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Oxalyl Hydroxamates as Reaction-Intermediate Analogues for Ketol-Acid Reductoisomerase

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ABSTRACT: *N*-Hydroxy-*N*-isopropyloxamate (IpOHA) is an exceptionally potent inhibitor of the *Escherichia coli* ketol-acid reductoisomerase. In the presence of Mg^{2+} or Mn^{2+}, IpOHA inhibits the enzyme in a time-dependent manner, forming a nearly irreversible complex. Nucleotide, which is essential for catalysis, greatly enhances the binding of IpOHA by the reductoisomerase, with NADPH (normally present during the enzyme's rearrangement step, i.e., conversion of a β-keto acid into an α-keto acid, in either the forward or reverse physiological reactions) being more effective than NADP. In the presence of Mg^{2+} and NADPH, IpOHA appears to bind to the enzyme in a two-step mechanism, with an initial inhibition constant of 160 nM and a maximum rate of formation of the tight, slowly reversible complex of 0.57 min^{-1} (values that give an association rate of IpOHA, at low concentration, of 5.9×10^4 M^{-1} s^{-1}). The rate of exchange of [^{14}C]IpOHA from an enzyme-[^{14}C]IpOHA-Mg^{2+}-NADPH complex with exogenous, unlabeled IpOHA has a half-time of 6 days (150 h). This dissociation rate (1.3×10^{-6} s^{-1}) and the association rate determined by inactivation kinetics define an overall dissociation constant of 22 pM. By contrast, in the presence of Mn^{2+} and NADPH, the corresponding association and dissociation rates for IpOHA are 8.2×10^4 M^{-1} s^{-1} and 3.2×10^{-6} s^{-1} (half-time = 2.5 days), respectively, which define an overall dissociation constant of 38 pM. In the presence of NADP or in the absence of nucleotide (both in the presence of Mg^{2+}), the enzyme-IpOHA complex is far more labile, with dissociation half-times of 28 and 2 h, respectively. In the absence of Mg^{2+} or Mn^{2+}, IpOHA does not exhibit time-dependent inhibition of the reductoisomerase. These results parallel the effects that divalent metals and nucleotide have on the rearrangement step of this enzyme, which is greater than 3-fold more rapid in the presence of NADPH than in the presence of NADP and absolutely dependent on Mg^{2+}, and strongly suggest that IpOHA is a potent inhibitor of ketol-acid reductoisomerase by virtue of its structural similarity to the rearrangement transition state.

Ketol-acid reductoisomerase (EC 1.1.1.86), the second common enzyme in the biosynthetic pathway for branched-chain amino acids, catalyzes the reversible conversion of a β-keto-α-hydroxy-α-alkyl acid to an α,β-dihydroxy-β-alkyl acid with concomitant oxidation of NADPH[1] to NADP. The physiological substrates and products are α-acetolactate and 2,3-dihydroxy-3-methylbutyrate (DHMB) for valine and leucine biosynthesis and α-aceto-α-hydroxybutyrate and 2,3-dihydroxy-3-methylpentanoate (DHMP) for isoleucine biosynthesis, respectively. The reaction likely proceeds in two steps with rearrangement of a β-keto-α-hydroxy-α-alkyl acid to an α-keto-β-hydroxy-β-alkyl acid reaction intermediate [3-hydroxy-3-methyl-2-oxobutyrate (HMOB) or 3-hydroxy-3-methyl-2-oxopentanoate (HMOP) for leucine and valine biosynthesis or isoleucine biosynthesis, respectively] followed by hydride transfer. Although attempts to isolate the α-keto acid reaction intermediate have been unsuccessful (Arfin & Umbarger, 1969; Chunduru et al., 1989), the two steps of the enzymic reaction (rearrangement and hydride transfer) can be distinguished on the basis of their metal ion requirements. The reductoisomerase will reduce the putative α-keto acid intermediate (HMOB or HMOP) with NADPH, and this reduction requires either Mg^{2+} or Mn^{2+}, in contrast to the oxidation of NADPH by a β-keto acid (acetolactate or acetohydroxybutyrate) which has an absolute specificity for Mg^{2+} (Chunduru et al., 1989). This is a rather unusual enzymic

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[1] Abbreviations: IpOHA, *N*-hydroxy-*N*-isopropyloxamate; NADP, oxidized nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; DHMB, 2,3-dihydroxy-3-methylbutyrate; DHMP, 2,3-dihydroxy-3-methylpentanoate; HMOB, 3-hydroxy-3-methyl-2-oxobutyrate; HMOP, 3-hydroxy-3-methyl-2-oxopentanoate; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid.

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reaction in that cobalamin, or other cofactors, is not required for the alkyl rearrangement reaction.

Recently, a possible analogue of the rearrangement transition state, 2-(dimethylphosphinoyl)-2-hydroxyacetic acid (Hoe 704), has been reported to be an exceptionally potent inhibitor of the reductoisomerase (Schultz et al., 1988). Although Hoe 704 was not specifically designed as an inhibitor of ketol-acid reductoisomerase, selective inhibition of this enzyme forms the basis of its herbicidal and bacteriostatic activities (Schultz et al., 1988). An increased general interest in the biosynthetic pathway for branched-chain amino acids has ensued with the identification of the first common enzyme, acetolactate synthase (EC 4.1.3.18), as the site of action of several new classes of commercial herbicides (Chaleff & Mauvais, 1984; Falco & Dumas, 1985; LaRossa & Schloss, 1984; Ray, 1984; Hawkes et al., 1989; Muhitch et al., 1987; Shaner et al., 1984; Schloss et al., 1988). These selective inhibitors of acetolactate synthase and ketol-acid reductoisomerase are extremely useful in studying the basic physiology and regulation of the biosynthetic branched-chain amino acid pathway in plants and microbes (LaRossa & Falco, 1984; LaRossa & Van Dyk, 1987; LaRossa et al., 1987; Van Dyk & LaRossa, 1986, 1987; Van Dyk et al., 1987; Rhodes et al., 1987; Schultz et al., 1988).

In this paper, we describe novel analogues of the transition state for the rearrangement step of ketol-acid reductoisomerase. The details of the interaction of one of these compounds, IpOHA, with the *Escherichia coli* enzyme, and features of the interaction that would suggest that this compound behaves as an analogue of the transition state for the rearrangement step, are presented here.

Materials and Methods

Materials. *N*-Isopropylhydroxylamine hydrochloride, *N*-methylhydroxylamine hydrochloride, benzaldehyde oxime, and sodium cyanoborohydride were obtained from Aldrich. [^{14}C]Oxalic acid was obtained from New England Nuclear. Hydroxylamine hydrochloride was obtained from Fischer. NADP, NADPH, and acetaldehyde oxime were obtained from Sigma. Magnesium chloride and manganese chloride, obtained from Alfa, were ultra puratronic grade. *N*-Ethylhydroxylamine and *N*-benzylhydroxylamine were prepared by reduction of their corresponding oximes with sodium cyanoborohydride by the method of Borch et al. (1971).

α-Keto Acids. HMOB and HMOP were prepared by a published procedure (Kiritani & Wagner, 1970) and purified by column chromatography on AG1-X8 (2.5 × 61 cm column, chloride form, flow rate 409 mL/h, 23-mL fractions collected). HMOB (2.6 mmol) was applied to the column as either the free acid or the sodium salt and eluted isocratically with 50 mM HCl (recovered in fractions 29–37 or 45–49, respectively). HMOP (1.6 mmol) was applied to the column as the sodium salt and eluted with a 2-L linear gradient of 0–1 M KCl (recovered in fractions 57–63). Analytically pure solutions (>95%) of HMOB and HMOP, by the criteria of enzymic assay with ketol-acid reductoisomerase, equivalent weight by titration with KOH, and ^{1}H NMR, were obtained after chromatography. Fractions containing HMOB were acidified by addition of HCl to pH 0.5, lyophilized, suspended in a small volume (50 mL) of diethyl ether, filtered, and then dried under N_2. HMOP, by contrast, could be efficiently extracted with diethyl ether (four times with 100-mL portions) directly after acidification of pooled chromatographic fractions with HCl. It is interesting to note that although the HMOP should be a racemic mixture, the enzyme is able to reduce both stereoisomers (>95%).

β-Keto Acids. (2*S*)-2-Hydroxy-2-methyl-3-oxobutyrate [(2*S*)-α-acetolactate] and (2*S*)-2-hydroxy-2-ethyl-3-oxobutyrate [(2*S*)-α-aceto-α-hydroxybutyrate] were prepared enzymically from pyruvate or an equimolar mixture of pyruvate and α-ketobutyrate, respectively, by use of *Salmonella typhimurium* acetolactate synthase II, Mg^{2+}, thiamin pyrophosphate, and FAD (Schloss et al., 1985; Abell et al., 1985). Acetohydroxybutyrate and acetolactate could be stored at −70 °C in 100 mM Tris-HCl buffer, pH 8.0, without noticeable decomposition for at least 1 year. The concentrations of stock solutions of acetolactate and acetohydroxybutyrate were determined enzymically before and after use to ensure that negligible decomposition (<5%) had occurred and maintained at 4 °C to minimize losses (half-time for decomposition at 25 °C is approximately 5 days, at pH 8.2). Stock solutions of racemic acetolactate could be prepared from 2-acetoxy-2-methyl-3-oxobutyric acid ethyl ester (Aldrich Chemical Co.) by suspending 1.032 g (5.0 mmol) to a final volume of 10 mL with 0.975 M NaOH and mixing vigorously until a neutral solution was obtained.

Synthesis of N-Hydroxy-N-isopropyloxamate. *N*-Hydroxy-*N*-isopropyloxamate (IpOHA) was prepared by alkaline hydrolysis of the methyl ester of *N*-hydroxy-*N*-isopropyloxamate. The methyl ester was prepared by dropwise addition over 1.5 h of 50 mL of a chloroform solution of *N*-isopropylhydroxylamine hydrochloride (60 mmol) and triethylamine (120 mmol) to a stirring 250-mL solution of methyloxalyl chloride (60 mmol) in diethyl ether at 4 °C. After being stirred 12 h at room temperature, the solution was filtered to remove precipitated salt, and the diethyl ether was removed under reduced pressure to afford a yellow-orange oil. The colorless methyl *N*-hydroxy-*N*-isopropyloxamate was collected upon distillation (bp 98–100 °C/0.25 mmHg), resulting in a final yield of 54%. The material in $CHCl_3$ consisted of two conformers (>100 mM), which yielded one form upon dilution (<10 mM), indicative of a monomer–dimer equilibrium: ^{1}H NMR ($CDCl_3$) δ 1.34 (d, 2.6 H), 1.44 (d, 3.4 H), 3.95 (s, 1.3 H), 4.1 (s, 1.72 H), 4.61 (q, 0.6 H), 4.71 (q, 0.4 H), 8.35 (br, 1 H). The potassium salt was obtained from hydrolysis of the ester by addition of 1 equiv of KOH to an aqueous 100 mM solution of ester. The extent of the hydrolysis reaction was monitored by ^{1}H NMR and the disappearance of the singlet resonances at 4.61 and 4.71 ppm.

Synthesis of N-Hydroxy-N-ethyloxamate. *N*-Ethylhydroxylamine was prepared by the $NaBH_3CN$ reduction of acetaldehyde oxime according to the procedure of Borch et al. (1971). The methyl ester of *N*-hydroxy-*N*-ethyloxamate was prepared by the same procedure described for *N*-hydroxy-*N*-isopropyloxamate except that 1 equiv of triethylamine was added. The colorless methyl *N*-hydroxy-*N*-ethyloxamate was collected upon distillation (bp 89–90 °C/0.15 mmHg), resulting in a final yield of 41%. The ^{1}H NMR spectrum in $CDCl_3$ corresponded to methyl *N*-hydroxy-*N*-ethyloxamate: δ 1.40 (t, 3 H), 3.6 (s, 3 H), 3.69 (q, 2 H). The potassium salt was obtained by hydrolysis of the ester.

Synthesis of N-Hydroxy-N-methyloxamate and N-Hydroxy-N-benzyloxamate. *N*-Benzylhydroxylamine was prepared by $NaBH_3CN$ reduction of benzaldehyde oxime according to the procedure of Borch et al. (1971). The methyl ester of *N*-hydroxy-*N*-benzyloxamate was prepared by a procedure similar to that described for *N*-hydroxy-*N*-isopropyloxamate, resulting in a cream colored solid (mp 102–104 °C). The proton NMR spectrum in $CDCl_3$ was consistent with that expected for *N*-hydroxy-*N*-benzyloxamate. *N*-Hydroxy-*N*-methyloxamate was prepared in a similar fashion,

and vacuum distillation gave a colorless oil (bp 92–94 °C/0.12 mmHg). The potassium salts of the two oxalyl hydroxamates were obtained by alkaline hydrolysis of the methyl esters.

Synthesis of N-Hydroxy-N-isopropyl[^{14}C]oxamate. [^{14}C]Oxalic acid (0.056 mmol, 4.1 mCi/mmol, DuPont-New England Nuclear), *N*-isopropylhydroxylamine (0.10 mmol), and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (0.058 mmol) in 8 mL of water were stirred for 15 h, and the pH of the solution was maintained between 4.8 and 5.3 by addition of 1 N HCl. The compound was purified by ion-exchange chromatography on an AG1-X8 column (bicarbonate form, 1.8 cm × 30 cm), and [^{14}C]IpOHA was eluted with an ammonium bicarbonate gradient (700 mL, 0–0.5 M, pH 7.6) resulting in a 24% yield, on the basis of counts recovered. The elution position corresponded to that of authentic, unlabeled IpOHA and was red upon addition of an aliquot to 1% $FeCl_3$ in 1 N HCl (a positive test for hydroxamates).

Purification of Ketol-Acid Reductoisomerase. The purification procedure was similar to that previously employed for acetolactate synthase isozyme II (Schloss et al., 1985) and isozyme III (Barak et al., 1988). *Escherichia coli* bearing plasmids encoding acetolactate synthase isozyme I (Newman et al., 1982), II (Blazey et al., 1981), or III (Lago et al., 1985) were used to purify both acetolactate synthase and ketol-acid reductoisomerase. As the level of the reductoisomerase expressed in *E. coli* is induced by acetolactate, these clones were enriched in the enzyme relative to wild-type cells (Arfin et al., 1969). The original ammonium sulfate steps of the acetolactate synthase purification protocol were modified to optimize the recovery of both synthase and reductoisomerase. The following protocol is representative. All steps were performed at 4 °C. A frozen cell paste (452 g) of *E. coli* K-12 MF-2361 (Newman et al., 1982) was suspended in 2 volumes of 0.1 M Tris-HCl (pH = p*K*, 8.1 at 25 °C), 10 mM EDTA, 1 mM DTT, and 0.1 mM FAD, and after thawing, the suspension was sonicated four times for 5-min intervals at 70% duty cycle (Heat Systems-Ultrasonics, Inc.). The suspension was centrifuged 5 h at 11 000 rpm to remove cellular debris. Ammonium sulfate (291 g/L) was added to the supernatant (42.5 g of protein; 1270 units) to yield 55% saturation, stirred 2.0 h, and then centrifuged 30 min in a Beckman GSA rotor at 12 000 rpm. The supernatant was adjusted with ammonium sulfate (92 g/L) to 65% saturation, stirred 2 h, and then centrifuged 30 min at 12 000 rpm in a GSA rotor. The pellet (6.59 g; 680 units) was resuspended in a minimal volume of buffer A [50 mM Tris-HCl (pH 8.1 at 25 °C), 1 mM $MgSO_4$, and 1 mM DTT] and applied to a 5 × 107 cm column of Sephacryl S-200 preequilibrated in buffer A. Fractions containing enzymic activity (38–50, 2.59 g, 576 units) were combined and applied to a DEAE-650M Fractogel column (2.4 × 50 cm) preequilibrated in buffer A. The DEAE Fractogel column was eluted with a 2-L linear gradient composed of 1 L of buffer A and 1 L of 0.4 M KCl in buffer A. Fractions containing enzymic activity (73–75, 0.38 g, 449 units) were pooled, concentrated to 20 mL, and applied to the S-200 column, preequilibrated with buffer A, and eluted with buffer A. The fractions from the S-200 column containing enzymic activity (0.2 g, 380 units) were combined and concentrated by pressure dialysis to a final concentration of 22–36 mg of protein/mL. A summary of the purification is listed in Table I. The purified protein could be stored frozen indefinitely (>1 year) without noticeable loss of enzymic activity. Increased stability of the reductoisomerase was observed in the presence of magnesium, which was included in all steps of the purification after the ammonium sulfate fractionation. The subunit molecular weight obtained from SDS–PAGE (8–25% gradient) was 55 000, which is nearly equivalent to that predicted by the nucleotide sequence of *ilvC* (54 000) (Wek & Hatfield, 1986). From the same batch of cells, 520 mg of acetolactate synthase isozyme I could be purified from the 0–55% saturation fraction from the ammonium sulfate fractionation step, with a specific activity of 32 units/mg (15% yield), by a protocol similar to that published for isozyme II (Schloss et al., 1985). The difference in specific activity between the acetolactate synthase isozyme I purified here and that reported previously, 26 units/mg (Eoyang & Silverman, 1984) and 34 units/mg (Grimminger & Umbarger, 1979), is probably due to differences in the methods used to determine the protein concentration [biuret in the current protocol and Grimminger and Umbarger (1979) vs the Coomassie blue method (Eoyang & Silverman, 1984)].

Table I: Purification of Ketol-Acid Reductoisomerase from *E. coli* K-12 MF-2361[a]

step	protein (g)	act. (units)	sp act. (units/mg)	yield (%)
extract	42.5	1270	0.030	100
50–65% ammonium sulfate pellet	6.59	680	0.097	53
Sephacryl S-200	2.59	576	0.22	45
DEAE 650M	0.380	449	1.18	35
second Sephacryl S-200	0.200	380	1.90	30

[a] 452 g of cell paste, wet weight.

Assays and Inactivation Kinetics. Purified ketol-acid reductoisomerase concentrations were determined by the biuret method (Lyane, 1957). Enzyme assays were conducted at 25 °C and pH 7.5. A standard reductoisomerase assay contained 100 mM Hepes–NaOH, pH 7.5, 10 mM $MgCl_2$, 200 μM NADPH, and 960 μM acetolactate. The oxidation of NADPH was monitored spectrophotometrically at 340 nm with an extinction coefficient for the reduced nucleotide of 6220 M^{-1} cm^{-1}. ^{1}H NMR was used to monitor the isomerization reaction. The time dependence of the inactivation of ketol-acid reductoisomerase by the oxalyl hydroxamates was examined by addition of enzyme to an assay mixture containing inhibitor or by preincubation of the enzyme with inhibitor for various time periods under assay conditions lacking various components (Schloss, 1989). When inactivation of the enzyme in the presence of Mn^{2+} was examined, HMOB was substituted for acetolactate in assay mixtures.

Stoichiometry of Inhibitor Binding. Enzyme (2.3 μM promoter) was preincubated with various concentrations of *N*-hydroxy-*N*-ethyloxamate (0.0–3.3 μM) for 10 min under assay conditions, except for the omission of acetolactate. An aliquot (10 μL) of the preincubation mixture was added to 1 mL of an assay mixture containing acetolactate, and the initial activity was determined.

Exchange Kinetics. A solution of ketol-acid reductoisomerase (0.2 mL, 0.11 mM promoter) was incubated with 0.21 mM [^{14}C]IpOHA (8.63 × 10^6 cpm/μmol) in 100 mM Hepes–NaOH, pH 7.4, 10 mM $MgCl_2$ or $MnCl_2$ (when present), and 400 μM nucleotide (NADP or NADPH, when present). After 2-h incubation at 25 °C, the enzyme–inhibitor complex was separated from unbound inhibitor by size exclusion chromatography by use of a 1.2 × 54 cm column containing Bio-Gel P-2 resin (Bio-Rad). The column was preequilibrated with 100 mM Hepes–NaOH, 10 mM metal chloride, and 200 μM nucleotide (if present in the incubation solution), eluted at 0.67 mL/min, and 0.67-mL fractions were

Table II: Steady-State Kinetic Parameters[a]

substrate	K_m (μM)	V/E_t (μmol min^{-1} mg^{-1})
acetolactate[b]	14 ± 2	2.1 ± 0.5
acetohydroxybutyrate[b]	2 ± 1	6.8 ± 0.1
HMOB[b]	281 ± 81	1.41 ± 0.07
HMOB[c]	46 ± 7	1.32 ± 0.09
HMOP[b]	207 ● 7	0.59
NADPH[b]	2.0 ± 0.5	2.1 ± 0.5

[a] All assays were run in 100 mM Hepes-NaOH, pH 7.4, containing 10 mM $MgCl_2$ or $MnCl_2$, at 25 °C. [b] 10 mM $MgCl_2$. [c] 10 mM $MnCl_2$.

Table III: Maximum Rate of Different Reactions Catalyzed by Ketol-Acid Reductoisomerase

reaction catalyzed[a]				
substrate	product	metal[b]	nucleotide	k_{cat} (s^{-1})
acetolactate	DHMB	Mg	NADPH[c]	1.8
acetolactate	DHMB	Mn	NADPH[c]	0[e]
acetolactate	HMOB	Mg		0[f]
acetolactate	HMOB		NADP[d]	0[f]
acetolactate	HMOB	Mg	NADP[d]	0[f]
DHMB	acetolactate	Mg	NADP[c]	0.0042
HMOB	acetolactate			0[f]
HMOB	acetolactate	Mg		0[f]
HMOB	acetolactate	Mg	NADP[d]	0.0015

[a] 100 mM Hepes-NaOH, pH 7.5, 1 mM substrate, 25 °C. [b] 10 mM. [c] 200 μM. [d] 1 mM. [e] <0.01 s^{-1}. [f] <0.0005 s^{-1} or $K_{eq} > 100$.

collected. The fractions containing the enzyme–inhibitor complex were pooled (5 mL), and unlabeled IpOHA was added to yield a final concentration of 2 mM. Aliquots (0.5 mL) were periodically passed through the Bio-Gel P-2 column to separate unbound inhibitor, and the fraction of radiolabel remaining in the complex was determined.

Data Processing. The MLAB data modeling program (Knott, 1979) was used to fit the following equations to data:

$$k' = k_4 + \frac{k_3(I/K_i)}{1 + I/K_i + S/K_m} \quad (1)$$

$$A = A_0 e^{-kt} \quad (2)$$

where k' is the first-order rate constant for transition between initial inhibition and final inhibition, k_4 is the rate constant for conversion of the tight complex to the initial complex, k_3 is the maximal rate constant for tight complex formation, I is the inhibitor concentration in the assay, K_i is the concentration of inhibitor giving 50% initial inhibition in enzyme-initiated assays at low substrate concentration, K_m is the protection constant (not necessarily equivalent to the Michaelis constant) for the ketol acid, S, against tight complex formation by I, A is the fraction of ^{14}C remaining in the enzyme–inhibitor complex at time t, A_0 is the fraction of ^{14}C in the complex initially, and k is the exchange rate of ^{14}C in the enzyme–inhibitor complex with free, unlabeled inhibitor [for discussion, see Schloss (1988, 1989)].

Results

Steady-State Kinetics. The steady-state parameters for the forward reaction (DHMB formation) at pH 7.5 are listed in Table II. Maximum rates were obtained at a metal concentration of 10 mM, in contrast to a 5 mM concentration of metal employed in a kinetic study of the enzyme by Chunduru et al. (1989). Relative rates for the overall reactions (forward or reverse) or the isomerization reactions are summarized in Table III. The enzyme will use only Mg^{2+} when acetolactate is the substrate, while reduction of the α-keto acid intermediate HMOB will proceed in the presence of Mg^{2+} or Mn^{2+}, similar

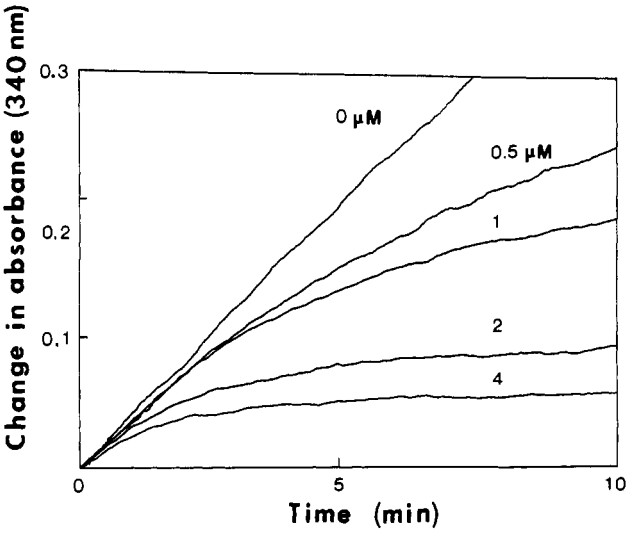

FIGURE 1: Assay time courses for *E. coli* ketol-acid reductoisomerase in the presence of micromolar concentrations of *N*-hydroxy-*N*-isopropyloxamate designated in the figure. The acetolactate concentration was 480 μM ($34K_m$).

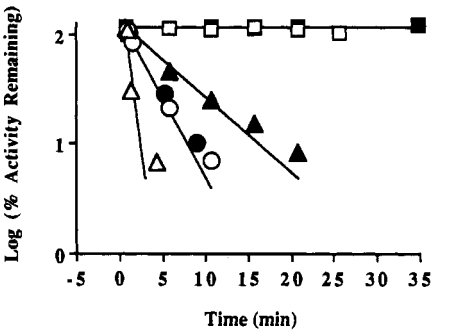

FIGURE 2: Effect of metal and nucleotide on *N*-hydroxy-*N*-isopropyloxamate binding. Enzyme (0.22 μM protomer) and IpOHA (3.3 μM) were preincubated without magnesium chloride or nucleotide (■), without magnesium but with 2 mM NADP (□), with 1 mM magnesium (▲), with 1 mM magnesium and 0.2 mM NADP (○), with 1 mM magnesium and 2 mM NADP (●), and with 1 mM magnesium and 0.2 mM NADPH (△).

to the observation of Chunduru et al. (1989). Rearrangement was observed independently by use of the putative intermediate HMOB, Mg^{2+}, and NADP. The rate of rearrangement was sensitive to the oxidation state of the nucleotide as the rate of conversion of HMOB to acetolactate was 30% that of the back-reaction, Table III. In the back-reaction, oxidation of DHMB to acetolactate, NADPH would presumably be formed prior to the rearrangement of HMOB to acetolactate.

Inhibition by Oxalyl Hydroxamates. Assay of ketol-acid reductoisomerase in the presence of IpOHA yielded nonlinear time courses with initial velocities that varied as a function of IpOHA (Figure 1). The inhibition was virtually complete at longer times if the concentration of IpOHA exceeded the concentration of enzyme employed in the assay. Time-dependent loss of enzyme activity in the presence of IpOHA was further examined by preincubating the enzyme (0.217 μM promoter) with IpOHA (3.3 μM) in assay buffer lacking substrate but containing various concentrations of metal and nucleotide (Figure 2). Aliquots (10 μL) were removed at various times, and the initial activity obtained upon dilution into an assay mixture (1 mL) was determined. In the presence of IpOHA the reductoisomerase lost activity in a pseudo-first-order manner. Magnesium or manganese was required for the time-dependent loss of activity independent of the presence or absence of nucleotide (NADP or NADPH). The

Table IV: Dissociation Rates for the Ketol-Acid Reductoisomerase–IpOHA Ternary and Quaternary Complexes

metal	[IpOHA] (mM)	nucleotide	half-time (h)	k (h^{-1})
Mg	2		2.0	0.35
Mn	2		<1	>0.69
Mg	2	NADP	28	0.025
Mg	0.2	NADP	28	0.024
Mg	2	NADPH	149	0.0046
Mn	2	NADP	<1	>0.69
Mn	2	NADPH	61	0.011

rate of inactivation was most rapid in the presence of both magnesium and NADPH (0.7 ± 0.2 min^{-1}), with the rate in the presence of magnesium and NADP (0.29 ± 0.02 or 0.33 ± 0.02 min^{-1} at 1 mM Mg^{2+} and 2 or 0.2 mM NADP, respectively) being somewhat slower and the rate in the presence of magnesium alone (0.16 ± 0.09 min^{-1}) being slowest (Figure 2).

Assay progress curves of reactions where the IpOHA concentration was varied at different acetolactate concentrations (not shown) were analyzed to obtain the association rate of IpOHA with the reductoisomerase in the presence of saturating nucleotide and divalent metal. The reciprocal of the rate constant for transition between the initial and final levels of inhibition was obtained from the time coordinant of the intersection point of tangential lines drawn to the initial and the final portions of assay progress curves, similar to those shown in Figure 1. Kinetic constants were obtained by fitting eq 1 (Materials and Methods) to data. The rate of inhibition by IpOHA in the presence of Mg^{2+} or Mn^{2+} obeyed saturation kinetics with maximum rates of 0.57 ± 0.13 or 0.59 ± 0.14 min^{-1}, respectively. In the presence of magnesium the initial inhibition constant, K_i, was 160 ± 90 nM, and the second-order rate constant for formation of the tight, enzyme–inhibitor complex was 5.9×10^4 M^{-1} s^{-1}, while in the presence of manganese the respective values were 120 nM and 8.2×10^4 M^{-1} s^{-1}. Binding of acetolactate was competitive with tight complex formation between IpOHA and enzyme in the presence of Mg^{2+} or Mn^{2+}.

The stoichiometry of inhibitor binding to ketol-acid reductoisomerase was obtained by titration of the enzyme with *N*-hydroxy-*N*-ethyloxamate (Morrison, 1969). Inhibition was directly proportional to the concentration of inhibitor, with complete inhibition obtained at 0.77 ± 0.05 mol of inhibitor/mol of protomeric unit (55 000 g). Extended incubation of the enzyme with inhibitor did not alter the results (not shown).

The rate of release of [^{14}C]IpOHA from various complexes with ketol-acid reductoisomerase is summarized in Table IV. In the absence of exogenous, unlabeled IpOHA, no loss of radiolabel from the enzyme complexes was observed over several days of incubation, demonstrating that loss of radiolabel from the complexes was exclusively due to exchange. Further, the rate of exchange was not sensitive to the concentration of exogenous IpOHA employed (Table IV), demonstrating that the exchange process was limited by the slow rate of [^{14}C]-IpOHA release. The [^{14}C]IpOHA released from the enzyme complex was chromatographically equivalent (Materials and Methods) to authentic IpOHA. Enzyme–[^{14}C]IpOHA–Mn^{2+} complexes were far more labile than analogous complexes containing Mg^{2+} in place of Mn^{2+}. In the absence of nucleotide, or in the presence of NADP, Mn^{2+}-containing complexes exchanged [^{14}C]IpOHA too rapidly to determine its rate of exchange accurately. In the presence of NADPH, however, a relatively stable complex with manganese was formed, with an exchange half-time of 61 ± 11 h. Exchange

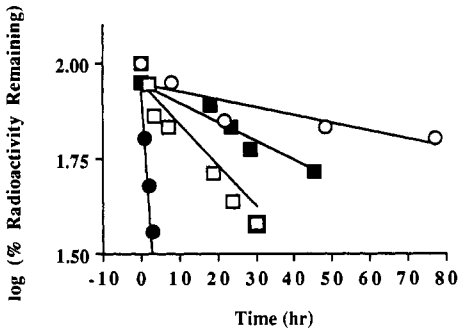

FIGURE 3: Loss of ^{14}C from enzyme-labeled IpOHA complexes expressed as the percent of ^{14}C remaining in the enzyme–inhibitor complex at various time intervals after addition of excess unlabeled inhibitor. The complexes examined include the magnesium ternary complex (●), the NADP–Mg complex (□), the NADPH–Mg complex (○), and the NADPH–Mn quaternary complex (■).

Table V: Effect of *N*-Alkyl Substitutions upon the Rate of Ketol-Acid Reductoisomerase Inactivation[a]

substituent on nitrogen	inactivation rate (s^{-1})
H	0.000 66
methyl	0.000 74
ethyl	0.001 0
isopropyl	0.001 9
benzyl	0.002 9

[a] All assays were run in 100 mM Hepes–NaOH, pH 7.4, containing 200 μM NADPH, 10 mM $MgCl_2$, 96 μM (2*S*)-acetolactate, 200 nM inhibitor, and 6.9 nM enzymic promoter at 25 °C. The rate of inactivation was determined from the reciprocal of the time coordinate of the intersection point of the tangential lines drawn to the initial and final velocities.

of [^{14}C]IpOHA from the enzyme–[^{14}C]IpOHA–Mn^{2+}–NADPH complex and from Mg^{2+}-containing complexes (Figure 3) appeared to follow first-order kinetics. In the absence of nucleotide, the Mg^{2+}-containing complex exchanged with a half-time of 2.0 ± 0.4 h. By contrast, the Mg^{2+}-containing complexes exchanged with half-times of 28 ± 7 and 149 ± 36 h in the presence of NADP and NADPH, respectively. The exchange rates in the presence of NADPH and Mg^{2+} or Mn^{2+} can be used together with the association rates determined by inactivation kinetics (5.9×10^4 and 8.2×10^4 M^{-1} s^{-1}, respectively) to calculate overall dissociation constants of 22 and 38 pM, respectively. Although, in the presence of NADPH, IpOHA binds only somewhat more tightly with Mg^{2+} than with Mn^{2+} (about 2-fold), in the presence of NADP the difference in affinity appears to be much greater (>28-fold, based on relative rates of release).

Comparison of the Relative Potency of Various N-Substituted Oxalyl Hydroxamates. Although not examined in detail, the rate of apparent inactivation of enzyme by several different oxalyl hydroxamates is presented in Table V. These inhibitors differ in the substituent present on the hydroxamate nitrogen. By the criteria of their association rates, more bulky substituents give more potent inhibition.

Inhibition of Ketol-Acid Reductoisomerase in Vivo. IpOHA is a potent bacteriostat. At a concentration of 100 nM, IpOHA prevents the growth of *E. coli* strain M152 for 30 h at 37 °C on minimal media supplemented with 0.2% glucose (Vogel & Bonner, 1956), under conditions where the doubling time was approximately 3 h. Longer term incubation (2 days) ultimately leads to growth of *E. coli* in the presence of IpOHA, indicating the growth effect is due to stasis and not lethality. In the presence of 1 mM valine and isoleucine, IpOHA (up to 1 mM) does not inhibit the growth rate of *E. coli*. As IpOHA (1 mM, up to 10 min) does not inhibit the

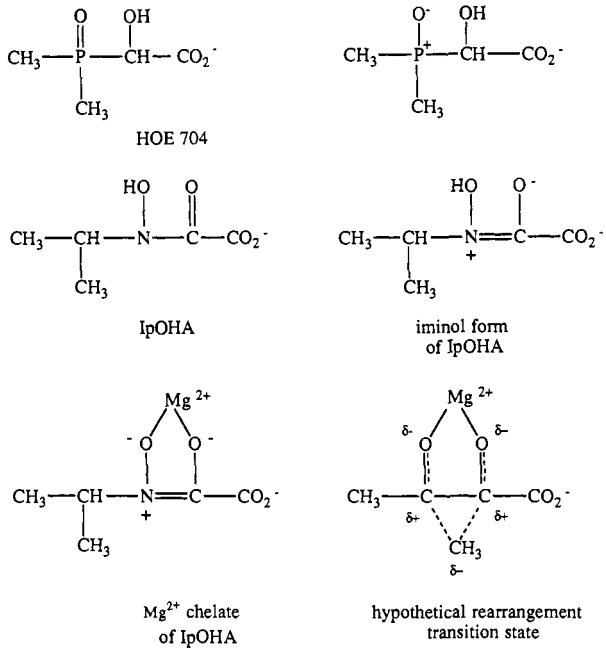

FIGURE 4: Structures of Hoe 704, IpOHA, and a hypothetical transition-state structure for the rearrangement reaction.

two other common enzymes of branched-chain amino acid biosynthesis, acetolactate synthase isozyme I, II (Schloss et al., 1985), or III (Barak et al., 1988) and dihydroxyacid dehydratase (Flint & Emptage, 1988), reversal of the growth effect by branched-chain amino acids indicates that it is solely due to the selective inhibition of ketol-acid reductoisomerase. IpOHA has also been observed to kill *Arabidopsis thaliana* seedlings (0.5 mM, although lower concentrations were considerably less effective) after germination in soft agar (Haughn et al., 1986), with substantial protection afforded by 1 mM valine, leucine, and isoleucine (V. A. Wittenbach, unpublished observations). Greenhouse studies indicate that IpOHA and its analogues have low herbicidal activity.

Discussion

There are a number of striking parallels between the rearrangement reaction catalyzed by ketol-acid reductoisomerase and its interaction with IpOHA. In catalyzing the isomerization of HMOB (the α-keto reaction intermediate for the physiological reaction) to acetolactate, the enzyme requires Mg^{2+} (with high, or absolute, preference over Mn^{2+}) and nucleotide. In a comparison of the back-reaction (conversion of NADP and DHMB to NADPH and acetolactate) to the isomerization of HMOB to acetolactate in the presence of NADP, the isomerization step is at least 3-fold faster in the presence of reduced nucleotide than with oxidized nucleotide. Tightest binding of IpOHA by enzyme requires the presence of both Mg^{2+} and NADPH. In the absence of metal, the complex between the reductoisomerase and IpOHA (if formed at all) is rapidly reversible. By contrast, in the presence of Mg^{2+}, the enzyme–Mg^{2+}–IpOHA complex releases IpOHA with a half-time of 2 h. Although a slowly reversible complex will form between enzyme and IpOHA in the presence of Mn^{2+}, the rate of release of IpOHA is substantially faster than that in the presence of Mg^{2+} (a half-time much less than 1 h). In the presence of both Mg^{2+} and NADPH, the rate of IpOHA exchange with unbound IpOHA has a half-time of about 6 days. If NADP is substituted for NADPH (Mg^{2+} present), the exchange half-time for IpOHA decreases to slightly more than 1 day. Nucleotide, its oxidation state, and metal ion have similar effects on IpOHA binding by ketol-acid reductoisomerase and the enzyme's ability to catalyze the rearrangement reaction, if not quantitatively equivalent effects under many conditions.

Despite the similarity between the behavior of IpOHA binding and the rearrangement reaction, it is unlikely that the potency of this inhibitor derives from its structural similarity to HMOB or HMOP (the intermediates of the physiological reactions). Although HMOB does not buildup under steady-state conditions with acetolactate and NADPH as substrates, it is unlikely that this is due to an exceptionally slow rate of release for this intermediate. When used as a substrate for the dehydrogenase reaction, there are sizable deuterium isotope effects on the reduction of HMOB or HMOP by $NADP^2H$ (Aulabaugh & Schloss, 1988). These isotope effects [$^D(V/K)$] demonstrate that HMOB or HMOP binds reversibly to enzyme up to the point of hydride transfer. Rapidly reversible binding by HMOB and HMOP contrasts with the nearly irreversible (half-time for release of 6 days) binding of IpOHA under these conditions. It is unlikely that the exceptionally potent inhibition of the reductoisomerase by IpOHA derives from its structural similarity to HMOB. Given the similar requirements of the rearrangement reaction and tight binding of IpOHA by ketol-acid reductoisomerase, it seems reasonable to conclude that IpOHA is actually an analogue of the rearrangement transition state (discrete from the α-keto acid reaction intermediates). IpOHA binds substantially tighter than acetolactate and HMOB, which have Michaelis constants that are 730000- and 13000000-fold larger than this inhibitor's dissociation constant. Although the predominant form of IpOHA does not resemble the likely transition state for the rearrangement reaction, the iminol form of this inhibitor has a number of structural features in common (Figure 4). During rearrangement of acetolactate to HMOB, both hydroxyl-bearing carbons would have partial carbonyl character, carbons 2 and 3 would have partial positive charge, and the migrating methyl group would have carbanion character. These features are mimicked in the iminol form of IpOHA by the full positive charge on nitrogen and the double bond between nitrogen and carbon, making both positions trigonal. In addition, to the extent that the metal participates in the rearrangement reaction and coordinates both oxygens to facilitate the reaction, the chelating properties of IpOHA (especially in its iminol form) should give enhanced binding.

Recently, Schultz et al. (1988) reported that an experimental herbicide from Hoechst, Hoe 704, is an exceptionally potent inhibitor of ketol-acid reductoisomerase ($K_i \approx 0.8$ μM). Similar to IpOHA, this inhibitor has characteristics of the rearrangement transition state. Since the bond between the phosphorus and oxygen is dative, it can be illustrated as shown in Figure 4, with positive charge on the phosphorus and negative charge on the oxygen. It is likely that both of these inhibitors, IpOHA and Hoe 704, are potent inhibitors of ketol-acid reductoisomerase due to their structural similarities to the transition state of the rearrangement reaction distinct from reaction intermediates of lower energy (such as HMOB or HMOP). However, unlike Hoe 704 (Schultz et al., 1988), IpOHA is a potent inhibitor of the growth of *E. coli* on minimal media. As branched-chain amino acids can prevent the growth inhibition, even at high concentrations of IpOHA, the inhibition is metabolically quite selective. IpOHA should prove to be a useful tool in studies of metabolic flux and the physiology of the branched-chain amino acid pathway.

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