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Cyclopropane-1,1-dicarboxylate is a slow-, tight-binding inhibitor of rice ketol-acid reductoisomerase

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Abstract

Ketol-acid reductoisomerase (EC 1.1.1.86) catalyses the second reaction in the biosynthesis of the branched-chain amino acids. The reaction catalyzed consists of two stages, the first of which is an alkyl migration from one carbon atom to its neighbour. The likely transition state is therefore a cyclopropane derivative, and cyclopropane-1,1-dicarboxylate (CPD) has been reported to inhibit the *Escherichia coli* enzyme. In addition, this compound causes the accumulation of the substrate of ketol-acid reductoisomerase in plants. Here, we investigate the inhibition of the purified rice enzyme. The cDNA was cloned, and the recombinant protein was expressed in *E. coli*, purified and characterized kinetically. The purified enzyme is strongly inhibited by cyclopropane-1,1-dicarboxylate, with an inhibition constant of 90 nM. The inhibition is time-dependent and this is due to the low rate constants for formation ($2.63 \times 10^5\ M^{-1}\ min^{-1}$) and dissociation ($2.37 \times 10^{-2}\ min^{-1}$) of the enzyme-inhibitor complex. Other cyclopropane derivatives are much weaker inhibitors while dimethylmalonate is moderately effective.

Keywords: Branched-chain amino acids; Cyclopropane-1,1-dicarboxylate; Enzyme inhibition; Herbicides; Ketol-acid reductoisomerase; Rice

1. Introduction

Plants and most micro-organisms have extensive biosynthetic capacities that allow them to survive on a small set of simple nutrients. In contrast, animals are heavily reliant on their diet for an extensive array of biochemical compounds and their precursors. For this reason, plants and micro-organisms contain numerous enzymes that are potential targets for bioactive compounds such as herbicides and antibiotics. An example is the enzymes involved the biosynthesis of the branched-chain amino acids; isoleucine and valine are synthesized in a parallel set of four reactions while an extension of the valine pathway results in leucine synthesis [1].

This pathway is the target for the sulfonylureas [2], the imidazolinones [3] and a variety of other herbicides [1], which all inhibit the first enzyme acetohydroxyacid synthase. The success of these herbicides has stimulated research into inhibitors of other enzymes in the pathway, including the second enzyme in the common pathway [4], ketol-acid reductoisomerase (KARI; EC 1.1.1.86) and two of those in the leucine extension [5,6]. The reaction catalyzed by KARI is shown in Fig. 1 and consists of two steps [7,8], an alkyl migration followed by an NADPH-dependent reduction. Both steps require a divalent metal ion, such as Mg^{2+}, Mn^{2+} or Co^{2+} but the alkyl migration is highly specific for Mg^{2+}. HOE 704 [9] and IpOHA [10] are potent competitive inhibitors of the enzyme and have been proposed to be mimics of the alkyl migration transition state (Fig. 1). A more obvious transition state mimic is a cyclopropane, and Gerwick et al. [11] presented some limited data to show that cyclopropane-1,1-dicarboxylate (CPD) inhibits *Escherichia coli* KARI. They also showed that application of CPD to various plant tissues caused accumulation of the substrate 2-acetolactate; these in vivo data strongly suggest that CPD can inhibit the plant enzyme.

The main focus of the work of Gerwick et al. [11] was to identify weeds that are resistant to acetohydroxyacid

Abbreviations: CPC, cyclopropanecarboxylate; CPD, cyclopropane-1,1-dicarboxylate; KARI, ketol-acid reductoisomerase

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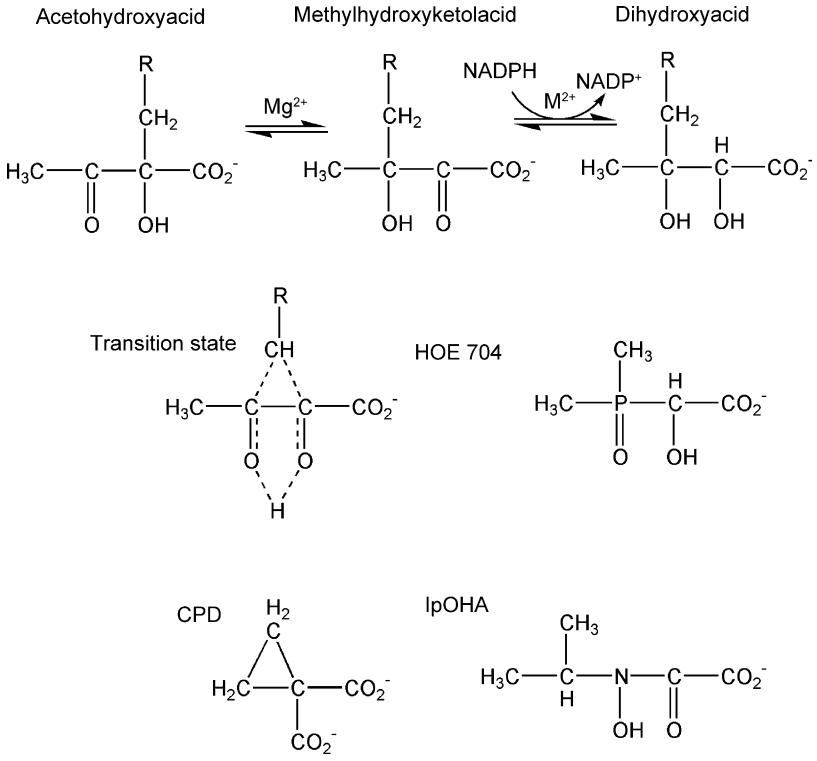

Fig. 1. The KARI reaction. The upper section shows the two-stage reaction catalyzed by KARI. An acetohydroxyacid (R = H or CH_3) undergoes an Mg^{2+}-dependent alkyl migration giving a methylhydroxyketolacid. This is then reduced to a dihydroxyacid by NADPH in the presence of a divalent metal ion. Below the reaction are shown the presumed transition state for the alkyl migration, and the structures of three KARI inhibitors.

synthase-inhibiting herbicides. Since that time, all related publications have been directed towards similar aims (e.g., [12]) and there has been no enzymological characterization of the effect of CPD on any plant KARI. The aims of this study are to elucidate the inhibition of a plant KARI by CPD.

2. Materials and methods

2.1. Materials

The rice KARI cDNA clone J013002N13 was obtained from the Rice Genome Resource Center, Japan. NADPH was obtained from Sigma and hydroxypyruvate was obtained from Fluka. CPD, other cyclopropane analogues and 2-hydroxy-2-methyl-3-ketobutyrate methyl ester were purchased from Aldrich. Alkaline hydrolysis was used to prepare 2-acetolactate from the latter compound, and *cis*-cyclopropane-1,2-dicarboxylate from its anhydride. The pET-30a plasmid and His·Bind resin were obtained from Novagen. Molecular biology reagents and analytical grade reagents were purchased from common chemical suppliers.

2.2. Cloning, expression and purification of rice KARI

The DNA sequence corresponding to mature KARI was amplified by PCR using the oligonucleotide primers 5′-aaaggatCC*ATG*GTCGCGGCGC-3′ and 5′-cccAaaTTtgaagctt*CTA*CGATGACTGCCGGAG-3′. In these sequences, lower case represents mismatched bases, underlining indicates the location of introduced *Bam*HI and *Hin*dIII restriction sites, and italics show the Met-54 codon or the reverse complement of the TAG stop codon. The PCR product was digested with *Bam*HI and *Hin*dIII and ligated into the pET-30a plasmid that had been digested with the same enzymes. The resultant expression plasmid was used to transform *E. coli* BL21(DE3) cells.

A single colony of these cells was inoculated into 20 ml of LB medium [13] containing 50 μg/ml kanamycin. The culture was incubated overnight at 37 °C and was used to inoculate each of two 500 ml volumes of LB medium containing 50 μg/ml kanamycin; the cultures were incubated at 37 °C with shaking. When an OD_{600} of 0.8 was reached, expression was induced by adding 0.5 mM isopropyl β-D-thiogalactoside to each culture; these were then incubated at room temperature (∼22 °C) for a further 4 h with shaking and the cells were harvested by centrifugation.

The frozen cell pellet was thawed, suspended in ice-cold purification buffer [20 mM Tris–HCl (pH 7.9)/500 mM NaCl] containing 5 mM imidazole and then treated with lysozyme (10 mg/g of cells for 30 min at 0 °C). The cells were disrupted by sonication, insoluble material was removed by centrifugation and the supernatant was passed through a 0.45 μm filter. The cell extract was applied to a 7 ml column of His·Bind resin (Novagen) that had been

charged by using 50 mM $NiSO_4$ then equilibrated with purification buffer containing 5 mM imidazole. The loaded column was washed with 23 ml of the same buffer, followed by 30 ml of purification buffer containing 25 mM imidazole, and then KARI was eluted with 30 ml of purification buffer containing 400 mM imidazole. Fractions containing the enzyme were pooled, concentrated to 2.5 ml by ultrafiltration and exchanged into 20 mM Na–Hepes buffer, pH 8.0 using a Pharmacia PD-10 column. The eluate was snap-frozen in liquid nitrogen and stored at −70 °C.

2.3. Enzyme and protein assays

KARI activity was measured by following the decrease in A_{340} at 30 °C in solutions containing 0.2 mM NADPH, 1 mM $MgCl_2$, substrate (2-acetolactate or hydroxypyruvate) and CPD or other inhibitors as required, in 0.1 M Tris–HCl, pH 8.0. The reaction was started by adding the enzyme except for inhibitor preincubation experiments, where the substrate was added last. Protein concentrations were estimated using the bicinchoninic acid method [14] and protein purity was assessed by SDS-PAGE [15].

2.4. Data analysis

Experimental data were analyzed by nonlinear regression (GraFit5, Erithacus Software) using one of the following equations.

Simple inhibition:

$$v_i = \frac{v_u}{(1 + [I]/K_i)} \quad (1)$$

where v_i is the rate at the inhibitor concentration $[I]$, v_u the uninhibited rate, and K_i is the inhibition constant.

Tight-binding inhibition:

$$v_i = \left(\frac{v_u}{2[E]}\right)(([E] - K_i - [I]) + (([E] + K_i + [I])^2 - 4[E][I])^{1/2}) \quad (2)$$

where v_i, v_u,$[I]$, and K_i are as for Eq. (1), while $[E]$ is the total enzyme concentration.

Slow-binding inhibition time-course:

$$[P] = v_s t + (v_o - v_s)\left(\frac{1 - \exp(-k^{app} t)}{k^{app}}\right) \quad (3)$$

where $[P]$ is the product concentration at time t, v_o and v_s are the initial and steady-state rates, respectively, and k^{app} is an apparent first-order rate constant.

Mechanism A, see Fig. 4 (linear regression):

$$k^{app} = k_{off} + k_{on}^{app}[I] \quad (4)$$

where k^{app} is the apparent first-order rate constant at the inhibitor concentration $[I]$, k_{off} the rate constant for release of I from the EI complex, and k_{on}^{app} is an apparent second-order rate constant for the formation of EI.

The following equations were used to calculate derived parameters, where K_i, k_{on}^{app}, and k_{off} are as defined in Eqs. (1)–(4), k_{on} is the true second-order rate constant for the formation of EI, $[S]$ is the substrate concentration and K_m is the Michaelis constant for this substrate.

$$k_{on} = k_{on}^{app}\left(1 + \frac{[S]}{K_m}\right) \quad (5)$$

$$k_{off} = k_{on} K_i \quad (6)$$

3. Results and discussion

3.1. Sequence and cloning of rice KARI

The Rice Genome Resource Center (http://cdna01.dna.affrc.go.jp) contains several clones that are annotated as KARI sequences and one of these (clone J013002N13, Accession number AK065295) was chosen for these studies. In plants, KARI is located in plastids to which it is targeted by an N-terminal peptide. Based on a protein sequence alignment with bacterial and plant KARIs, we determined that the mature rice protein would start at or near Met-54. The only plant KARI sequence that has been confirmed by activity measurements on the encoded protein is that from spinach [16]. Comparing that sequence with the rice clone that we have used, there is 83% identity of amino acids. If the presumed plastid-targeting region is excluded, the identity is 86%. The majority of the differences are conservative substitutions. Similar results were obtained for a probable *Arabidopsis thaliana* sequence [17], with 81 and 87% identity, respectively. All active site residues identified by three-dimensional structure determination of the spinach enzyme [18] are fully conserved.

To construct an expression plasmid, we removed the DNA encoding the plastid-targeting sequence (159 base-pairs) by PCR, and cloned the rest of the cDNA into the pET-30a vector. The protein encoded by this plasmid consists of the first 50 residues derived from pET-30a, followed by the rice KARI residues Met-54 to the C-terminal Ser-578. The deduced molecular weight of this recombinant protein is 62831 Da.

3.2. Expression, purification and activity

Expression of recombinant rice KARI in *E. coli* was successful and most of the protein was found in the soluble fraction of cell extracts. Immobilized metal affinity chromatography yielded a product that contained insignificant amounts of impurities, as judged by SDS-PAGE. The yield of recombinant rice KARI from a 1 l culture was 45 mg with a specific activity (measured with saturating 2-acetolactate) of 1.33 U/mg. The only recombinant plant KARI characterized previously is that from spinach [16]. That enzyme was obtained in a yield of 13 mg from 2 l of culture, with a specific activity of 1.4 U/mg when assayed with 2-acetolactate.

3.3. Kinetic properties

The K_m values for the two substrates are 42.0 ± 2.9 μM (2-acetolactate) and 7.18 ± 1.02 μM (NADPH) while that for the magnesium ion cofactor is 3.24 ± 0.32 μM (data not shown). Assays of enzymic activity were also performed using the substrate analogue hydroxypyruvate, which participates in the reductive half-reaction only (Fig. 1). The K_m for this substrate is 893 ± 32 μM while the specific activity is 1.97 ± 0.23 U/mg (data not shown). The high K_m for hydroxypyruvate (compared to 2-acetolactate) is advantageous for the studies reported below so this substrate was utilized in all further experiments.

3.4. Inhibition by CPD

Gerwick et al. [11] reported that the inhibition of *E. coli* KARI is time-dependent. When assays were performed by adding rice KARI to reaction mixtures containing CPD, time-dependent inhibition was observed (Fig. 2A). To characterise the steady-state inhibition constant, KARI was preincubated for 10 min with NADPH, Mg^{2+} and CPD, then the reaction was initiated with hydroxypyruvate. Under these conditions, the change in A_{340} was found to be linear with time; however, the simple hyperbolic inhibition curve of Eq. (1) does not fit well to the rates that were obtained. It should be noted that the inhibitory concentrations of CPD extend into the low micromolar range, and this is comparable to the active site concentration, which was estimated from the amount of protein added to be 0.49 μM in this experiment. Therefore, a compensation for tight-binding must be applied [19]. When the data were analyzed with Eq. (2) for tight-binding inhibition, a good fit was obtained (Fig. 3) with a K_i of 90.3 ± 13.3 nM. The reported [11] apparent K_i (I_{50}) of CPD for *E. coli* KARI is 4.7 μM.

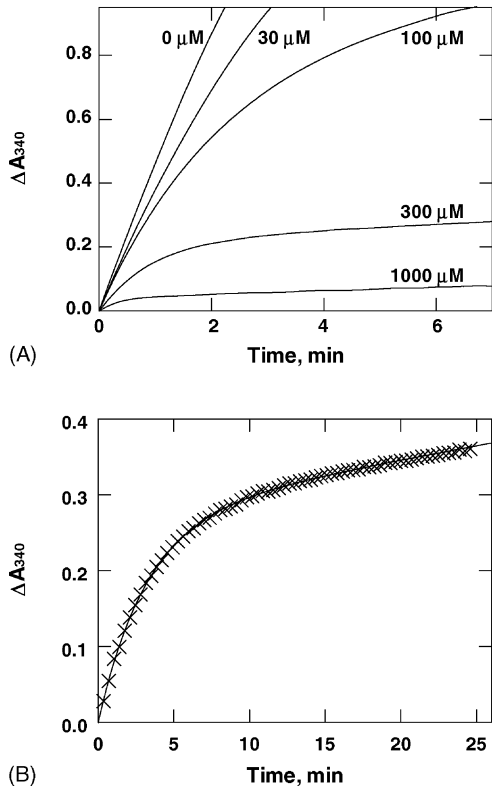

Fig. 2. Slow-binding inhibition of KARI by CPD. *Panel A:* reaction mixtures (1 ml) were prepared containing 1 mM hydroxypyruvate, 0.2 mM NADPH, 1 mM $MgCl_2$ and the indicated concentration of CPD in 0.1 M Tris–HCl buffer, pH 8.0. After equilibration at 30°, 5 μl of KARI (0.14 U) was added and the change in A_{340} was followed at 30°. *Panel B:* shows a similar experiment, using 5 μM CPD, 3 mM hydroxypyruvate, and 0.021 U of KARI. The line is the best fit of Eq. (3) to the data (v_o = 0.0844 ± 0.0009 A_{340} min^{-1}, v_s = 0.0037 ± 0.0001 A_{340} min^{-1}, k^{app} = 0.3115 ± 0.0038 min^{-1}).

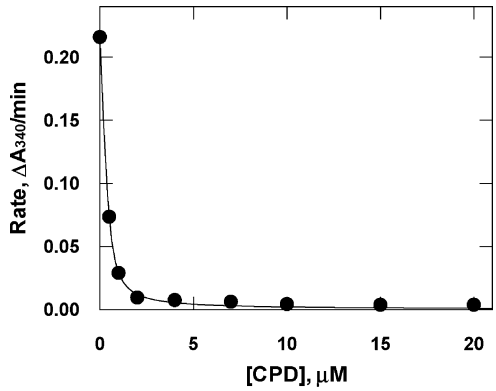

Fig. 3. Inhibition of KARI by CPD. Reaction mixtures (0.99 ml) were prepared containing 0.2 mM NADPH, 1 mM $MgCl_2$ and the indicated concentration of CPD in 0.1 M Tris–HCl buffer, pH 8.0. After equilibration at 30°, 0.066 U of KARI were added and the samples were preincubated at 30° for 10 min. Hydroxypyruvate (10 μl, 1 mM final concentration) was added and the initial rate of NADPH oxidation was measured. The solid line is the best fit of Eq. (2) to the data (v_u = 0.216 ± 0.003 A_{340} min^{-1}, K_i = 0.0903 ± 0.0133 μM, [E] = 0.49 ± 0.05 μM).

The time-dependent competitive inhibition illustrated in Fig. 2A can arise from three basic mechanisms [20,21] as illustrated in Fig. 4. In Mechanism A, the binding of inhibitor to EI is intrinsically slow. In Mechanism B, inhibitor binding is rapid but this is followed by a slow transition to a second complex, EI*. In Mechanism C, there is a slow equilibrium between two enzyme forms and the form to which inhibitor binds (E*) is not the catalytically competent form (E). In each case, the formation of product follows a time-course governed by Eq. (3); the three mechanisms are most easily distinguished by the relationship between k^{app} and the inhibitor concentration [21]. For Mechanism A, there is a linear dependence while for Mechanisms B and C, there is an hyperbolic dependence. The difference between Mechanisms B and C is that k^{app} increases with the inhibitor concentration for the former and decreases for the latter.

A series of experiments, similar to those illustrated in Fig. 2, were conducted and analyzed using Eq. (3); a typical example at 5 μM CPD is shown in Fig. 2B. The dependence

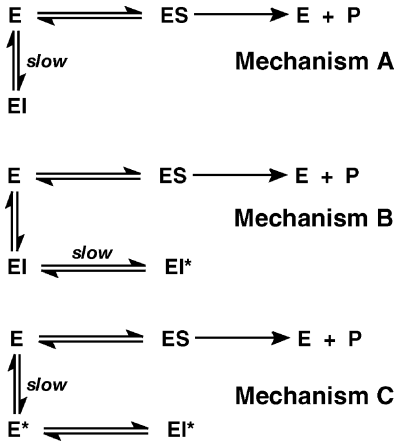

Fig. 4. Mechanisms for slow-binding competitive inhibition. In Mechanism A, the binding of inhibitor to EI is intrinsically slow. In Mechanism B, inhibitor binding is rapid but this is followed by a slow transition to a second complex, EI*. In Mechanism C, there is a slow equilibrium between two enzyme forms and the form to which inhibitor binds (E*) is not the catalytically competent form (E).

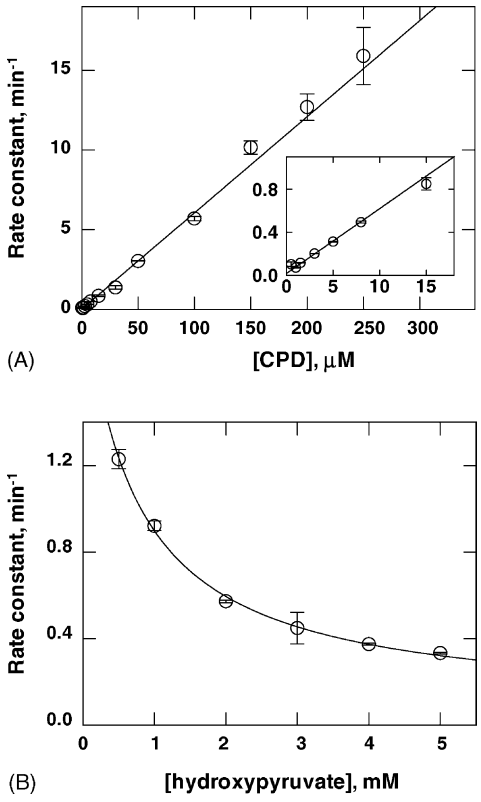

Fig. 5. Slow-binding competitive inhibition of rice KARI. *Panel A:* shows the results of the quantitative analysis of experiments similar to those shown in Fig. 2B.The values of k^{app} obtained over a range of CPD concentration are illustrated in the main graph, with an expansion of the region up to 15 μM shown in the inset. For CPD concentrations of 50 μM and above, the amount of KARI was increased to 0.18 U. The line is the best fit of Eq. (4) to the data ($k_{off} = 0.0161 \pm 0.0031$ min^{-1}, $k_{on}^{app} = 0.0604 \pm 0.0008$ μM^{-1} min^{-1}). *Panel B:* shows a similar experiment in which the CPD concentration was fixed at 7 μM while varying the concentration of hydroxypyruvate. The line is the best fit of Eq. (7) to the data ($k_{on} = 2.82 \pm 0.19 \times 10^5$ M^{-1} min^{-1}, $k_{off} = 7.28 \pm 4.26 \times 10^{-2}$ min^{-1}, $K_m = 717 \pm 151$ μM).

of k^{app} upon the CPD concentration, over the range 0.5–250 μM is shown in Fig. 5A. The rapidity of the onset of inhibition precluded reliable estimates of k^{app} at higher CPD concentrations. Over this range, k^{app} is a linear function of the inhibitor concentration indicating that Mechanism A governs the interaction.

The fitted straight line allows estimates of the rate constants for inhibitor binding and release (Eqs. (4) and (5)). From the slope of the best fit line in Fig. 5A, the value of k_{on} was estimated to be $2.63 \pm 0.08 \times 10^5$ M^{-1} min^{-1}. In principle, k_{off} can be determined from the intercept on the ordinate of the line in Fig. 5A ($1.61 \pm 0.31 \times 10^{-2}$ min^{-1}). However, this estimate may be unreliable because the line passes very close to the origin. Therefore, an alternative estimate was calculated using Eq. (6) in conjunction with the values of K_i and k_{on} determined above. This gave $k_{off} = 2.37 \pm 0.36 \times 10^{-2}$ min^{-1}, which is consistent with that calculated from the ordinate intercept in Fig. 5A. Repetition of this experiment gave $k_{on} = 3.08 \pm 0.21 \times 10^5$ M^{-1} min^{-1} and $k_{off} = 2.88 \pm 0.46 \times 10^{-2}$ min^{-1}.

Combining Eqs. (4) and (5) shows that there should be an inverse hyperbolic relationship between k^{app} and the concentration of hydroxypyruvate, as shown by Eq. (7).

$$k^{app} = k_{off} + \frac{k_{on}}{1 + [S]/K_m} \quad (7)$$

This dependence was confirmed (Fig. 5B) and allowed independent estimates of k_{on} ($2.82 \pm 0.19 \times 10^5$ M^{-1} min^{-1}), k_{off} ($7.28 \pm 4.26 \times 10^{-2}$ min^{-1}) and K_m (717 ± 151 μM) in good agreement with the values reported above.

3.5. Other inhibitors

A range of compounds related to CPD were tested as inhibitors. Using hydroxypyruvate at a concentration of 1 mM, no inhibition was observed by 1-aminocyclopropanecarboxylate (1-aminoCPC), 1-methylCPC, 2-methylCPC, cyclopropane-1,2-dicarboxylate or the dimethyl ester of CPD when each was added at a concentration of 1 mM. Inhibition, but no time-dependence, was observed for 1-hydroxyCPC ($K_i = 5.6 \pm 0.4$ μM), 1-aminocarbonylCPC (20.3 ± 5.4 μM), and 1-cyanoCPC (58.5 ± 13.7 μM). Dimethylmalonate is a good inhibitor ($K_i = 716 \pm 82$ nM) that shows slow-binding kinetics with a linear dependence of k^{app} upon the inhibitor concentration, over the range 13–1300 μM. From these data, the value of k_{on} was estimated to be $2.74 \pm 0.10 \times 10^4$ M^{-1} min^{-1}, while k_{off} was calculated to be $1.95 \pm 0.23 \times 10^{-2}$ min^{-1}. The rate constant for dimethylmalonate release is very similar to that for CPD, while the value for k_{on} is nearly 10-fold lower. Thus, the weaker inhibition by dimethylmalonate (eight-fold) is due almost entirely to its lower rate constant for binding to KARI. Ethylmalonate is also a slow-binding inhibitor, with a K_i of 1.92 ± 0.20 μM.

4. Conclusions

The observation that CPD causes accumulation of 2-acetolactate in plant tissues [11,12] is explained by the inhibition of plant KARI that we have characterized here. We had originally thought that the inhibitory activity of CPD is due to its structural similarity to the probable cyclopropyl transition state. However, other cyclopropane derivatives are relatively weak inhibitors, with the most potent (1-hydroxyCPC, $K_i = 5.6 \pm 0.4$ μM) being more than 60 times less effective than CPD. Moreover, no other cyclopropane tested displayed the time-dependent inhibition that is observed with CPD. The compound with kinetics that most closely resembles those of CPD is dimethylmalonate, which can be considered to be a mimic of CPD with the 2,3-bond cleaved. The only other compound tested that showed time-dependent inhibition is ethylmalonate, another ring-opened mimic of CPD. These observations lead us to suggest that the main contributor to potent and time-dependent inhibition is the presence of geminal carboxyl groups, which could occupy two coordination positions of Mg^{2+} in the active site. In this context, we note that the two potent KARI inhibitors HOE 704 and IpOHA each may form a bidentate complex with Mg^{2+} [10] and both show time-dependent inhibition of plant KARI [22] with a linear dependence of k^{app} upon the inhibitor concentration.

As far as we are aware, there are no published studies on the effectiveness of CPD as a herbicide. Preliminary work in this laboratory has suggested that it is substantially less potent in vivo than its effects on KARI in vitro might suggest. However, now that we have identified the geminal carboxyl groups as a key structural feature for inhibition of KARI, the way is opened for a synthetic program of herbicide discovery based on this molecular framework.

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