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Mechanism of Ketol Acid Reductoisomerase—Steady-State Analysis and Metal Ion Requirement†

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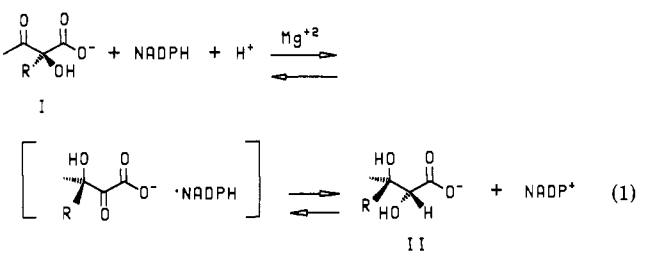
Received June 6, 1988; Revised Manuscript Received August 11, 1988

ABSTRACT: Ketol acid reductoisomerase is an enzyme of the branched-chain amino acid biosynthetic pathway. It catalyzes two separate reactions: an acetoin rearrangement and a reduction. This paper reports on the purification of the enzyme from a recombinant *Escherichia coli* and on the steady-state kinetics of the enzyme. The kinetics of the reaction were determined for the forward and reverse reaction by using the appropriate chiral substrates. At saturating metal ion concentrations the mechanism follows an ordered pathway where NADPH binds before acetolactate. The product of the rearrangement of acetolactate, 3-hydroxy-3-methyl-2-oxobutyrate, is shown to be kinetically competent as an intermediate in the enzyme-catalyzed reaction. Starting with acetolactate, Mg^{2+} is the only divalent metal ion that will support enzyme catalysis. For the reduction of 3-hydroxy-3-methyl-2-oxobutyrate, Mn^{2+} is catalytically active. Product and dead-end inhibition studies indicate that the binding of metal ion and NADPH occurs randomly. In the forward reaction direction, the deuterium kinetic isotope effect on V/K is 1.07 when acetolactate is the substrate and 1.39 when 3-hydroxy-3-methyl-2-oxobutyrate is the substrate.

The enzyme acetohydroxy acid reductoisomerase (2,3-dihydroxyisovalerate:$NADP^+$ oxidoreductase; EC 1.1.1.86) is found in the branched-chain amino acid biosynthetic pathway. It catalyzes the reaction indicated in eq 1. The two natural substrates for the enzyme differ in the identity of the R group that undergoes migration. In compound I, when R is methyl, biosynthesis proceeds to ultimately produce either valine or leucine. For the compound where R is ethyl, the ultimate



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product is isoleucine. The enzyme reaction is unique in that an intramolecular alkyl transfer occurs without the intervention of cobalamin or any other organic cofactor. The enzyme

† Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for the support of this research.

0006-2960/89/0428-0486$01.50/0

activity is dependent on the presence of a divalent magnesium ion (Armstrong & Wagner, 1961).

The reductoisomerase from *Salmonella typhimurium* is a tetramer of identical subunits having a molecular weight of 220 000. This tetramer catalyzes both the migration and reduction steps of the chemical transformation of acetolactate to dihydroxyisovalerate (II, eq 1). Umbarger partially purified the enzyme from *Escherichia coli* (Umbarger et al., 1960) and obtained a homogeneous preparation from *S. typhimurium* (Arfin & Umbarger, 1969). Using this enzyme, he characterized the basic aspects of the reaction. Hoefler also reported the purification of the enzyme from *S. typhimurium* (Hoefler et al., 1975). Shematek investigated part of the steady-state kinetic mechanism of the enzyme from this source (Shematek et al., 1973). Initial velocity patterns for the forward reaction in the presence and absence of products indicated an ordered bi-bi mechanism. The steady-state parameters for the reverse reaction were not reported.

The stereochemistry of the reaction is known. That (2*S*)-acetolactate (I, R = Me) and (2*S*)-acetohydroxybutyrate (I, R = Et) were the substrates for the reaction was established by the work of Crout (Armstrong et al., 1974, 1983) and, independently, Hill (Hill et al., 1979). The product diol resulting from the reaction of acetolactate has the 2*R* configuration, while that from acetohydroxybutyrate has the 2*R*,3*R* configuration (Hill & Yan, 1971; Crout & Whitehouse, 1972). Using ^{13}C-labeled acetolactate, Crout showed that the 2-methyl group that undergoes migration occupies the *pro-R* position in the product 2,3-dihydroxy-3-methylbutyrate (Crout et al., 1980). In acetohydroxybutyrate, the migrating ethyl group retains its stereochemistry at the methylene carbon in the product diol (Cahill et al., 1980). The reductoisomerase is able to catalyze the reduction of ketopantoate to produce pantoate (the intermediate in coenzyme A biosynthesis) which again requires that the reduction half-reaction produce a 2-(*R*)-hydroxy acid (Primerano & Burns, 1983). Finally, the reduction step requires the transfer of the *pro-S* hydrogen from NADPH[1] (Arfin & Umbarger, 1969).

The expression of the gene (ilvC) for the reductoisomerase is regulated by an activator protein encoded by the ilvY gene. The substrates for the reductoisomerase are bound by the Y protein, thereby inducing the synthesis of the enzyme. Other substrate analogues are capable of inducing the synthesis of the reductoisomerase, although at reduced levels (Arfin et al., 1969). The genes for *E. coli* ilvC and ilvY have recently been cloned into a multicopy plasmid (Wek & Hatfield, 1986) and the sequences determined. Since the plasmid carries both genes, the concentration of the activator protein Y is never limiting.

There is current interest in the biosynthetic pathway of the branched-chain amino acids and in the mechanism of reductoisomerase. The sulfonyl urea and imidazolinone herbicides are known to inhibit the biosynthesis of these amino acids (Chaleff & Mauvais, 1984; Shaner et al., 1984). The point of inhibition is acetolactate synthase, the enzyme that precedes reductoisomerase in the pathway. Our interest in the reductoisomerase enzyme centers on the chemical mechanism for the alkyl migration step. We have taken advantage of the mode of expression of ilvC to induce recombinant bacteria to synthesize large amounts of enzyme. In this paper, we report the purification of the enzyme from this source as well as steady-state kinetic analyses of the forward and reverse reactions. We have also determined the kinetics of the enzyme reaction using the intermediate 3-hydroxy-3-methyl-2-oxobutyrate. Finally, we present the results of kinetic isotope effect studies on the reduction aspect of the enzyme reaction.

Experimental Procedures

Materials

General. Lead tetraacetate was purchased but was recrystallized from glacial acetic acid and dried in vacuo prior to use. 3-Hydroxy-2-butanone (acetoin) from Aldrich was distilled under vacuum, and 1-naphthol (Aldrich) was sublimed as needed for acetoin determinations. The Affi-Gel Blue and PAGE reagents were obtained from Bio-Rad. Ethyl 2-acetoxy-2-ethylacetoacetate was synthesized by the procedure of Kiritani (Kiritani & Wagner, 1970) using the recrystallized lead tetraacetate and was twice vacuum distilled. B-side deuteriated NADPH was prepared with glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Sigma) by the procedure of Viola (Viola et al., 1979). For ^2H KIE studies, this [^2H]NADPH and NADPH were prepared identically and purified over AGMP-1 (200–400 mesh) ion-exchange resin (Northrup & Duggleby, 1987). The purified material had an A_{260}/A_{340} of 2.3.

(2S)-2-Hydroxy-2-methyl-3-oxobutanoic Acid [(2S)-Acetolactate]. This compound was synthesized by the procedure of Armstrong (Armstrong et al., 1983). The intermediate, 2(*S*)-methyl 2,3-dimethyl-2-hydroxybut-3-enoate, obtained by quinine resolution was analyzed by 400-MHz ^1H NMR in the presence of the chiral shift reagent tris[3-[(heptafluoropropyl)hydroxymethylene]-(+)-camphorato]europium(III). Integration of the vinyl hydrogens indicated on enantiomeric purity of 90%. Following ozonolysis of this compound, the specific rotation was 8.44° (c = 3, ethanol) indicating an optical purity of 88% for the methyl ester of the correct enantiomeric substrate for reductoisomerase. This is based on the rotation of the 2*R* compound of −9.6° (c = 3, ethanol) as reported by Hill (Hill et al., 1979). ^1H NMR ($CDCl_3$) δ 4.80 (b s, 1 H), 3.75 (s, 3 H), 2.24 (s, 3 H), 1.56 (s, 3 H). For use, the methyl ester was hydrolyzed at room temperature by using 2 equiv of NaOH for 5 min followed by the addition of an excess of 1 M Tris, pH 7.5. The final pH of the substrate solution was about 8. ^1H NMR of the hydrolysis solution (carried out in D_2O for this analysis) indicated complete hydrolysis with no evidence of side reactions when the procedure was followed as described up to the addition of the Tris solution. The specific rotation of the substrate solution was 45°, indicating an optical purity of 87% (Hill et al., 1979). The solution was prepared immediately before use. Enzymatic analysis using reductoisomerase and an excess of NADPH indicated >95% purity of the substrate. While in use, the substrate was kept at 0 °C and assayed before and after sets of kinetic runs. There was no evidence of decomposition. To check for decomposition and/or racemization of the substrate, the hydrolysis was carried out as above and the pH adjusted to 9.5. After 4 h at 0 °C followed by 2 days at −20 °C, the rotation was 37.9°, indicating an optical purity of 73%. When the rotation of this solution was followed as a function of time at room temperature, the ro-

[1] Abbreviations: ACES, 2-[(2-amino-2-oxoethyl)amino]ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADP, oxidized nicotinamide adenine dinucleotide phosphate; AS, ammonium sulfate; AL, 2-hydroxy-2-methyl-3-oxobutyrate (acetolactate); DMB, 2,3-dihydroxy-3-methylbutyrate; HKIV, 3-hydroxy-3-methyl-2-oxobutyrate; NMR, nuclear magnetic resonance spectroscopy; KIE, kinetic isotope effect; c_f, commitment of substrate to partition forward; c_r, commitment of substrate to partition backward; K_{ii}, inhibition constant reflected as an intercept effect; K_{is}, inhibition constant reflected as a slope effect.

tation decreased according to first-order kinetics. The rate constant for this loss of optical rotation was 0.0092 (0.0006) min^{-1}.

Sodium 2,3-Dihydroxy-3-methylbutanoic Acid (DMB). Methyl 3,3-dimethylacrylic acid (33 mmol) in 20 mL of CH_2Cl_2 was treated dropwise with a solution of *m*-chloroperbenzoic (34 mmol) in 20 mL of CH_2Cl_2. After 20 h at room temperature, the mixture was filtered and the solvent removed. The oily solid residue was triturated with hexane/$CHCl_3$ (4/1 v/v) and filtered. After removal of the solvent from the filtrate, the epoxy ester was distilled (bp 74 °C/12–15 mmHg). ^1H NMR ($CDCl_3$) δ 3.70 (s, 3 H), 3.26 (s, 1 H), 1.40 (s, 3 H), 1.35 (s, 3 H). The ester function was hydrolyzed in a minimum volume of aqueous NaOH and acidified with concentrated HCl, and the two-phase system was extracted with ether. The ether layer was dried and the solvent removed. Water was added and the solution refluxed for 4 h. The water was removed in vacuo and the acid treated with triethylamine in water. The salt was purified by ion-exchange chromatography. A column (2.5 × 30 cm) was packed with AG1-X8 (bicarbonate form), and the salt (20 mM) was applied to the column. The product was eluted with a triethylammonium bicarbonate (TEAB) gradient (0.02–0.2 M, pH 7.0). The fractions containing the diol were detected by oxidizing aliquots with periodic acid and then adding 2,4-dinitrophenylhydrazine. The sustained appearance of a red color indicated the presence of the diol. The requisite fractions were pooled and lyophilized, and the resulting triethylammonium salt was converted to the sodium form by use of AG50. ^1H NMR (D_2O) δ 3.80 (s, 1 H), 1.10 (s, 6 H). ^{13}C NMR (D_2O) δ 179.3, 74.0, 73.2, 25.4, 25.3.

(2R)-2,3-Dihydroxy-3-methylbutanoic Acid [(2R)-DMB]. The racemic acid was prepared as above. The acid was resolved as the quinine salt by fractional crystallization from ethanol at 4 °C using 1.1 mol equiv of quinine. After five recrystallizations the specific rotation and melting point of the needlelike crystals were in agreement with those reported (Hill & Yan, 1971). The acid was recovered by dissolving the quinine salt in hot water and adjusting the pH to 10 with 2 M NaOH. The resulting solution was filtered and extracted with chloroform to remove residual quinine. The aqueous layer was applied to an AG50 column (H^+ form) and eluted with 6 column volumes of water. The specific rotation of this acid was −15.8° (lit. −16°) (Hill & Yan, 1971). Triethylamine was added to an aqueous solution of the resolved diol acid, and the resulting salt was converted to the sodium form using AG50. ^1H NMR (D_2O) δ 3.70 (s, 1 H), 1.20 (s, 6 H). On the basis of the reported rotation, the diol acid contains 9% of the 2*S* enantiomer. The concentration of DMB was determined enzymatically by using reductoisomerase and a 100-fold molar excess of NADP at pH 10.0 (CAPS buffer).

3-Hydroxy-3-methyl-2-oxobutanoic Acid (HKIV). Ethyl 3-methyl-2-oxobutanoate was treated with a small amount of anhydrous HBr, heated to 60 °C, and then treated dropwise with 1 equiv of Br_2 while stirring. The red color following the addition of a drop of Br_2 was allowed to dissipate before the next drop was added. The resulting crude ethyl 3-bromo-3-methyl-2-oxobutyrate was treated dropwise with an aqueous solution containing 1 equiv of K_2CO_3. The aqueous mixture was saturated with NaCl and extracted with ether. The ether layer was dried (Na_2SO_4) and the ether removed in vacuo. The residual clear oil was distilled and the ethyl 3-hydroxy-3-methyl-2-oxobutanoate collected (bp 92–94 °C/13 mmHg). ^1H NMR ($CDCl_3$) δ 4.32 (q, J = 7.2 Hz, 2 H), 3.23 (s, 1 H), 1.48 (s, 6 H), 1.35 (t, J = 7.1 Hz, 3 H). ^{13}C NMR ($CDCl_3$) δ 198.4, 162.4, 76.2, 62.4, 26.1, 13.9. For use, the ester was hydrolyzed for 5 min in an aqueous solution containing 1.1 equiv of KOH. ^1H NMR analysis indicated complete reaction with no evidence of any side reactions. The hydrolyzed compounds was diluted immediately with assay buffer and used. Enzymatic analysis using reductoisomerase and an excess of NADPH indicated >95% purity. No decomposition was observed during the course of the day providing the solution was kept at 0 °C. Fresh material was prepared daily.

Methods

General. In some experiments, the concentration of acetolactate was determined after decarboxylation to form acetoin. Typically, 0.4 mL of reaction mixture containing acetolactate was quenched with 0.40 mL of 0.25 M H_2SO_4. After 30 min at 37 °C, acetoin was determined by the Westerfield method (Westerfield, 1945). In our assays we could determine acetolactate concentrations as low as 10 μM by this procedure.

Steady-state kinetic analysis was performed at 25 °C in a thermostated cell holder. For all assays, the standard buffer ACES/Tris/ethanolamine (0.1 M/0.052 M/0.052 M, I = 0.1) (Ellis & Morrison, 1982) was used at the indicated pH. The enzyme was slightly more active (1.2-fold) in phosphate buffer. The kinetic data were fit to the standard equations using Cleland's programs (Cleland, 1979) which had been adapted to BASIC by Dr. C. Falzone.

The bacterial strain was obtained from Ron Wek at the University of California, Irvine, and maintained on LB agar containing 100 μg/mL ampicillin. P-I laboratory conditions and procedures were consistently maintained. The enzyme was assayed by the procedure of Arfin (Arfin & Umbarger, 1969) in which the rate of the reaction is measured by following the decrease in absorbance at 340 nm as NADPH is oxidized. Protein was determined by the procedure of Lowry (Lowry et al., 1951). The SDS–PAGE was carried out by the procedure of Laemmli (1970). All protein purification steps were carried out at 4 °C.

The association constants for acetolactate and 3-hydroxy-3-methyl-2-oxobutyrate (HKIV) with Mg^{2+} and Mn^{2+} were determined according to the procedure described by O'Sullivan (O'Sullivan & Smithers, 1979). The method relies on the competition in binding metal ion between the molecule of interest and 8-hydroxyquinoline, the concentration of the latter complex being determined spectrophotometrically. For our determination, we varied the concentration of metal from 0 to 2 mM and the concentration of acetolactate or HKIV from 0 to 10 mM while the concentration of 8-hydroxyquinoline was constant at 0.25 mM in the standard buffer of the enzyme assays. Plots of A_{360} vs $[Mg^{2+}]$ or $[Mn^{2+}]$ were the same whether AL or HKIV were present or not. We determine that the association constant for either metal with AL or HKIV can not be greater than 24 M^{-1}. The association constant for Mn^{2+} binding to NADPH was taken from the work of Dalziel (Kuchel et al., 1980) and Coleman (1972). Dalziel reports a K_a of 943 M^{-1} at pH 7.0 and ionic strength 0.15. Coleman reports a K_a of 2560 M^{-1} at pH 8.05 and ionic strength 0.1. Dalziel also reports an association constant of 179 M^{-1} for formation of a Mg–NADPH complex at pH 7 and ionic strength 0.15. Using the change in the formation constant for NADPH–Mn as a function of pH, we estimate the K_a for formation of a NADPH–Mg complex at the pH of our kinetic assays to be 210 M^{-1}; the corresponding value for NADP is 157 M^{-1}. Coleman indicates that the affinity for binding metals to NADPH appears to involve the dianion of the 2′-phosphate. The correction for the change in pH is based upon a pK_a for the 2′-phosphate of 6.3 as measured by the chemical

shift of the ^{31}P NMR resonances of this phosphate as a function of pH. The estimated value for the association constant should be close to the true value. When we use this value of the K_a to calculate the concentrations of uncomplexed metal and nucleotide, the apparent substrate inhibition observed in the absence of this correction is eliminated. Morrison indicates that none of the components of our buffer system will bind either of the metal ions to a significant extent (Ellis & Morrison, 1982).

^1H NMR Analysis of the Enzyme Reaction. We investigated the products of the reaction using NMR. The reaction was set up in an NMR tube containing 10 mM each of NADP and NADPH, 40 mM each (2*S*)-acetolactate and (2*R*)-DMB, 4 mM $MgCl_2$, and 5 units of the enzyme in 1.8 mL of 90% D_2O. An imidazole buffer (50 mM) system was used to maintain the pH at 7.0. Spectra were obtained at 300 MHz approximately every 2 h for a total of 8 h. Since the concentrations of the substrates are not at equilibrium, there is a net conversion of the acetolactate to DMB. Equilibrium was reached after about 4 h as judged by comparing the integrals of the methyl peaks for acetolactate and DMB. After 10 h, an aliquot of the reaction mixture was removed and assayed, demonstrating that the enzyme was still active. HKIV, acetolactate, and DMB were then added, and a spectrum was recorded after each addition. Comparing these spectra with those obtained during the enzyme reaction allowed us to assign specific peaks to specific compounds. The peak corresponding to the methyl groups of HKIV (δ 1.53) overlapped the peak for the 2-methyl group of acetolactate (δ 1.51). The two methyl groups of DMB were separate (δ 1.27), as was the methyl group adjacent to the ketone group of acetolactate (δ 2.38). The hydrogen on carbon 2 of DMB (δ 3.93) was not resolved from the peaks arising from the ribose rings of NADPH and NADP. By comparing the integration of the peaks at δ 2.38 and 1.53, we conclude that HKIV is less than 5% of the amount of acetolactate present at equilibrium.

Bacterial Growth and Enzyme Overexpression. The bacteria was transferred from an agar slant to 100 mL of LB media containing 100 μg/mL ampicillin and incubated overnight in a shaking water bath at 37 °C. Thirty milliliters of this culture was used to inoculate 3 L of growth media (LB broth and ampicillin). At the time of inoculation, sodium 2-ethyl-2-hydroxybutyrate was added via sterile filtration to a final concentration of 6 mM. A fresh addition of this sodium salt was made 4 h later. After a total of 6 h of growth, sodium 2-ethyl-2-hydroxyacetoacetate was added to a final concentration of 6 mM. Growth was allowed to proceed for 4.5 h at which time the medium was centrifuged. The bacterial pellet was washed with 50 mM phosphate buffer, pH 8, and the centrifuged bacteria were frozen at −20 °C. In general, the bacteria from two such fermentations were used in the following purification.

Enzyme Purification. The bacterial pellet was suspended in 3 volumes of a buffer of 0.01 M KH_2PO_4, pH 8.0, containing 3 mM mercaptoethanol, 4 mM $MgCl_2$, 0.05 mM EDTA, and 20 μg/mL phenylmethanesulfonyl fluoride and sonicated in 30-mL batches at 60% power for 4 min by using a Branson 200 sonicator. The solution was centrifuged at 13000*g* for 1 h. The pellet was then suspended in 1 volume of the previous buffer and subjected to a second sonication and centrifugation. To the combined supernatants was added $^1/_{10}$ volume of 5% streptomycin sulfate. After 15 min the solution was centrifuged at 13000*g* for 40 min. The supernatant was then treated with $^1/_{10}$ volume of 2% (w/v) cetyltrimethylammonium bromide and immediately centrifuged at 13000*g* for 40 min. The detergent is not soluble in water at room temperature and so must be warmed slightly before adding it to the enzyme solution at 4 °C. The supernatant was made 40% saturated with $(NH_4)_2SO_4$ (AS) by adding the appropriate volume of saturated AS containing 3 mM mercaptoethanol and 0.5 mM EDTA (pH 7.5). After sitting for 45 min, the solution was centrifuged at 14000*g* for 1 h. The supernatant was then made 65% saturated in AS and after sitting for 45 min was centrifuged at 14000*g* for 1 h. The precipitate was dissolved in 0.01 M KH_2PO_4, pH 6.0, buffer containing 0.5 mM EDTA, 3 mM mercaptoethanol, and 4 mM $MgCl_2$ to a concentration of 2 mg of protein/mL. This solution was then passed through an Affi-Gel blue column (2.5 × 20.0 cm, 100-mL bed volume) equilibrated in the same buffer. We found it convenient to divide the enzyme solution into portions containing 100 units each at this stage and treat each portion separately. After a portion was added to the column, nonspecifically bound protein was washed off with 200 mL of the pH 6 buffer. Elution of the reductoisomerase was accomplished with a linear 600-mL gradient of 0–1 M NaCl in the pH 6 phosphate buffer. The reductoisomerase eluted after about 250 mL. The column was washed with 6 M urea and then pH 6 buffer before the next enzyme portion was added. The active fractions were combined, made 65% saturated in ammonium sulfate, and centrifuged at 13000*g* for 1 h. The pellet contained the pure enzyme and was stored at 4 °C under a small volume of 65% saturated AS. The complete purification can be performed in 2 days. We detect no decrease in enzyme activity over a period of 6 months. For use in kinetic analysis, the enzyme was diluted in assay buffer and passed through a Sephadex G-25 column with assay buffer.

Table I: Purification of Ketol Acid Reductoisomerase[a]

fraction	total units	sp act.	yield (%)
crude extract	1966	0.34	
streptomycin sulfate	1990	0.34	100
cetyltrimethylammonium bromide	1532	0.40	77
40–65% saturated $(NH_4)_2SO_4$ pellet	1336	0.70	67
combined eluates of Affi-Gel Blue	1311	2.50	64

[a] The steps are those given under Methods section. The enzyme activity was measured in 0.1 M phosphate buffer containing 4 mM $MgCl_2$ at 25 °C. Protein was determined by the procedure of Lowry. Racemic acetolactate was used as the substrate.

Results

Enzyme Purification. The data on the purification are summarized in Table I. Because of the ability to overexpress the enzyme, the reductoisomerase is present as about 12% of the total protein in the crude cell extract. Given such a level, the purification was quite straightforward. In our hands, 2-ethyl-2-hydroxybutyrate was as effective as acetolactate for the induction of the enzyme. We have optimized the purification to some extent. Omission of the cetyltrimethylammonium bromide precipitation results in a slightly less pure preparation after the affinity column. Pure enzyme in this case can be obtained after a DEAE Sephadex A-50 ion-exchange column. However, this column takes longer than the centrifugation and does not markedly improve the overall yield. Although the enzyme was produced from a well-characterized plasmid, we carefully checked the molecular weight of the purified enzyme. SDS–PAGE (in both 11% and 8% gels) indicated the enzyme had the correct subunit molecular weight (53 000) (Arfin & Umbarger, 1969). The specific activity of our enzyme preparation is 2.5 as compared to the value 1.91 reported by Arfin (Arfin & Umbarger, 1969) for the enzyme

from *S. typhimurium*. These specific activities were determined under the same conditions, and although the enzymes are from different sources, the amino acid compositions appear to be quite similar (Wek & Hatfield, 1986).

Substrate Stability. Acetolactate is known to be unstable as a salt in aqueous solution (Crout & Hedgecock, 1979; Hill et al., 1979). We investigated the stability of acetolactate under the conditions of the enzyme assay. The conditions used to hydrolyze the methyl ester led to at most a 2–5% loss of optical activity. The salt was reasonably stable at near neutral pH when kept at 0 °C or lower. At most, a 15% loss of optical activity was observed after 2 days under normal storage conditions. At room temperature, the rate of loss of optical activity was slow relative to the time required for an enzyme assay, although extended incubations at a pH greater than 9.5 could suffer significant loss of optical activity. Aside from the racemization reaction, there are a variety of other spontaneous reactions one could envision that would destroy acetolactate. When a room temperature aqueous solution of sodium acetolactate was studied by ^{1}H NMR, no detectable degradation was evident over about 30 min. The singlet corresponding to the 2-methyl group did not appear to change, and no new peaks were observed. We are confident that under the conditions of our assay no significant decomposition of acetolacetate occurred.

The 2-keto-3-hydroxy-3-methylbutyrate is also somewhat unstable. The preparation of this compound reported by Snell (Radhakrishnan & Snell, 1960) leads to a very heterogeneous product (Crout & Hedgecock, 1979) apparently because the neutralization of the bromination reaction leads to considerable decarboxylation. We modified that preparation and were able to obtain pure product. When the hydrolysis of the ethyl ester of this compound was followed for 45 min in an NMR tube, no significant decomposition was observed at room temperature. Hydrolysis was complete within 5 min. The only peaks present could be accounted for by the structure of HKIV and ethanol.

Steady-State Kinetic Analysis at Constant Metal Ion Concentration. Preliminary studies established that the pH maximum for V/K was about 8.2. We analyzed the effect of racemic acetolactate as opposed to chiral acetolactate on the steady-state kinetics. We observed parallel lines when the concentration of (2*S*)-acetolactate in the racemic and resolved substrate solutions was used in double-reciprocal plots. This pattern is characteristic of competitive inhibition by the 2*R* enantiomer (Cleland et al., 1973). The same situation applied in the reverse direction with racemic and (2*R*)-DMB. The inhibition constants for the incorrect enantiomer were 81 μM and 9 μM for acetolactate and DMB, respectively. When Mg^{2+} was treated as the variable substrate at saturating levels of acetolactate (2 mM) and NADPH (0.2 mM), substrate inhibition was observed. The maximum initial rate was obtained when the concentration of Mg^{2+} was 4.0 mM. To simplify the kinetic analysis, we fixed the Mg^{2+} concentration at this level and investigated the kinetic mechanism. When the NADPH concentration was varied at a fixed variable concentration of acetolactate, nonlinear double-reciprocal plots resulted. Using the association constant for formation of a metal–nucleotide complex given under Experimental Procedures, we determined the concentrations of NADPH and Mg that were uncomplexed and the concentrations of the metal–nucleotide complex. Linear double-reciprocal plots resulted when the concentration of uncomplexed metal was held at 4 mM and the reciprocal of the concentration of uncomplexed NADPH was plotted. The analysis was extended to include product inhibition studies and an analysis of the kinetics of the reverse reaction. The apparent steady-state kinetic parameters are listed in Table II. The results of the product inhibition studies are summarized in Table III. The product inhibition studies clearly establish an ordered addition of NADPH and acetolactate and an ordered dissociation of DMB and NADP and thus confirm the same basic mechanism proposed by Shematek (Shematek et al., 1969) for the enzyme from *S. typhimurium*. We then investigated the kinetics associated with the reduction of HKIV by the enzyme. Unlike acetolactate and 2,3-dihydroxy-3-methylbutyrate, this substrate lacks a chiral center. The apparent steady-state parameters determined for this substrate are listed in Table II. Since HKIV is kinetically competent as an intermediate in the reaction, we investigated how this intermediate would partition during reaction. In the presence of Mg^{2+} and NADPH, HKIV was added with the enzyme. When the change in concentration of NADPH had reached 100 μM, the reaction was quenched with 0.25 M H_2SO_4 and the amount of AL formed was determined as acetoin (the decarboxylation product of AL). No acetoin was detected.

Table II: Steady-State Kinetic Parameters[a]

parameter	value	parameter	value
V_f/E_T	21 (0.2) s^{-1}	K_{NADP}	65 (1.9)
K_{AL}	36 (1.2)	$K_{i,NADP}$	94 (5.9)
$K_{i,AL}$	100 (2.2)	V_f/E_T[b]	37 (0.01) s^{-1}
K_{NADPH}	8.4 (0.2)	K_{HKIV}	240 (0.08)
$K_{i,NADPH}$	9.7 (0.3)	$K_{i,HKIV}$	110 (0.01)
V_r/E_T	0.51 (0.004)	K_{NADPH}[b]	14 (0.01)
K_{diol}	6.5 (0.2)	$K_{i,NADPH}$[b]	6.3 (0.01)
$K_{i,diol}$	9.4 (0.6)		

[a] All assays were run in ACES/Tris/ethanolamine standard buffer at pH 8.2 containing 4 mM $MgCl_2$ at 25 °C. All Michaelis constants are in micromolar units. The appropriate chiral substrate was used in each case. The forward reaction direction (subscript f) is that which produces NADP. Standard errors are indicated in parentheses. The constants were derived by fitting initial rate data with the equation for a sequential mechanism. [b] For this value, HKIV was used as the variable substrate.

Table III: Product Inhibition Patterns[a]

variable substrate	fixed substrate[b]	inhibitor	K_{is}	K_{ii}	pattern
acetolactate	NADPH (0.02)	NADP	1.42	1.0	NC
acetolactate	NADPH (0.02)	DMB	0.62	7.2	NC
NADPH	acetolactate (0.16)	NADP	0.25		C
NADPH	acetolactate (0.08)	DMB	0.25	1.4	NC
NADPH	acetolactate (1.40)	DMB		2.2	UC

[a] All assays were performed at 25 °C in the standard buffer at pH 8.5. The concentration of $MgCl_2$ was held at 4 mM. The inhibition constants have units of millimolar. The pattern designation is noncompetitive (NC), competitive (C), or uncompetitive (UC). The constants were obtained by fitting the initial rate data to the appropriate equation. Racemic DMB was used in the inhibition studies. [b] The millimolar concentration of the fixed substrate is given in parentheses.

Analysis Based on the Haldane Relationship. To check the internal consistency of the data, the apparent steady-state parameters were substituted into the equation for the Haldane expression for an ordered mechanism. The Haldane expression for this mechanism is (Cleland, 1963)

$$K_{eq}^{app} = V_f K_{i,NADP} K_{DMB} / V_r K_{i,NADPH} K_{AL}$$

Shematek reports a value of 0.75 for the equilibrium constant of this reaction at pH 7.5 (Shematek et al., 1973). Correction of this value to pH 8.0 (the pH of our kinetic analysis) indicates K_{eq} should be 0.24. Using our apparent steady-state values for the reaction in the Haldane expression produces a K_{eq} value of 71, in substantial disagreement with the value

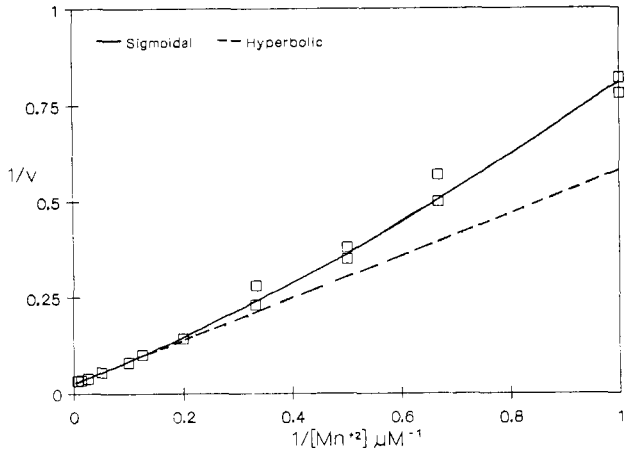


FIGURE 1: The double reciprocal plot for the reduction of HKIV catalyzed by Mn^{2+}. The concentrations of HKIV and NADPH were fixed at 5.5 times their K_m values. The solid curve is the best fit of the data obtained by using the program SIGMOIL (Cleland, 1979). The dashed line is obtained by fitting the data to HYPER.

reported. We then determined the value of K_{eq} for the conditions of our assay. Our procedure involved the addition of the two substrates and two products to a cuvette and determining how the absorbance at 340 nm changed upon the addition of enzyme. We subsequently altered the concentrations of NADPH and NADP until addition of enzyme failed to produce a change in absorbance over a period of 30 min. The equilibrium constant we determined by this procedure was 88 (*12*), in reasonable agreement with the value calculated from the Haldane. This value is based on the concentration of uncomplexed nucleotides as are our kinetic data. The reason for the discrepancy between the values of the equilibrium constant as determined by us and Arfin is not clear. One possibility is that Arfin did not correct for complexation between the metal and the nucleotides and therefore did not use the correct concentrations in his equilibrium calculation.

Alternate Metal Ions. The reaction requirement for Mg^{2+} is somewhat unusual. We investigated whether other divalent and trivalent metal ions could substitute for magnesium in the reaction. It is apparent that a metal ion is absolutely required for reaction as there is no discernible change in the A_{340} in the absence of Mg^{2+}. We attempted to substitute Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, Cu^{2+}, and Co^{3+} for Mg^{2+}. At concentrations up to 10 mM in each metal ion, no change in the A_{340} could be detected when the incubation included acetolactate and NADPH in our standard buffer at pH 8.0 (indicating less than 0.2% activity). However, with acetolactate as substrate, manganese did behave as a competitive inhibitor with respect to magnesium (this is the only metal we tested as an inhibitor). The K_{is} for Mn^{2+} is 7.7 μM compared to an apparent K_m for Mg^{2+} of 0.42 mM (determined at saturating levels of AL and NADPH). When we investigated the reduction of HKIV, Mn^{2+} was found to be as effective as Mg^{2+} in catalyzing this part of the reaction. The concentration of HKIV was varied at different fixed concentrations of Mn^{2+} while the NADPH was maintained at saturating levels (0.12 mM). The data were adequately described by a sequential mechanism (K_{Mn} = 11 μM, K_{HKIV} = 0.25 mM, V/E_T = 41 s^{-1}). The K_{Mg} under the same conditions is 0.42 mM. We investigated whether Mn^{2+} would catalyze the conversion of HKIV to acetolactate. In the presence of saturating levels of NADP and HKIV at pH 8.25, Mn^{2+} did not support the formation of acetolactate. Starting from DMB and NADP, Mn^{2+} did catalyze the formation of NADPH; however, no acetolactate (measured as acetoin) could be detected.

Table IV: Inhibition Constants for Determining Metal Nucleotide Order[a]

variable substrate[b]	inhibitor	K_{is}	K_{ii}
acetolactate	α-methyllactate	26 (4)	
Mg^{2+}	Mn^{2+}	7.7 (1)	
NADPH	3-aminopyridine–NADP	48 (15)	
Mg^{2+}	NADP	31 (10)	43 (4)
Mg^{2+}	3-aminopyridine–NADP	200 (10)	390 (50)
NADPH	Mn^{2+}	20 (5)	13 (1)
NADPH	α-methyllactate		32 (3)

[a] All assays were performed at 25 °C in the standard buffer at pH 8.2. All inhibition constants are in micromolar units with standard errors given in parentheses. [b] For these determinations, the nonvaried substrates were fixed at the following concentrations: [acetolactate] = 0.4 mM, [NADPH] = 0.12 mM, and [Mg^{2+}] = 4.0 mM.

When Mn^{2+} was varied over a wide concentration range at less than saturating concentrations of HKIV and NADPH (5.5 times the K_m value of each), the resulting plot was distinctly nonhyperbolic (Figure 1). It is clear from the plot that the data correspond to the curve describing a sigmoidal response of initial rate to changes in Mn^{2+} concentrations. The $K_{0.5}$ is 17 μM. The Hill coefficient for the curve has a value of 1.08. In an effort to further characterize the nonhyperbolic response of initial rate to changes in the concentration of Mn^{2+}, we looked at other inhibition patterns.

The inhibition patterns are presented in Table IV. The 3-aminopyridine–NADP is not a substrate for the reverse reaction and is a competitive inhibitor with respect to NADPH. The α-methyllactate is a very good competitive inhibitor with respect to acetolactate. On the basis of the inhibition patterns for Mn^{2+} and 3-aminopyridine–NADP, we can distinguish whether an ordered addition of metal and NADPH or a random addition of these two substrates is correct. The patterns observed are indicative of a random addition of NADPH and metal ion to the free enzyme. Since α-methyllactate is an uncompetitive inhibitor with respect to NADPH, it is clear the binding site for acetolactate is formed only when the metal ion and the reduced nucleotide are bound to the enzyme.

Deuterium Kinetic Isotope Effect Studies. The effect of deuterium substitution upon the rate of the enzyme reaction was studied in the forward reaction direction. The data were analyzed by the programs developed by Cleland for KIE's on both V and V/K, V/K only, and V only (Cleland, 1977). In the forward reaction direction, stereospecifically labeled [^{2}H]NADPH was used at a fixed concentration while the concentrations of partially resolved (2*S*)-acetolactate or HKIV were varied. For each substrate, the best fit to the data assumed on isotope effect on V/K only. For acetolactate the $^D(V/K)$ = 1.07 (0.03) and for HKIV $^D(V/K)$ = 1.39 (0.10). Since for acetolactate the incorrect enantiomer is known to be a competitive inhibitor with respect to the correct enantiomer, the ^DV value for the partially resolved acetolactate cannot be obtained directly (Grimshaw & Cleland, 1980). The ^DV value for HKIV could not be greater than 1.13 (0.03) according to the fit of the data to the equation for a KIE on V and V/K.

Discussion

Although the enzyme has been purified from a variety of sources, the enzyme from *E. coli* has not been reported. The specific activity of the enzyme from *S. typhimurium* is about 2, and this enzyme has a remarkably similar amino acid composition to the enzyme from *E. coli* (Wek & Hatfield, 1986). The enzyme we obtain from the recombinant bacteria

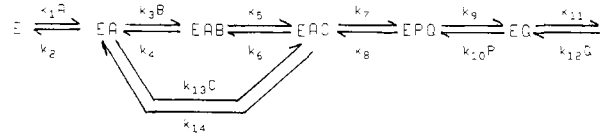
has the same molecular weight and a slightly higher specific activity than the enzyme from *S. typhimurium*. The enzyme appears homogeneous by SDS–PAGE. Chromatography of the enzyme from the final step of Table I on either hydroxyapatite or Sephadex A-50 does not increase the specific activity.

The steady-state rate data and the results of the product inhibition studies indicate that the *E. coli* enzyme follows an ordered reaction mechanism where NADPH binds first and acetolactate binds second at saturating concentrations of metal ion. A more sensitive test for an ordered mechanism is obtained by measuring the $^{D}(V/K)$ for one substrate as a function of the concentration of the second substrate (Cook & Cleland, 1981). The small $^{D}(V/K)$ for acetolactate precludes the use of this test. The ordered mechanism is in agreement with the mechanism proposed for the enzyme from *S. typhimurium* (Shematek et al., 1973). In fact the values for the steady-state constants for the enzyme from these two sources are very similar. We additionally report that the proposed enzyme intermediate, 3-hydroxy-3-methyl-2-oxobutyrate, is kinetically competent in the reaction. The V/E_T for this substrate at saturating NADPH and Mg^{2+} concentrations is about 1.7 times larger than the V/E_T for the partially resolved acetolactate [this is true even when we correct for inhibition of the enzyme by the small amount of (2*R*)-acetolactate present in our substrate stock]. The reverse reaction, oxidation and isomerization of 2,3-dihydroxy-3-methylbutyrate, is very slow under the conditions of our assay. It is easy to understand why the initial reports on the enzyme reaction indicated that it was irreversible and why the kinetics of the reverse reaction have not been examined until now.

The behavior of the metal ion in the reaction is somewhat unusual. It is the uncomplexed metal and nucleotide that are the true substrates for the reaction. In accord with this is the fact that double-reciprocal plots of initial rate vs concentration of the magnesium–NADPH complex do not produce a single straight line. In addition, linear double-reciprocal plots result when the concentration of uncomplexed NADPH is considered. On the basis of the inhibition data, there is a random addition of metal ion and nucleotide to the enzyme. This ternary complex is then competent in the binding of acetolactate. We report a slight curvature in the double-reciprocal plot using Mn^{2+} as the variable substrate at less than saturating levels of NADPH. At saturating levels of NADPH, linear double-reciprocal plots are found. The apparent cooperativity may simply be a manifestation of the randomness in the binding of metal and nucleotide to the enzyme. Shematek reports a slight cooperativity in the binding of NADPH (Shematek et al., 1973). These data are based on the equilibrium binding of the nucleotide to the enzyme as monitored by fluorescence changes. It is not clear from the data whether or not Mg^{2+} was present during these studies. If the metal was present at less than saturating levels, the apparent cooperativity could be due to a slight difference in NADPH binding affinity dependent on the binding status of Mg to the enzyme. This would then indicate that the random addition of substrates was responsible for the nonlinearity observed in the steady-state kinetics. If, on the other hand, equilibrium binding studies do show distinct cooperativity, then that must account for the nonlinear kinetics. These studies are currently under way.

There is another odd aspect of the requirement for the metal ion in the reaction. The two different reactions catalyzed by the enzyme—isomerization and reduction—can be separated by using different metal ions. Mg^{2+} is competent in both the isomerization and reduction. Mn^{2+} will only catalyze the

Scheme I: Kinetic Mechanism of Ketol Acid Reductoisomerase at Saturating Metal Ion Concentration



reduction step. This occurs in both reaction directions. On the basis of our crude estimate by NMR and a more quantitative measurement using radiolabeled material (Arfin & Umbarger, 1969), HKIV is not a free intermediate when Mg^{2+} is used in the catalysis. However, in the presence of Mn^{2+} and diol, NADPH is produced while no acetolactate is observed. Likewise, Mn^{2+} will not catalyze the isomerization of HKIV to acetolactate in the presence of NADP. Mg^{2+} is competent in producing acetolactate in each case indicated. To our knowledge, this behavior is unique in enzymology. In most enzyme reactions where a magnesium ion is required, manganese will also be active. The different kinetic behavior with these two metals implies that the isomerization reaction requires a very precise orientation of the β-keto-α-hydroxy acid with respect to the metal and that this requirement for a precise orientation is relaxed for the reduction reaction. This might indicate that the isomerization reaction leads to a change in the chelation structure between the metal and the substrate. This argument requires that the metal interact directly with the substrate. This seems reasonable since no reaction, either isomerization or reduction, will occur in the absence of a metal ion. However, the possibility that the metal merely adjusts the conformation of the enzyme and does not interact directly with the substrate cannot be discounted.

The kinetic isotope effect studies show relatively small $^{D}(V/K)$ values for both acetolactate and HKIV. The $^{D}(V/K)$ for acetolactate is 1.07 and that for HKIV is 1.39, both determined in the reaction direction where NADPH is oxidized. Although we have not determined the kinetic mechanism for the reduction of HKIV, we assume that this follows an ordered mechanism. If the enzyme reaction occurs in two steps, isomerization followed by reduction, then the minimal mechanism at saturating metal ion concentrations can be represented by Scheme I (here A is NADPH, B is acetolactate, and C is 3-hydroxy-3-methyl-2-oxobutyrate). The $^{D}(V/K)$ is given by $(^{D}k_7 + c_f + c_r{}^{D}K_{eq})/(1 + c_f + c_r)$ (Schimerlik et al., 1977). For acetolactate c_f is given by $k_7/k_6(1 + k_5/k_4)$ while for HKIV c_f is k_7/k_{14}. The c_r is the same for both substrates and is given by k_8/k_9. The absence of an isotope effect on V/K for acetolactate indicates that reduction is not rate limiting for this substrate. Since a small but significant $^{D}(V/K)$ is observed for the reduction of the proposed intermediate, this indicates some step preceding reduction is rate limiting for acetolactate (k_7 much greater than k_6 or k_5). The result of this combination of rate constants would mean that the concentration of the intermediate HKIV in the steady state is very small and could account for the fact that none of the intermediate is released from the enzyme. As required by this combination of rate constants, it is apparent from the partitioning experiments that, in the presence of NADPH and magnesium ion, HKIV is reduced to DMB much faster than it isomerizes to acetolactate.

From the data presented, it appears that the enzyme ketol acid reductoisomerase catalyzes the conversion of acetolactate to 2,3-dihydroxy-3-methylbutyrate by a two-step mechanism. The first step is an alkyl migration that forms 3-hydroxy-3-methyl-2-oxobutyrate which is subsequently reduced to the diol. On the basis of the steady-state kinetic parameters and

deuterium KIE studies for acetolactate and HKIV, the reduction is not the rate-limiting step in the overall reaction. This study suggests that some step which precedes reduction is rate limiting in the forward reaction direction. Two likely candidates are the alkyl migration step or an isomerization of the metal chelate of the intermediate HKIV. These possibilities need to be investigated by other KIE studies on the natural and alternate substrates.

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