge, as described in the Experimental section.

Under these conditions, no enzyme activity was recovered after inhibition by IpOHA, even after 7 days of incubation in the presence of excess acetohydroxy acid substrate. A control carried out in the absence of inhibitor showed only a 5% loss of enzyme activity. In contrast, for Hoe 704 about 55% of the initial

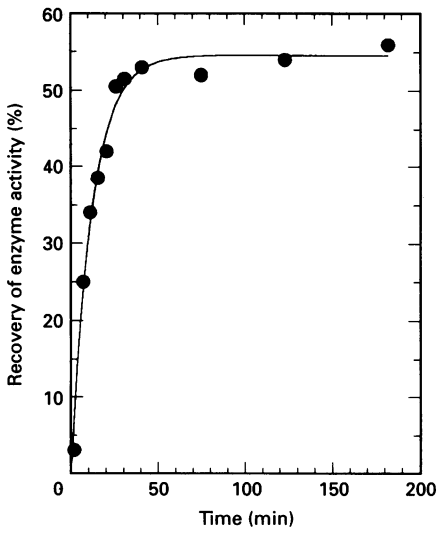


Figure 3 Restoration of enzyme activity by an excess of 2-aceto-2-hydroxybutyrate after inhibition by Hoe 704

Enzyme (0.879 nmol) was first incubated with Hoe 704 (1.55 nmol) in a volume of 100 μl containing 250 nmol of NADPH and 2.5 μmol of Mg^{2+}. After 20 min incubation at 25 °C, a 1000-fold excess of 2-aceto-2-hydroxybutyrate (1 μmol) over inhibitor was added. Aliquots of 10 μl were withdrawn at various time intervals, and recovery of enzyme activity was assayed in 1 ml of 50 mM Tris/HCl, pH 8.2, containing 3 mM $MgCl_2$, 0.25 mM NADPH and 0.6 mM 2-aceto-2-hydroxybutyrate. The continuous line was obtained by non-linear least-squares regression analysis of the data using the equation:

$$\text{Recovery of enzyme activity (\%)} = V_{max.}^{restored}(1 - e^{-k_{-0}t})$$

where $V_{max.}^{restored}$ is the maximal value of enzyme activity recovered, and k is the re-activation rate constant, for the following values of the best-fit parameters: $V_{max}^{restored} = 54.6 \pm 1.2\%$; $k_{-0} = 0.082 \pm 0.006$ min^{-1}.

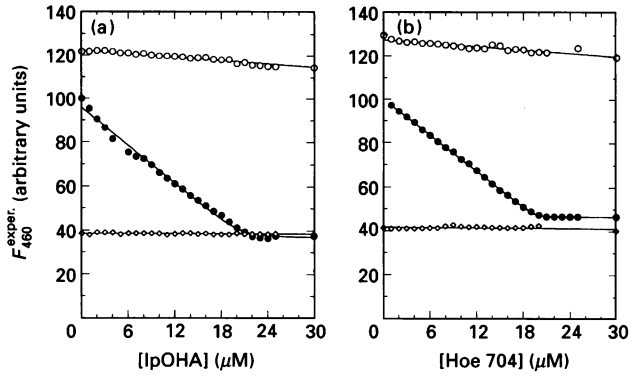


Figure 4 Fluorimetric titration of binding of IpOHA and Hoe 704 to acetohydroxy acid isomeroreductase

Titrations were effected in 200 μl of 50 mM Tris/HCl, pH 8.2, containing 65 μM NADPH, 3 mM Mg^{2+} and 20.4 μM enzyme (●). Controls were performed without enzyme (◇) or without Mg^{2+} (○). The excitation wavelength was 370 nm and the emission wavelength was 460 nm. After stepwise addition of IpOHA (**a**) or Hoe 704 (**b**) (0.2 μl additions of 1 mM inhibitor solution), the emitted fluorescence, $F_{460}^{exper.}$, was recorded. In the case of the inhibitor-containing assays, and since saturation occurred at low [IpOHA] and [Hoe 704], the binding data were analysed on the basis of a tight-binding hypothesis. Then $F_{460}^{exper.}$ is given by:

$$F_{460}^{exper.} = F_0 - \Delta F_{460}^{max.} \frac{c_0 + i_0 + K_d - \sqrt{[(c_0 + i_0 + K_d)^2 - 4\,c_0 i_0]}}{2\,c_0}$$

where c_0 and i_0 are the total concentrations of enzyme–NADPH–Mg^{2+} complex and ligand (IpOHA or Hoe 704) respectively, K_d is the equilibrium dissociation constant of the encounter complex (eqn. 7), F_0 is the initial fluorescence and $\Delta F_{460}^{max.}$ is the maximum change of fluorescence achieved at saturation. For the experiments conducted in the presence of inhibitors, the continuous lines were obtained by non-linear least-squares analysis of the data to the above equation for the following values of the best-fit parameters. For IpOHA, $K_d = 0.078 \pm 0.06$ μM; $c_0 = 20.5 \pm 0.4$ μM; $\Delta F_{460}^{max.} = 59.3 \pm 1.9\%$; $F_0 = 96.8 \pm 1\%$. For Hoe 704, $K_d = 0.011 \pm 0.05$ μM; $c_0 = 19.6 \pm 0.1$ μM; $\Delta F_{460}^{max.} = 53.5 \pm 0.3\%$; $F_0 = 99.9 \pm 0.1\%$.

enzyme activity was readily recovered upon acetohydroxy acid substrate addition (Figure 3). The re-activation process followed first-order kinetics. Moreover, prolonged incubations, up to 7 days, did not increase the level of recovery of enzyme activity. Also, varying the temperature of the incubation assays (between 4 °C and 37 °C) did not affect the percentage of enzyme re-activation, but influenced the velocity of the reactivation process. For example, at 25 °C, the half-time of the re-activation process was of the order of 7 min (Figure 3). At 4 °C and 37 °C this time was of the order of 25 min and 3 min respectively (results not shown). Strictly, the off rate determined at 25 °C for Hoe 704 from the data in Figure 3 ($k_{-0} = 1.6 \times 10^{-3}$ s^{-1}) should be subtracted from $k_{obs.}$ in eqn. (6). However, the effect of the correction will be minor across most of the range of conditions described in Table 3. For the off rate and on rate previously determined for Hoe 704, one can calculate a K_i of 70 nM. A similar value (K_i of 300 nM) has been reported for inhibition of the *S. cerevisiae* enzyme by Hoe 704 [30].

Steady-state kinetic studies with the restored enzyme yielded apparent K_m values for acetohydroxy acid substrates, NADPH and Mg^{2+} that were identical with those previously determined for the native enzyme [12] (results not shown). We conclude that, at least for Hoe 704, a dissociation of the enzyme–inhibitor complex had occurred.

Since only part of the initial enzyme activity can be recovered, the results for Hoe 704 indicate a permanent inactivation of the enzyme after incubation with this compound and its subsequent removal. A similar phenomenon has been observed during inhibition of maize (*Zea mays*) acetohydroxy acid synthase by chlorsulfuron or imazaquin [11]. As Hawkes [23] has pointed out, this peculiar behaviour might be important to account for the potency of acetohydroxy acid synthase to behave as an efficient target for herbicides, because once dissociated from the enzyme, the inhibitor will release the enzyme in an inactive state and will be reused to inactivate new enzyme molecules. As for acetohydroxy acid synthase, the results of Figure 3 therefore suggest the existence of transitions between metastable conformational states of acetohydroxy acid isomeroreductase. Slow conformational transitions of enzymes in response to changes in concentrations of substrates or modifiers have been well documented [31–35].

Fluorescence analysis

Binding of IpOHA and Hoe 704 to the enzyme molecules was directly assessed by conducting fluorescence measurements. Consistent with previous results [12], there was a marked enhancement of NADPH fluorescence in the presence of enzyme (Figure 4), previously assigned to the formation of a binary complex between enzyme and nucleotide [12]. Moreover, the fluorescence level of this complex was slightly lower in the presence of Mg^{2+} than in its absence (Figure 4), suggestive of the formation of a ternary enzyme–NADPH–Mg^{2+} complex. Under these conditions, there was a considerable decrease of the emitted fluorescence upon adding increasing amounts of IpOHA or Hoe 704 (Figure 4). A saturation behaviour was observed, suggesting the formation of an enzyme–NADPH–Mg^{2+}–inhibitor complex.

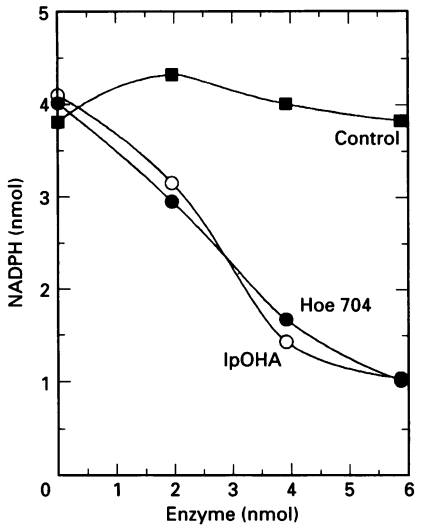


Figure 5 Gel filtration of preformed enzyme–NADPH–Mg^{2+} complexes in the absence or presence of IpOHA or Hoe 704

Various amounts of enzyme were incubated for 20 min at 25 °C with 300 nmol of Mg^{2+}, 4 nmol of NADPH, and 10 nmol of IpOHA (○) or Hoe 704 (●) in a total volume of 100 μl. Incubations were also effected under the same conditions in the absence of either inhibitor (■, control). These mixtures were then injected on a HiLoad 16/60 Superdex column equilibrated in 25 mM potassium phosphate, pH 7.5. Elution disclosed two peaks of absorbance. One corresponded to an elution volume of 78 ml and contained the free enzyme and the enzyme–NADPH–Mg^{2+}–inhibitor complex. The other was eluted much later (107 ml) and contained free Mg^{2+}, inhibitor and NADPH. Of these low-M_r molecules, only NADPH contributed significantly to the absorbance at 280 nm. After column elution, free NADPH was calculated from the area of the peak corresponding to an elution volume of 107 ml and plotted as a function of the initial amount of enzyme.

Remarkably, at saturating [IpOHA] or [Hoe 704] the fluorescence level decreased to that measured for NADPH free in solution. Experiments conducted in the absence of Mg^{2+} showed only a weak decrease of fluorescence after addition of these compounds suggesting that IpOHA and Hoe 704 bind primarily to the enzyme–NADPH–Mg^{2+} complex. Also, controls effected without enzyme did not reveal any interaction between NADPH, Mg^{2+} and either of the two inhibitors (Figure 4).

To quantify the binding process, non-linear-regression analyses of the data in Figure 4 were performed, assuming that encounter complex formation occurred as defined in eqn. (1), in which, here, E corresponds to the enzyme–NADPH–Mg^{2+} complex. Formation of the encounter complex is governed by the equilibrium constant K_d:

$$K_d = \frac{k_{-0}}{k_0} = \frac{(c_0 - x)(i_0 - x)}{x} \quad (7)$$

where c_0 and i_0 are the total concentrations of enzyme–NADPH–Mg^{2+} complex and ligand (IpOHA or Hoe 704) respectively, and x is the concentration of bound ligand. Figure 4 shows that, owing to the very tight binding of IpOHA and Hoe 704, the value of K_d was poorly defined, consistent with the finding that these two compounds behaved as nearly irreversible inhibitors of the plant acetohydroxyacid isomeroreductase. Also, the maximum variation of fluorescence was reached for a concentration of inhibitor that was not significantly different from the total concentration of monomeric enzyme unit, indicating a stoichiometric binding of both IpOHA and Hoe 704 to the plant enzyme, a finding that is also consistent with the data of Figure 1.

Gel-filtration analysis

One possibility to account for the above fluorescence data is that binding of IpOHA or Hoe 704 to the enzyme–NADPH–Mg^{2+} complex induced a considerable modification of the accessibility of the bound NADPH to solvent molecules. Another possibility is that binding of IpOHA or Hoe 704 was accompanied by the release of NADPH from the enzyme. To distinguish between these two alternatives, a gel-filtration analysis was carried out. Different amounts of enzyme (0–60 μM) were incubated for 20 min at 25 °C with IpOHA (0.1 mM) or Hoe 704 (0.1 mM) or without inhibitor, in the presence of 3 mM Mg^{2+} and 40 μM NADPH. Then each of these reaction mixtures was injected on a HiLoad 16/60 Superdex column. In the absence of IpOHA or Hoe 704, the elution profile obtained with the preformed enzyme–Mg^{2+}–NADPH complex disclosed two peaks of absorbance at 280 nm: the enzyme was eluted as the high-M_r (115000), as reported previously [12], and NADPH was eluted much later, at the same V_e/V_0 as for NADPH run alone as a control. The peak area on the profiles corresponding to free NADPH was calculated, and plotted as a function of enzyme concentration in the incubation mixtures (Figure 5). This area was independent of enzyme concentration (Figure 5, control), indicating that the enzyme–Mg^{2+}–NADPH complex readily dissociated upon gel filtration, in agreement with our previous results showing that NADPH binds reversibly to the enzyme and enzyme–Mg^{2+} complex (K_d of the order of 2 μM; [12]). In contrast, when the preincubation mixtures contained IpOHA or Hoe 704, an increase of the enzyme concentration was associated with a decrease of the area corresponding to free NADPH (Figure 5), suggesting that in the presence of Mg^{2+} and inhibitor, NADPH remained tightly bound to the enzyme. We conclude that the slow increase in inhibition observed with IpOHA and Hoe 704 did not result from displacement of NADPH from the enzyme by these compounds.

DISCUSSION

In agreement with the findings of Wittenbach et al. [5], we observed that, although inhibitors of acetohydroxy acid isomeroreductase, such as IpOHA and Hoe 704, translocate within plants, their herbicidal activity is very low compared with that of inhibitors of acetohydroxy acid synthase. To explain this paradox, we have investigated the mechanism of interaction of purified spinach chloroplast acetohydroxy acid isomeroreductase with these compounds. Fluorescence measurements indicated that both inhibitors bind avidly to the enzyme in the presence of Mg^{2+} and NADPH, inducing marked conformational changes close to the nucleotide-binding site. By monitoring the time course of enzyme inhibition in the simultaneous presence of substrate and inhibitors, we observed that, as for the corresponding enzyme purified from *E. coli* [3,4], IpOHA and Hoe 704 act as slow tight-binding inhibitors for the plant enzyme. An analysis of these kinetic data [27,28] disclosed, however, that binding of Hoe 704 occurs 11 times faster than that of IpOHA. Since the amount of IpOHA needed to give a herbicidal effect was about 15-fold higher than that of Hoe 704, this comparison suggests that the herbicidal potency of these compounds is correlated with their rates of association with the plant enzyme. Strikingly, IpOHA binds considerably faster to *E. coli* acetohydroxy acid isomeroreductase ($k_0 = 5.9 \times 10^4$ $M^{-1} \cdot s^{-1}$; [1]) than to the plant enzyme (Table 3). This would help to explain the observation that, in contrast with plants, the growth of *E. coli* cells is extremely sensitive to inhibition by this compound [1]. Clearly, the herbicidal potency was not correlated with the ease of recovery of enzyme activity from the inhibited enzyme

complexes, since for inhibition by IpOHA it has not been possible to recover any enzyme activity, even after several days of incubation with an excess of substrate, whereas for Hoe 704 about 55% of the initial enzyme activity was recovered in a few minutes.

On the basis of activity (Figure 1) and fluorescence (Figure 4) measurements, we observed that both IpOHA and Hoe 704 bind in a stoichiometric manner to the plant acetohydroxy acid isomeroreductase. Furthermore, for both compounds the progress curves for NADPH oxidation in the presence of substrate and inhibitor can be well fitted with a simple exponential function (Figure 2). Since Hoe 704 is a racemic compound, these results suggest that both enantiomers bind to, and inhibit, the plant enzyme. These results are in accordance with the fact that the *E. coli* enzyme is able to reduce both enantiomers of 2,3-dihydroxy-3-methylbutyrate, a putative α-keto acid intermediate [4], indicating that stereospecificity of the acetohydroxy acid isomeroreductase could be lost after the isomerization of the (*S*) enantiomer of the α-keto acid substrates.

Besides the very slow inhibition kinetics, it is emphasized that both IpOHA and Hoe 704 behaved as competitive inhibitors with respect to acetohydroxy acid substrates, particularly because in plants [3,5,36] and bacteria [2] inhibition *in vivo* of acetohydroxy acid isomeroreductase by these compounds leads to a massive accumulation of acetohydroxy acid substrates. Furthermore, we must consider that the inhibitor compounds are presented *in vivo* to the target enzyme simultaneously with the enzyme substrates. Thus because their binding rate constants are so low, what seems important is not so much the fact that these compounds display an exceptional affinity for the enzyme, but that the time needed for the system to attain full inhibition may become excessively long, that is, much longer than the time necessary for *de novo* synthesis of new enzyme molecules. (From the M_r of the spinach enzyme subunit, this time is expected to be of the order of a few minutes [37].) Under such conditions, the degree of enzyme inhibition that can be obtained at a given inhibitor concentration is not dependent on the concentration of the target enzyme, but instead is a function of enzyme substrate concentration. We suggest that this would account for the observation that herbicidal activity is only obtained at inhibitor concentrations in the micromolar range for Hoe 704 and IpOHA. Although the potent herbicide sulfometuron methyl is also a slow-binding inhibitor of acetohydroxy acid synthase [38], it must be stressed that this compound acts as an 'extraneous' site inhibitor (i.e. the herbicide-binding site is outside the active site of the enzyme) [5]. Then, in contrast with the reaction-intermediate analogues of acetohydroxy acid isomeroreductase, the rate of inhibitor binding to acetohydroxy acid synthase is independent of substrate concentration, and the degree of enzyme inhibition is not negated by substrate concentration and/or accumulation, but depends principally on the enzyme concentration.

As Schloss [38] has pointed out, from the standpoint of inhibitor design for agricultural or pharmaceutical applications, a low rate of dissociation is desirable, as it may be expected to enhance the effectiveness of an inhibitor, while a low rate of association is not, as it delays the time required for inhibition at a given concentration *in vivo*. The latter feature may be of particular importance for acetohydroxy acid isomeroreductase and the slow-binding competitive inhibitor compounds such as IpOHA and Hoe 704 because the apparent K_m of the plant enzyme for its acetohydroxy acid substrates is the micromolar range [12]. Presumably, if the rate constants for association of compounds such as IpOHA and Hoe 704 with plant acetohydroxy acid isomeroreductase were near those expected for a diffusion-controlled encounter of enzyme and inhibitor (10^8–10^9 $M^{-1} \cdot s^{-1}$; [39,40]), then these compounds would exhibit much better herbicidal activity than experimentally observed.

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REFERENCES

1 Bryan, J. K. (1980) in The Biochemistry of Plants, vol. 5 (Miflin, B. J., ed.),. pp. 403–452, Academic Press, New York
2 Armstrong, F. B. and Wagner, R. P. (1961) J. Biol. Chem. **236**, 2027–2032
3 Schulz, A., Spönemann, P., Köcher, H. and Wengenmayer, F. (1988) FEBS Lett. **238**, 375–378
4 Aulabaugh, A. and Schloss, J. V. (1990) Biochemistry **29**, 2824–2830
5 Wittenbach, V. A., Aulabaugh, A. and Schloss, J. V. (1990) in Proceedings of the Seventh International Congress of Pesticide Chemistry (IUPAC), Hamburg (Frehse, H., ed.), pp. 151–160, VCH Publishers, Weinheim and New York
6 Dumas, R., Joyard, J. and Douce, R. (1989) Biochem. J. **262**, 971–976
7 Dumas, R., Lebrun, M. and Douce, R. (1991) Biochem. J. **277**, 469–475
8 Shaner, D. L., Anderson, P. C. and Stidham, M. A. (1984) Plant Physiol. **76**, 545–546
9 Schloss, J. V. (1989) in Target Sites of Herbicide Action (Böger, P. and Sandmann, G. eds.), pp. 165–245, CRC Press, Boca Raton, FL
10 Subramanian, V., Loney, V. and Pao, L. (1989) in Prospects for Amino Acid Biosynthesis Inhibitors of Crop Protection and Pharmaceutical Chemistry, Monograph no. 42 (Copping, L. G., Dalziel, J. and Dodge, A. D., eds.), pp. 97–100, British Crop Protection Council, Farnham
11 Durner, J., Gailus, V. and Böger, P. (1991) Plant Physiol. **95**, 1144–1149
12 Dumas, R., Job, D., Ortholand, J.-Y., Emeric, G., Greiner, A. and Douce, R. (1992) Biochem. J. **288**, 865–874
13 Dumas, R., Curien, G., DeRose, R. and Douce, R. (1993) Biochem. J. **294**, 821–828
14 Curien, G., Dumas, R. and Douce, R. (1993) Plant Mol. Biol. **21**, 717–722
15 Arfin, S. M. and Umbarger, H. E. (1969) J. Biol. Chem. **244**, 1118–1127
16 Shematek, E. M., Arfin, S. M. and Diven, W F. (1973) Arch. Biochem. Biophys. **158**, 132–138
17 Hofler, J. G., Decedue, C. J., Luginbuhl, G. H., Reynolds, J. A. and Burns, R. O. (1975) J. Biol. Chem. **250**, 877–882
18 Chunduru, S. K., Mrachko, G. T. and Calvo, K. C. (1989) Biochemistry **28**, 486–493
19 LaRossa, R. A. and Schloss, J. V. (1984) J. Biol. Chem. **259**, 8753–8757
20 Krampitz, L. O. (1948) Arch. Biochem. Biophys. **17**, 81–85
21 Scopes, R. K. (1974) Anal. Biochem. **59**, 277–282
22 Schloss, J. V. and Aulabaugh, A. (1990) Z. Naturforsch. **45c**, 544–551
23 Hawkes, T. R. (1993) Proc. 1993 Br. Crop Protect. Conf. – Weeds, **2**, 723–730,
24 Morrison, J. F. (1969) Biochim. Biophys. Acta **185**, 269–286
25 Cha, S. (1975) Biochem. Pharmacol. **24**, 2177–2185
26 Walsh, C. T. (1984) Annu. Rev. Biochem. **53**, 493–535
27 Tian, W.-X. and Tsou, C.-L. (1982) Biochemistry **21**, 1028–1032
28 Liu, L. and Tsou, C.-L. (1986) Biochim. Biophys. Acta **870**, 185–190
29 Morrison, J. F. (1982) Trends Biochem. Sci. **7**, 102–105
30 Hawkes, T. R. and Edwards, L. S. (1990) in Biosynthesis of Branch Chain Amino Acids (Barak, Z., Chipman, D. M. and Schloss, J. V., eds.), pp. 413–424, VCH Publishers, Weinheim and New York
31 Frieden, C. (1970) J. Biol. Chem. **245**, 5788–5799
32 Ricard, J., Buc, J. and Meunier, J.-C. (1977) Eur. J. Biochem. **80**, 581–592
33 Ricard, J., Meunier, J.-C. and Buc, J. (1974) Eur. J. Biochem. **49**, 195–208
34 Storer, A. C. and Cornish-Bowden, A. (1977) Biochem. J. **165**, 61–69
35 Neet, K. E. and Ainslie, G. R. (1980) Methods Enzymol. **64**, 192–226
36 Wittenbach, V. A., Rayner, D. R. and Schloss, J. V. (1992) in Biosynthesis and Molecular Regulation of Amino Acid in Plants (Singh, B., Flores, H. E. and Shannon, J. C., eds.), pp. 69–88, American Society of Plant Physiologists
37 Lewin, B. (1990) Genes, 4th edn., Oxford University Press, New York, and Cell Press, Cambridge, MA
38 Schloss, J. V. (1988) Acc. Chem. Res. **21**, 348–353
39 Fersht, A. (1985) Enzyme Structure and Mechanism, pp. 147–149, W. H. Freeman and Company, New York
40 Job, D., Jones, P. and Dunford, H. B. (1993) J. Phys. Chem. **97**, 9259–9262

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