Acetolactate Synthase and Ketol-Acid Reductoisomerase: Targets for Herbicides Obtained by Screening and *de novo* Design*

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Z. Naturforsch. **45c,** 544–551 (1990); received December 19, 1989

Acetolactate Synthase, Ketol-Acid Reductoisomerase, Sulfonylurea Herbicides, Sulfometuron Methyl, Branched-Chain Amino Acid Biosynthesis

Several major classes of herbicides, discovered by conventional screening techniques, have been found to inhibit the first common enzyme of branched-chain amino acid biosynthesis, acetolactate synthase, as their mode of action. These herbicides seem to bind to an evolutionary vestige of a quinone-binding site, extraneous to the active site, that is present due to the evolutionary history of this enzyme. Besides their herbicidal effect on sensitive plants, these compounds can effect stasis in the growth of bacteria and yeast. Recently is has been reported that an experimental herbicide from Hoechst, Hoe 704, that was discovered by conventional screening techniques, inhibits the second common enzyme of branched-chain amino acid biosynthesis [Schultz *et al.*, FEBS Lett. **238,** 375–378 (1988)]. We have also recently designed novel reaction-intermediate analogs (*e.g. N*-isopropyl oxalylhydroxamate) that are exceptionally potent (K_i = 22 pM; half-time for release approximately six days) and selective inhibitors of the second common enzyme, ketol-acid reductoisomerase. Both of these selective inhibitors of the second common enzyme will kill sensitive plants, but will only inhibit the growth (without killing) of bacteria. The effects in bacteria parallel those obtained by mutations in the relevant genes, where loss of either the first or second common enzyme in the pathway gives an organism that is auxotrophic for branched-chain amino acids, but does not result in a conditionally lethal phenotype. Higher plant mutants have only been obtained to date that are deficient in functional leucine-specific gene products (as yet uncharacterized), threonine deaminase (isoleucine specific), and dihydroxyacid dehydratase (common). The phenotypes of these mutants, at least at the level of cell culture, are similar to those of their bacterial counterparts, in that auxotrophy, but not conditional lethality, is obtained. These results highlight the potential non-equality of the enzymes of branched-chain amino acid biosynthesis as targets in herbicide design.

Introduction

The three enzymes common to the biosynthesis of all three branched-chain amino acids (valine, leucine, and isoleucine) are acetolactate synthase (EC 4.1.3.18), ketol-acid reductoisomerase (EC 1.1.1.86), and dihydroxyacid dehydratase (EC 4.2.1.9). Other enzymes involved in the biosynthesis of branched-chain amino acids include threonine deaminase (EC 4.2.1.16), which is specific to isoleucine biosynthesis, 2-isopropylmalate synthase (EC 4.1.3.12), 3-isopropylmalate dehydratase (EC 4.2.1.33), and 3-isopropylmalate dehydrogenase (EC 1.1.1.85), which are specific to leucine biosynthesis, as well as branched-chain amino acid utilizing transaminases (*e.g.* EC 2.6.1.42). A renewed interest in the biosynthetic pathway for branched-chain amino acids has ensued with the identification of the first common enzyme, acetolactate synthase, as the site of action of several new commercial herbicides [1–7]. The concurrent identification of structurally diverse herbicides by screening activities at several agrochemical companies raises the question whether other enzymes involved in the biosynthesis of branched-chain amino acids may be suitable targets for herbicides. Is acetolactate synthase unique in either the phytotoxicity associated with its inhibition, or in the ease of selection of inhibitors for this enzyme?

* Based on a paper presented in a symposium entitled: "Herbicides Active in the Chloroplast", August 13 to 15, 1989, in Monheim, Bundesrepublik Deutschland.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/0500–0544 $ 01.30/0

Acetolactate Synthase

The reactions catalyzed by the first common enzyme in the biosynthesis of branched-chain amino acids, acetolactate synthase, are illustrated in Fig. 1. Homologous condensation of two mole-

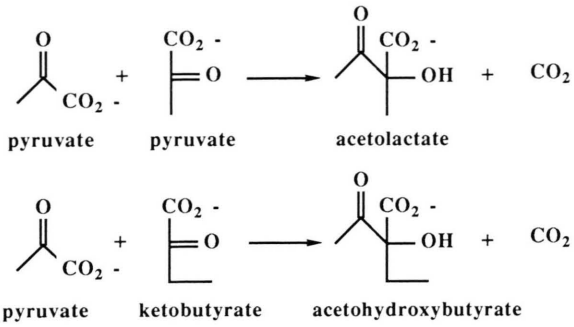

Fig. 1. The physiological reactions catalyzed by acetolactate synthase.

cules of pyruvate gives α-acetolactate and CO_2, as the first step in valine and leucine biosynthesis, or heterologous condensation of pyruvate and α-ketobutyrate gives α-aceto-α-hydroxybutyrate and CO_2, as the first common step in isoleucine biosynthesis. Given the reactions catalyzed, it is not surprising that the enzyme has an absolute requirement for thiamine pyrophosphate and a divalent metal, that physiologically is probably magnesium. However, one long standing curiosity of this enzyme is its requirement for flavin adenine dinucleotide (FAD), despite the absence of an apparent redox role for this cofactor [8]. With the bacterial acetolactate synthase, the requirement for FAD is absolute, and flavin mononucleotide (FMN) cannot substitute for FAD [9, 10]. The absolute requirement for FAD is all the more curious in that the rate of the enzymic reaction is insensitive to the oxidation state of the cofactor, and substitution of FAD by flavin analogs with different redox potentials (such as 8-chloro-FAD or 5-deaza-FAD) are of little consequence [9]. As an unusual flavoprotein, an unusual selectability in herbicide screening would seem to be a likely possibility.

For detailed mechanistic studies and examination of the molecular details of the enzyme's interaction with herbicides, the bacterial enzyme is the only practical alternative. Although the enzyme is rather labile, the abundance of the three major isozymes of acetolactate synthase from enteric bacteria, isozyme I (ALS I, *ilvBN* encoded) [11, 12], isozyme II (ALS II, *ilvGM* encoded) [13], or isozyme III (ALS III, *ilvIH* encoded) [14], in *E. coli* clones makes the purification of large quantities (gram) of high specific activity enzyme possible [9, 10, 15, 16]. These enzymes share many of the properties of the enzymes from higher plants such as sensitivity to inhibition by branched-chain amino acids (ALS I and ALS III, but not ALS II), inhibition by various herbicides (most notably ALS II, to a lesser extent ALS III, and even less so ALS I), partitioning ratios between the two physiological reactions (ALS II and ALS III, but not ALS I) [17–19], and obvious sequence homology with the enzymes from yeast [20], *Arabidopsis thaliana*, and tobacco [21]. Of the bacterial isozymes, ALS II has served as a paradigm for the mechanism of acetolactate synthase and its interaction with herbicides. This enzyme is composed of two large subunits of molecular weight 59,300 (*ilvG* encoded) and two small subunits of molecular weight 9700 (*ilvM* encoded), with a combined molecular weight of 138,000 ($\alpha_2\beta_2$) [9, 10, 13].

The likely catalytic sequence for ALS II is illustrated in Fig. 2. Addition of pyruvate to the thiazole ring of thiamine pyrophosphate gives the lactyl-thiamine pyrophosphate intermediate. Decarboxylation of this intermediate gives the eneamine form of hydroxyethyl-thiamine pyrophos-

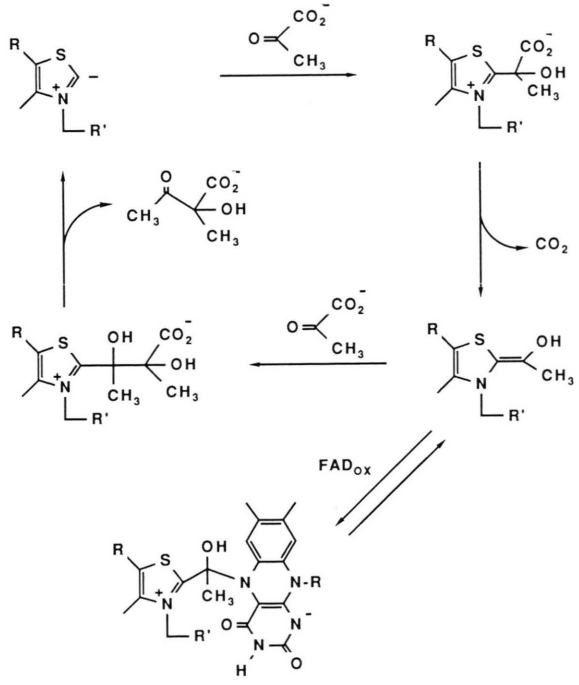

Fig. 2. The likely catalytic cycle of acetolactate synthase for the homologous condensation of two pyruvates.

phate. At this point the normal catalytic sequence would be addition of a second molecule of pyruvate to form an acetolactyl-adduct (or addition of ketobutyrate in the heterologous reaction). However, ALS II appears to digress somewhat from the catalytic sequence at this point, as quenching of flavin absorbance observed upon initiation of the enzymic reaction may suggest attack on the isoalloxazine ring of FAD by the hydroxyethyl intermediate [22]. This possibility seems attractive, as acetolactate synthase appears to have evolved from the redox active flavoprotein pyruvate oxidase, or at least a similar oxidative enzyme [23]. In any case, attack of the flavin would be a reversible side step in the catalytic cycle, and would be consistent with the slightly faster rate observed (about 10%) with the reduced form of the enzyme. Release of acetolactate (or acetohydroxybutyrate) would regenerate the free thiazole ring and complete the catalytic cycle. Direct evidence for the hydroxyethyl intermediate of the ALS II reaction has been obtained by chemical quench with C-1-, C-2-, or C-3-labelled [^{14}C]pyruvate or [^{14}C]thiamine pyrophosphate [24]. Addition of the first pyruvate to ALS II is highly committed, as rather small carbon isotope effects are observed for the decarboxylation of C-1 [^{13}C]pyruvate [25]. Further, even at low pyruvate concentrations the reaction rate is directly proportional to substrate concentration, despite the fact that two molecules of pyruvate are involved in the reaction [9, 10]. Thus, the saturation and inhibition kinetics are likely to be dominated by the addition of the second pyruvate (or ketobutyrate for the heterologous condensation).

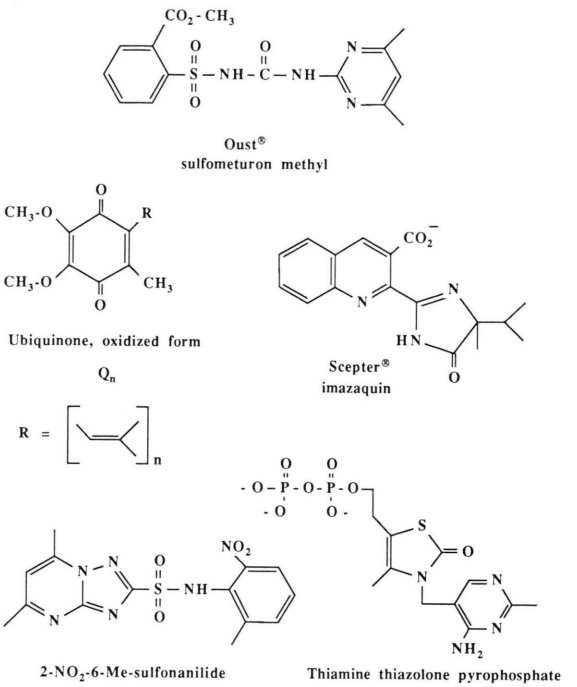

Fig. 3. Various inhibitors of acetolactate synthase.

Inhibitors of Acetolactate Synthase

Several major classes of herbicides inhibit acetolactate synthase as their mode of action, DuPont's sulfonylurea herbicides [1–5], American Cyanamid's imidazolinone herbicides [5–7], and Dow's triazolo pyrimidine or sulfonanilide herbicides [5]. The structures of representatives of each class, sulfometuron methyl, imazaquin, and 1,2,4-triazolo-(1,5-a)-2,4-dimethyl-3-(N-sulfonyl-(2-nitro-6-methylaniline))-1,5-pyrimidine (2-NO_2-6-Me-sulfonanilide), respectively, are illustrated in Fig. 3. Each of these compounds are potent, time-dependent inhibitors of ALS II. All three are biphasic in their interaction, in that there is an initial, rapidly formed complex that slowly converts to a less readily reversible form of inhibition. Sulfometuron methyl and 2-NO_2-6-Me-sulfonanilide are nearly, but not completely, competitive with pyruvate in their initial interaction, while imazaquin is more nearly non-competitive [26]. As the slowly reversible inhibition is not observed in the absence of pyruvate, these inhibitors presumably bind to the enzyme after the addition of the first pyruvate, and sulfometuron methyl and 2-NO_2-6-Me-sulfonanilide, but not imazaquin, interfere with binding of the second pyruvate. The slow increase in inhibition does not result from displacement of thiamine pyrophosphate or FAD from the enzyme by sulfometuron methyl. The rate of exchange of ^{14}C-labelled, enzyme-bound thiamine pyrophosphate with exogenous cofactor is actually retarded by sulfometuron methyl, and extended incubation of the enzyme with the herbicide does not reduce the stoichiometry of bound cofactor [26, 27]. Sulfometuron methyl perturbs the flavin absorption spectrum under turnover or non-turnover conditions [22]. It also has a less dramatic effect on the

quenching of flavin absorbance associated with the initiation of the enzymic reaction than it does on the net rate of the enzymic reaction. The herbicide appears to alter the "equilibrium" of the side reaction illustrated in Fig. 2, such that less of the hydroxyethyl intermediate adds to flavin, by about a factor of two. Sulfometuron methyl and 2-NO_2-6-Me-sulfonanilide appear to bind most tightly to the hydroxyethyl-intermediate form of the enzyme, while imazaquin binds most tightly to both the hydroxyethyl and acetolactyl forms of the enzyme. The herbicides interact most tightly, although not exclusively, with the enzyme in mid-catalytic stride.

This rather unusual mode of inhibition, coupled with the lack of an obvious binding site for these herbicides (not the thiamine pyrophosphate, FAD, pyruvate, or ketobutyrate sites) posed an enzymological dilemma in explaining the molecular details of these interactions. Fortunately, Grabau and Cronan [23] had determined the gene sequence for *poxB,* that encodes an enzyme mechanistically related to acetolactate synthase, pyruvate oxidase. Pyruvate oxidase has substantial sequence homology to acetolactate synthase, indicating a common evolutionary heritage for these two enzymes. Unlike acetolactate synthase, pyruvate oxidase uses its essential FAD cofactor in a more conventional way, for redox chemistry. This perhaps explains the origin of the FAD in acetolactate synthase. But, of greater interest, relative to the inhibition of acetolactate synthase by herbicides, pyruvate oxidase also binds one additional cofactor no longer required by acetolactate synthase, ubiquinone-40 or Q_8 (Fig. 3, with $n = 8$ [28]). The interaction of Q_8 with pyruvate oxidase is also tightest in the presence of pyruvate, similar to the effect that substrate has on the interaction of herbicides with acetolactate synthase. It would appear that a residual quinone site on acetolactate synthase is the mysterious site of accommodation for the various herbicides. Further, this would help explain the observation that although reduction of the FAD of ALS II has little effect on the rate of the enzymic reaction it has a rather dramatic effect on the enzyme's sensitivity to herbicides, reducing its sensitivity approximately 4-fold. Although sulfometuron methyl, imazaquin, and 2-NO_2-6-Me-sulfonanilide, have no effect on pyruvate oxidase, water-soluble quinones such as Q_0 and Q_1 ($n = 0$ and 1, respectively, Fig. 3) are good inhibitors of ALS I, ALS II, and ALS III [26]. Similar to the effects of herbicides, Q_0 is a time-dependent inhibitor of ALS I and ALS III. Further, Q_0 can displace radiolabelled sulfometuron methyl from ALS II, demonstrating that its binding site overlaps that of the herbicide. It would appear that the herbicide site of acetolactate synthase is somehow derived from the quinone site of pyruvate oxidase. Since the mechanism of pyruvate oxidase involves the transfer of electrons from pyruvate to flavin, and then to quinone, an ordered orientation is likely with quinone in the outermost position. The effect that sulfometuron methyl has on thiamine pyrophosphate release, suggests that a similar binding arrangement would exist for acetolactate synthase, and that the herbicides would inhibit the enzyme by preventing product release and/or completion of the catalytic cycle. As these inhibitors would bind in large part, or exclusively for some herbicides, outside of the enzyme's active site, these inhibitors could be referred to as "extraneous site inhibitors".

By way of contrast, thiamine thiazolone pyrophosphate (Fig. 3) is a mechanism based inhibitor of acetolactate synthase [29]. It is an exceptionally potent inhibitor by virtue of its structural similarity to the ene-amine form of the hydroxyethyl-thiamine pyrophosphate reaction intermediate (Fig. 2). Unlike the sulfonylurea herbicides, thiamine thiazolone pyrophosphate does not require the presence of pyruvate to form a nearly irreversible complex with ALS II, but does require a divalent metal. With the apo-enzyme the cofactor analog inactivates the enzyme in a pseudo-first order fashion, without a kinetically significant initial, reversible complex. If the enzyme is first activated by thiamine pyrophosphate and magnesium, however, the enzyme is inhibited by the analog only as rapidly as the cofactor dissociates (a half-time of about 5 min). Thiamine thiazolone monophosphate, or the unphosphorylated cofactor analog, does not exhibit potent inhibition of ALS II. As the pyrophosphate cannot get into microbes or plants, and thiamine thiazolone is not pyrophosphorylated *in vivo,* thiamine thiazolone pyrophosphate is of no value as a potential herbicide or bacteriostat, despite the fact that it is a much better inhibitor of acetolactate synthase than any of the herbicides.

Branched-Chain Amino Acid Biosynthesis

Selection of acetolactate synthase as a herbicidal target appears to have occurred at least in part due to its unusual binding site with a rather high degree of structural promiscuity. What are the prospects of the other enzymes of branched-chain amino acid biosynthesis (Fig. 4) for the design of herbicides? One way to assess the physiological consequences of completely inhibiting an enzyme is to select for a mutant that lacks that enzyme. A few auxotrophs have been selected in higher plants for branched-chain amino acids. Tobacco cells have been selected that are auxotrophic for isoleucine [30–32], and tobacco and carrot cells have been selected that are auxotrophic for both isoleucine and valine [32, 33]. These auxotrophs are deficient in threonine deaminase (step *1I*, Fig. 4) and dihydroxyacid dehydratase (step *3C*, Fig. 4), respectively. As valine can be transaminated to ketoisovalerate, it will allow for the biosynthesis of leucine in the absence of any of the three common enzymes of the branched-chain amino acid pathway (steps *1C*, *2C*, and *3C*, Fig. 4). Tobacco plants have been successfully obtained that are auxotrophic for isoleucine from the mutant cells [31]. Leucine requiring plant cells have also been obtained, but the missing enzyme has yet to be identified (steps *1L*, *2L*, or *3L*, Fig. 4) [34, 35]. The auxotrophic cells appear to exhibit no acute toxicity upon starvation for branched-chain amino acids, suggesting that inhibition of these enzymes (threonine deaminase and dihydroxyacid dehydratase) would not quickly lead to the death of a plant. Unfortunately, for the first two of the three

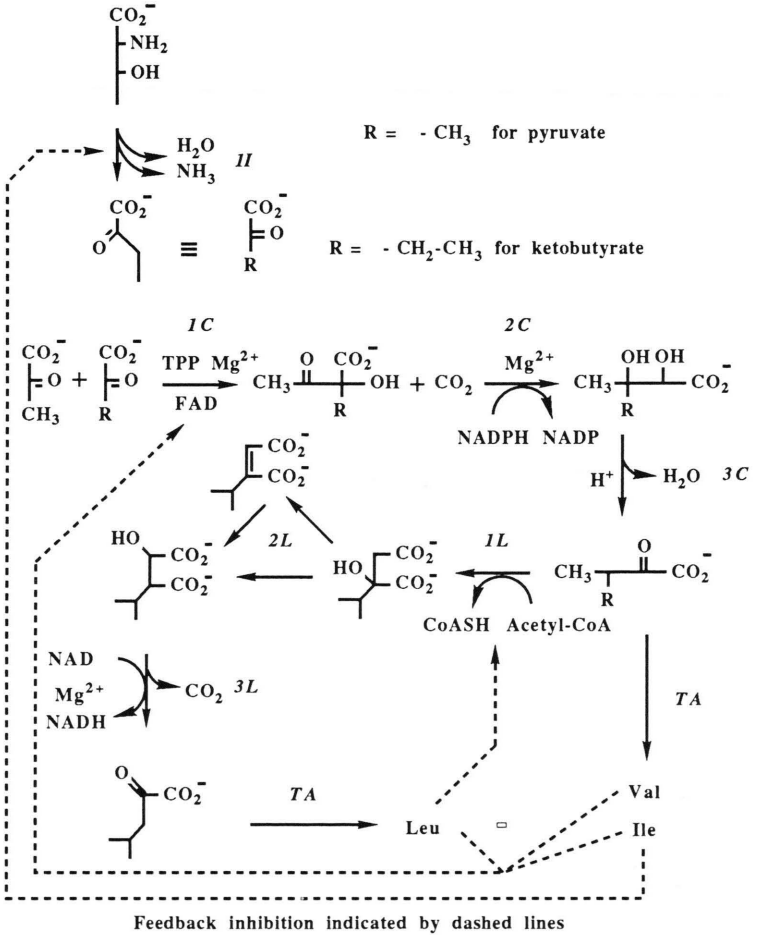

Fig. 4. The biosynthetic pathway for branched-chain amino acids. Common steps in the biosynthesis of all three branched-chain amino acids are designated by *1C*, acetolactate synthase; *2C*, ketol-acid reductoisomerase, *3C*, dihydroxyacid dehydratase, and *TA*, branched-chain amino acid transaminase; the step specific to isoleucine biosynthesis by *1I*, threonine deaminase (or threonine dehydratase); the steps specific to leucine biosynthesis by *1L*, 2-isopropylmalate synthase; *2L*, 2-isopropylmalate dehydratase (or isopropylmalate isomerase), *3L*, 3-isopropylmalate dehydrogenase. Feedback inhibition of the enzymes by the end products of the pathway, branched-chain amino acids, is indicated in the figure by dashed arrows.

common enzymes of branched-chain amino acid biosynthesis, acetolactate synthase (step *1C*) and ketol-acid reductoisomerase (step *2C*) no mutants have been identified. It would be particularly interesting to see if a mutant lacking acetolactate synthase would have a phenotype that exhibited acute toxicity upon starvation for branched-chain amino acids.

In bacteria, unlike plants, inhibition of acetolactate synthase causes stasis rather than lethality. This matches the phenotype of mutants lacking acetolactate synthase [36]. However, the potency of growth inhibition of bacteria by the sulfonylurea herbicides seems to be due in large part to the toxicity of α-ketobutyrate build-up rather than simple starvation [37–42]. It is unclear how potent these compounds would be as bacteriostats divorced from the secondary effects of metabolic blockade. If substrate build-up and its associated toxicity is important to the effects seen in bacteria and plants, then there is no reason to expect equivalent effects from the inhibition of different enzymes in the same pathway.

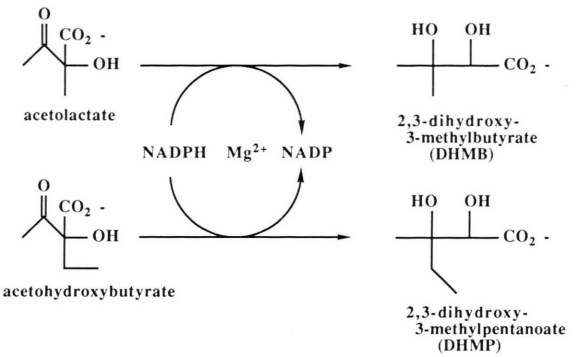

Fig. 5. The physiological reactions catalyzed by ketol-acid reductoisomerase.

Ketol-Acid Reductoisomerase

Of the three common enzymes of branched-chain amino acid biosynthesis, ketol-acid reductoisomerase remains as an enticing possibility. Inhibition of acetolactate synthase is known to give rise to herbicidal effects. Mutants lacking dihydroxyacid dehydratase seem to survive starvation for branched-chain amino acids without exhibiting acute toxicity [35]. With this in mind we set out to design inhibitors for the reductoisomerase. The physiological reactions catalyzed by the reductoisomerase are illustrated in Fig. 5. This is a rather unusual reaction in that alkyl migration takes place with magnesium and NADPH as the only cofactors. The reaction is likely to proceed in two steps, with alkyl migration preceding a simple dehydrogenase reaction. There are no deuterium isotope effects with either acetolactate or acetohydroxybutyrate and NADP^{2}H [43, 44]. Similarly, no deuterium isotope effects are observed on the reverse reaction with [α-^{2}H]2,3-dihydroxy-3-methylbutyrate or [α-^{2}H]2,3-dihydroxy-3-methylpentanoate and NADP. However, if the putative α-keto reaction intermediates (Fig. 6) and NADP^{2}H are used, substantial deuterium isotope effects are observed. Rearrangement independent of the dehydrogenase reaction can also be observed by starting with the α-keto reaction intermediates in the presence of NADP and magnesium. This rate is approximately one third the rate of k_{cat} in the reverse overall reaction. Also, although when acetolactate or

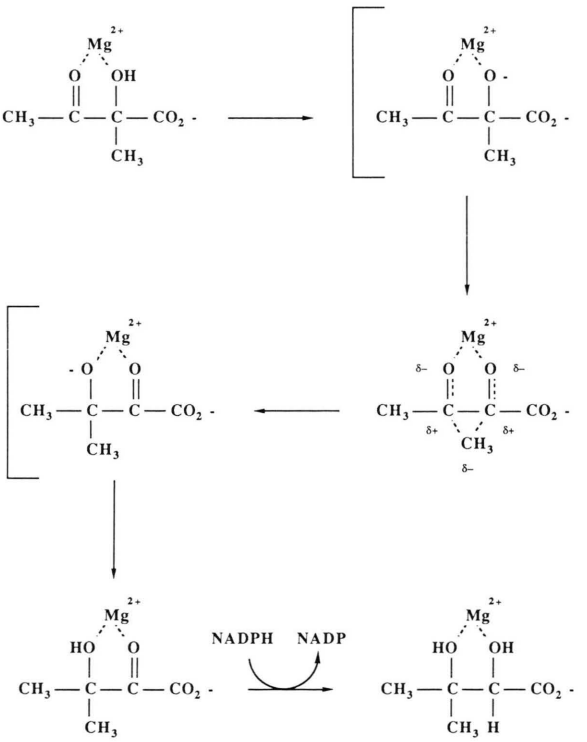

Fig. 6. A detailed reaction mechanism for ketol-acid reductoisomerase.

acetohydroxybutyrate are used as substrate only magnesium will satisfy the metal requirement, when the α-keto reaction intermediates are synthesized and used as substrates for the dehydrogenase reaction either magnesium or manganese will support the reaction [45]. A detailed mechanism for the ketol-acid reductoisomerase is proposed in Fig. 6. The rearrangement reaction is enclosed in brackets. Coordination of the α-hydroxy ketoacid would lower the pK of the hydroxyl to facilitate formation of the alkoxide. This would then proceed through a transition state with partial positive charge on the two oxygen-bearing carbons, partial negative charge on both of the oxygens, partial negative charge on the migrating alkyl moiety, and trigonal character at both oxygen-bearing carbons. The alkoxide of the α-keto acid could then be protonated prior to the hydride transfer step. Coordination of the carbonyl by metal would polarize it to facilitate its reduction.

As the α-keto acid intermediate shows deuterium isotope effects, it is not a tightly-bound intermediate. It must be reversibly bound up to the point of hydride transfer. Thus, in the design of a potent inhibitor for this enzyme, the rearrangement transition state needs to be considered. One structure that would have similarity to the rearrangement transition state is an oxalyl hydroxamate [16]. Although, in the absence of metal, the inhibitor would resemble the α-keto acid intermediate, when chelated to metal the inhibitor would be predominantly in the iminol form as illustrated for N-isopropyl oxalylhydroxamate (IpOHA) in Fig. 7. In its iminol form the nitrogen would have positive charge, both oxygens could have negative charge, and the positions equivalent to the oxygen-bearing carbons of the transition state would be trigonal. Also, to the extent that metal chelation is important, the oxalyl hydroxamate is a good chelator. Recently, another compound that has structural similarity to the rearrangement transition state, dimethylphosphinoyl-2-hydroxyacetic acid (Hoe 704, Fig. 7), has been reported to inhibit (K_i = 0.8 μM) ketol-acid reductoisomerase [46]. Both compounds are time-dependent inhibitors of the *E. coli* ketol-acid reductoisomerase [16, 46]. IpOHA is an exceptionally potent inhibitor with an association rate of 5.9×10^4 M^{-1} s^{-1}, a half-time for release in the presence of Mg^{2+} and NADPH of 6 days, and an overall K_i of 22 pM. The enzyme-

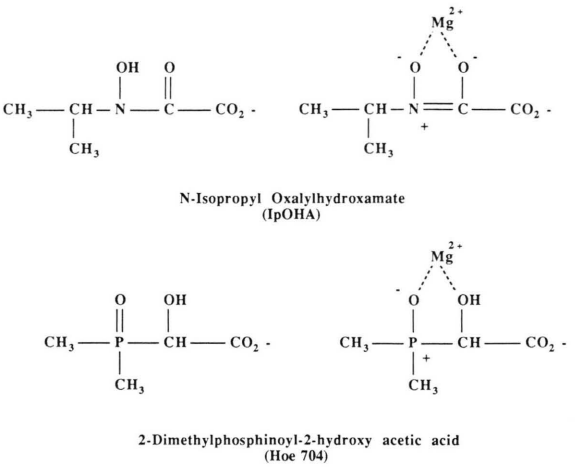

Fig. 7. The structures of IpOHA and Hoe 704.

IpOHA complex is far more labile under conditions that do not favor the rearrangement reaction, consistent with the expected behavior if IpOHA is an analog of the rearrangement transition state. In the absence of metal a tight complex does not form between the reductoisomerase and IpOHA. In the presence of Mg^{2+} alone IpOHA is released with a half-time of 2 h, whereas in the presence of Mn^{2+} its rate of release is too rapid to measure. With Mg^{2+} and NADP, it has a half-time for release of 28 h, whereas substitution of Mn^{2+} for Mg^{2+} lowers the half-time for release to less than 1 h.

IpOHA [16], but not Hoe 704 [46], is a potent inhibitor of the growth of *E. coli*. At 100 nM, IpOHA will prevent the growth of *E. coli* for up to 30 h on minimal media. The inhibition is selective as 1 mM IpOHA does not inhibit the growth of *E. coli* in the presence of branched-chain amino acids, and IpOHA does not inhibit the two other common enzymes of branched-chain amino acid biosynthesis, acetolactate synthase or dihydroxyacid dehydratase. However, both IpOHA and Hoe 704 are only modest herbicides [16, 46]. Although 0.5 mM IpOHA will kill *Arabidopsis thaliana* in soft agar with substantial protection afforded by branched-chain amino acids, lower concentrations are ineffective. These results, together with the relatively high application rates required for Hoe 704, suggest that although inhibition of ketol-acid reductoisomerase can be herbicidal, it is not likely to be as effective as inhibition of acetolactate synthase.

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