Purification and Properties of the Acetohydroxy Acid Isomeroreductase of *Salmonella typhimurium*

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SUMMARY

α-Acetohydroxy acid isomeroreductase, which catalyzes both the second step specific to valine biosynthesis and the third step specific to isoleucine biosynthesis, has been purified from derepressed *Salmonella typhimurium*. The enzyme appears to be homogeneous in the ultracentrifuge and by immunodiffusion in agar. The molecular weight, as determined by sedimentation equilibrium ultracentrifugation, is approximately 220,000. The enzyme is stereospecific for the B side of NADPH.

At pH 7.5, the optimal pH for the isomeroreductase, the reversibility of the reaction cannot be shown, but at higher values the reaction is readily reversible. The pH optimum of the reverse reaction is about 9.4.

The purified enzyme catalyzes the isomerization of α-keto-β-hydroxyisovalerate to α-acetolactate and the NADPH-linked reduction of α-keto-β-hydroxyisovalerate to α,β-dihydroxyisovalerate as well as the over-all conversion of α-acetolactate to α,β-dihydroxyisovalerate. The ratio of these activities remains constant during purification and the coenzyme and Mg++ requirements suggest that the isomerase and reductase activities are inherent properties of the enzyme. However, experiments with labeled substrate indicate that α-keto-β-hydroxyisovalerate is not a free intermediate of the over-all reaction.

The biosynthesis of valine and that of isoleucine proceed by a similar sequence of reactions, four of which are catalyzed by enzymes common to both sequences (1). One of these enzymes catalyzes the conversion of α-acetolactate to α,β-dihydroxyisovalerate and α-aceto-α-hydroxybutyrate to α,β-dihydroxy-β-methylvalerate the second step in valine biosynthesis and the third step in isoleucine biosynthesis, respectively (2).

CH₃-C-CH-COOH + NADPH + H⁺ ⇌
CH₃-C-COOH + NADP⁺

CH₃-C-CH-COOH + NADPH + H⁺ ⇌
C₆H₄

This conversion requires both an alkyl migration from 1 carbon atom to an adjacent one and a NADPH-dependent reduction. The enzyme catalyzing this reaction has been partially purified from *Escherichia coli* (2, 3), *Salmonella typhimurium* (4), *Neurospora crassa* (5), and *Phaseolus radiatus* (6).

The most generally accepted mechanism for the over-all conversion of the acetohydroxy acids to the α,β-dihydroxy acids is an initial acyloin rearrangement to the α-keto-β-hydroxy acids followed by reduction to the dihydroxy acids (7). However, a mechanism in which the acetohydroxy acids are first reduced and then undergo a pinacol type rearrangement or a concerted isomerization and reduction have not been ruled out. Previous studies with partially purified isomeroreductases from several sources have shown that neither anglyceric or tiglyceric acids, the direct reduction products, nor the α-keto-β-hydroxy acids, the rearranged but unreduced compounds, are substrates for the isomeroreductase under the conditions in which it is normally assayed (2-4, 8).

Crude extracts from a variety of organisms also convert α-keto-β-hydroxyisovalerate and α-keto-β-hydroxy-β-methylvalerate to the corresponding dihydroxy acids. This latter...
activity has been termed \( \alpha \)-keto-\( \beta \)-hydroxy acid reductase and has been separated from the isomeroreductase (3). The partially purified reductase from both \( N. \) crassa and \( E. \) coli shows no metal ion requirement and can utilize either NADPH or NADH as hydrogen donor. Armstrong and Wagner (9) reported that a partially purified isomeroreductase from \( S. \) typhimurium exhibited no reductase activity, but upon storage lost activity in the isomeroreductase assay with concomitant appearance of reductase activity. They suggested, therefore, that the reductase might be a modified form of the isomeroreductase which had lost the ability to isomerize the acetohydroxy acids.

In an effort to obtain information concerning the mechanism of action of this enzyme, we have begun a study of the physical and chemical properties of the isomeroreductase from \( S. \) typhimurium. This paper describes a procedure for the preparation of highly purified isomeroreductase and a more detailed study of some of the catalytic properties of the purified enzyme. The results described herein show that the enzyme has distinct \( \alpha \)-keto \( \beta \)-hydroxy acid isomerase and reductase activities in addition to the isomeroreductase activity previously recognized. The ratios of these activities to each other remain essentially constant throughout purification and all are absolutely dependent upon Mg\(^{++}\). Although these activities seem to be inherent properties of the isomero-reductase, other evidence presented below strongly suggests that the \( \alpha \)-keto-\( \beta \)-hydroxy acids are not free intermediates in the over-all conversion of the acetohydroxy acids to the dihydroxy acids.

**EXPERIMENTAL PROCEDURE**

**Reagents**—\( \alpha \)-Acetolactate was synthesized and prepared for use as a substrate as described by Kranpitz (10). \( \alpha \)-Aceto-\( \alpha \)-hydroxybutyrate was prepared in an exactly analogous manner except that the ethyl-substituted acetooacetic ester was used. \( \alpha \)-\( \beta \)-Dihydroxyisovalerate and \( \alpha \)-\( \beta \)-dihydroxy-\( \beta \)-methylvalerate were synthesized according to Sjolander et al. (11). These compounds were prepared by Reef Laboratories (Lafayette, Indiana). \( \alpha \)-Keto \( \beta \)-hydroxyisovalerate was prepared according to Radhakrishnan, Wagner, and Snell (3). The following were obtained commercially: oxidized and reduced NADP and NAD (Sigma and P-L Biochemicals); \( \alpha \)-methylacetate and isovalerate (Eastman Organic Chemicals); \( \alpha \)-hydroxybutyrate, \( \alpha \)-hydroxy-\( \alpha \)-methylbutyrate, \( \alpha \)-hydroxyisovalerate, \( \beta \)-hydroxyisovalerate, and 3-hydroxy-3-methyl-2-butanone (K and K Laboratories); \( \alpha \)-ketoisovalerate and \( \alpha \)-keto-P-valerate (Sigma); lead acetate, creatine hydrate, and \( \alpha \)-naphthol (Fisher); cetyl trimethyl ammonium bromide (Matheson, Coleman, and Bell); enzyme grade (\( \text{N} \)\( \text{I} \)\( \text{L} \))\( \text{SO}_4 \) (Mann); glucose 6-phosphate, di-sucrose, citrate, glucose 6-phosphate dehydrogenase, isocitric dehydrogenase, and glutamate dehydrogenase (Calbiochem). Carboxymethyl and DEAE-cellulose were purchased from Bio-Rad and Sephadex G-10 from Pharmacia.

\( ^3 \)C-\( \alpha \)-Acetolactate and \( ^3 \)H-\( \alpha \)-acetolactate, both labeled specifically in the \( \alpha \)-methyl group, were prepared by New England Nuclear following the procedure of Krampitz (10) and with radioactive methyl iodide in the condensation with acetooacetic ester. Small amounts of radioactive impurities were present in the samples but they were readily removed by chromatography on a DEAE-Sephadex column (1.2 \( \times \) 10 cm) equilibrated with 0.01 \( \mu \) potassium phosphate, pH 7.5. The radioactive contaminants were not adsorbed and the \( \alpha \)-acetolactate was eluted after 80 ml of starting buffer had passed through the column.

**Assay of \( \alpha \)-Acetohydroxy Acid Isomeroreductase Activity**—Five micromoles of \( \alpha \)-acetohydroxy acid, 2 \( \mu \) moles of MgCl\(_2\), 0.05 \( \mu \) mole of NADPH, 100 \( \mu \) moles of potassium phosphate (pH 7.5), and enzyme in a final volume of 0.5 ml were incubated at 30°. The NADPH was added last and the change in absorbance at 340 m\( \mu \) measured with a Gilford model 2000 multiple sample absorbance recorder. One unit of isomeroreductase activity is defined as the amount required to oxidize 1 \( \mu \) mole of NADPH per min under the standard assay conditions.

**Assay of \( \alpha \)-Keto-\( \beta \)-hydroxy Acid Reductase Activity**—Reductase activity at pH 7.5 was assayed essentially as described by Radhakrishnan et al. (3). Ten micromoles of \( \alpha \)-keto-\( \beta \)-hydroxyisovalerate, 0.1 \( \mu \) mole of NADPH, 100 \( \mu \) moles of potassium phosphate (pH 7.5), and enzyme in a final volume of 0.5 ml were incubated at 30°. The NADPH was added last and the change in absorbance at 340 m\( \mu \) measured with a Gilford model 2000 multiple sample absorbance recorder. One unit of reductase activity is defined as the amount required to oxidize 1 \( \mu \) mole of NADPH per min under these conditions. For the assay of reductase activity at pH 9.4, the potassium phosphate was replaced by 100 \( \mu \) moles of carbonate-bicarbonate buffer and 2 \( \mu \) moles of MgCl\(_2\) were added to the reaction mixture.

**Assay of \( \alpha \)-Keto-\( \beta \)-hydroxy Acid Isomerase Activity**—The conversion of \( \alpha \)-keto-\( \beta \)-hydroxyisovalerate to \( \alpha \)-acetolactate was followed by measuring acetoin formed after deoxycarboxylation as described below.

**Other Assays**—\( \alpha \)-Acetolactate was determined quantitatively after conversion to acetoin by incubating with 5% H\(_2\)SO\(_4\) at 37° for 30 min. Acetoin, in turn, was assayed by the Westerfeld method (12). The dihydroxy acids were determined by the procedure of Adelberg (13). \( \alpha \)-Keto-\( \beta \)-hydroxyisovalerate was determined as the 2,4-dinitrophenylhydrazine derivative. Protein was measured by the procedure of Lowry et al. (14). Radioactive samples were dissolved in Bray's scintillation fluid (15) and counted in a Nuclear-Chicago scintillation counter, model 725.

**Immunization**—Purified isomeroreductase, 3.1 mg, emulsified in 1 ml of Freund's complete adjuvant (Difco) was injected subcutaneously into male Purdue Dutch rabbits weighing 1.5 kg. Blood was collected prior to immunization, 3 weeks after injection, and at 10-day intervals thereafter. Serum, obtained by centrifugation, was fractionated with ammonium sulfate (0 to 50%), dialyzed against 0.9% NaCl, and stored at -20°.

**Ouchterlony Technique**—Immunodiffusion analysis by the method of Ouchterlony (16) was performed in 1% agar containing 0.9% NaCl. Plates were incubated at room temperature for 48 hours and then at 4° for 10 days.

**Ultracentrifuge Studies**—Sedimentation velocity and equilibrium experiments were carried out with a Spinco model E ultracentrifuge equipped with schlieren optics, interference optics, and a rotor temperature indicator control unit. Sedimentation equilibrium experiments were performed with a multichannelled cell and the meniscus depletion method of Yphantis (17). All fringe displacements were corrected for window distortion with water blanks.
Friedkin (18) and by gel filtration on a Sephadex G-10 column (1.4 × 50 cm) equilibrated with 0.1 M potassium phosphate, pH 7.0. To determine the stereospecificity of the isomeroreductase with respect to the pyridine nucleotide the following procedures were used. NADPH-4-3H was prepared by incubating 0.3 μmoles of NADP-4-3H, 7.5 μmoles of MgCl₂, 6 μmoles of NADP, 300 μmoles of potassium phosphate (pH 7.0), and 0.04 μg (0.07 unit) of pig heart isocitric dehydrogenase in a final volume of 3 ml at 37° until all of the NADP was reduced. The isocitric dehydrogenase was inactivated by heating at 70° for 2 min and the NADPH-4-3H was reoxidized by adding 15 μmoles of NADPH-4-3H and 10 μl of purified isomeroreductase and reincubating at 37°. NADPH-4-3H was prepared and reoxidized in a similar fashion except that theReaction was performed by adding 6 μmoles of glucose 6-phosphate and 0.01 mg (1.4 units) of yeast glucose 6-phosphate dehydrogenase and the MgCl₂ was present only during the reoxidation step.

Since the NADP-4-3H was prepared by reduction with sodium borohydride-3H it was possible that some labeling of positions 2 and 6 of the pyridine ring had also occurred. An estimate of the amount of tritium present at position 4 was obtained by preparing NADPH-4-3H with the isocitrate dehydrogenase system as described above and reoxidizing with bovine heart glutamate dehydrogenase. Since the NADP-4-3H was prepared by reduction with sodium borohydride-3H it was possible that some labeling of positions 2 and 6 of the pyridine ring had also occurred. An estimate of the amount of tritium present at position 4 was obtained by preparing NADPH-4-3H with the isocitrate dehydrogenase system as described above and reoxidizing with bovine heart glutamate dehydrogenase, an enzyme stereospecific for the B side of the pyridine ring (19). The reoxidation was performed by adding 32 μmoles of α-ketoglutarate, 600 μmoles of ammonium acetate, and 0.04 mg (0.12 unit) of bovine heart glutamate dehydrogenase.

After reoxidation was complete, the reaction mixtures were heated at 70° for 2 min and 20 μmoles of the appropriate dihydroxy acid or of glutamic acid were added to the mixtures. The heat-precipitated protein was removed by centrifugation and the supernatant fluids were evaporated to dryness under reduced pressure. They were then dissolved in minimal amounts of distilled water and chromatographed on a Sephadex G-10 column, 1.4 × 50 cm, equilibrated with distilled water; 0.5-ml fractions were collected. NADP was recovered in Fractions 40 to 47. The dihydroxy acids as well as glutamic acid were eluted between Fractions 60 and 70. The glutamic acid was further purified by chromatography on Dowex 1-acetate (20). The dihydroxy acids were adsorbed on Dowex 1-formate columns and eluted with 1 N formic acid.

### RESULTS

**Enzyme Purification**

A summary of the results of a typical purification procedure is given in Table I. Unless otherwise stated all of the steps were carried out at 0-4°. All buffers contained 10⁻³ M MgCl₂ and 3 × 10⁻³ M mercaptoethanol.

**Growth of Cells and Preparation of Crude Extract**—The enzymes of isoleucine and valine biosynthesis are subject to multivalent repression by isoleucine, valine, and leucine (21). It is advantageous to increase the specific activity of the crude extract by using an organism auxotrophic for isoleucine and valine and growing the cells on a limiting amount of one of these amino acids. The isomeroreductase is highly derepressed when the auxotrophs are grown on limiting amounts of valine. The organism used in these studies was strain MD10 of S. typhimurium, which lacks transaminase B. Derepression of 6- to 8-fold over wild type levels was routinely achieved by growing the organism at 37° with vigorous aeration in Davis-Mingioli medium (22), in which citrate was omitted and the glucose concentration increased to 0.5%, supplemented with 5 × 10⁻⁴ M L-leucine, 5 × 10⁻⁴ M L-isoleucine, and 1.5 × 10⁻⁴ M glyceryl-L-valine. Under these conditions the concentration of valine is the factor limiting growth. The cells were harvested by centrifugation at 10,000 × g after 10 to 14 hours of growth. The cell pellets were resuspended in one-tenth the growth volume of 0.05 M potassium phosphate, pH 8.0, and again centrifuged. The washed cells were used immediately for preparation of the extracts or were stored frozen at ~20° prior to use.

Cells were suspended in 3 volumes of 0.01 M potassium phosphate, pH 7.0, and disrupted in 40-ml batches by sonic oscillation with a 75-watt Branson Sonifier at full power for 5 min. Cell debris was removed by centrifugation at 10,000 × g for 30 min.

**Treatment with Cetyl Trimethyl Ammonium Bromide**—One-fourth volume of 2% (w/v) cetyl trimethyl ammonium bromide (because of the limited solubility of cetyl trimethyl ammonium bromide, the solution was warmed and then used at room temperature) was added slowly with stirring to the crude extract (in an ice bath). After stirring for 15 min, the suspension was centrifuged at 25,000 × g for 40 min and the residue was discarded.

**Treatment with Lead Acetate**—A 0.1 M lead acetate solution was added slowly with constant stirring to the cetyl trimethyl ammonium bromide solution.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total activity</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>144</td>
<td>113</td>
<td>3528</td>
<td>0.032</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>Cetyl trimethyl ammonium bromide supernatant</td>
<td>165</td>
<td>100</td>
<td>1140</td>
<td>0.006</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Pb(C₂H₅O₂)₂ supernatant</td>
<td>171</td>
<td>102</td>
<td>708</td>
<td>0.144</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fractionation</td>
<td>10</td>
<td>80</td>
<td>290</td>
<td>0.333</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>Carboxymethyl cellulose fractionation</td>
<td>48</td>
<td>50</td>
<td>39</td>
<td>1.282</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Crystals</td>
<td>2</td>
<td>38</td>
<td>20.8</td>
<td>1.827</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Re crystallization</td>
<td>2</td>
<td>29.4</td>
<td>15.4</td>
<td>1.910</td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>

* The substrate is α-acetolactate.
* The ratio refers to the rate with α-acetolactate as substrate compared to the rate with α-acetolactate as substrate.
ammonium bromide supernatant to give a final concentration of 0.01 M lead acetate. The suspension was immediately centrifuged at 25,000 × g for 30 min and the precipitate was discarded.

Ammonium Sulfate Fractionation—(NH₄)₂SO₄, saturated at 4° and adjusted to pH 7.5 with NH₄OH, was added to the lead acetate supernatant to give a final saturation of 40%. After standing for 30 min, the precipitate was centrifuged and discarded. The supernatant fluid was brought to 60% saturation by a further addition of saturated (NH₄)₂SO₄ solution. After standing for 1 hour, the precipitate was collected by centrifugation and dissolved in a minimal volume of 0.01 M sodium acetate, pH 5.6.

Carboxymethyl Cellulose Chromatography—Carboxymethyl cellulose was washed (23) and equilibrated with 0.01 M sodium acetate, pH 5.6, and packed into a column (2.5 × 35 cm). The 0 to 60% ammonium sulfate fraction was placed on this column and, after adsorption of the protein, the column was washed with 1 to 2 column volumes of 0.01 M sodium acetate (pH 5.6) and then eluted with a linear gradient of sodium acetate. The mixing chamber contained 275 ml of 0.01 M sodium acetate, pH 5.6, and the reservoir contained 275 ml of 0.22 M sodium acetate, pH 5.6. The flow rate was 30 to 40 ml per hour. The enzyme was eluted after about 250 ml of the gradient had passed through the column and was usually contained in 30 to 50 ml.

Crystallization from Ammonium Sulfate—Crystallization was achieved by the general method of Kohn and Jakoby (24). The pooled carboxymethyl cellulose fractions were made 65% saturated with (NH₄)₂SO₄ by the addition of a saturated solution and after standing for 1 hour the precipitate was collected by centrifugation. The precipitate was consecutively extracted with small volumes of a solution containing 0.05 M potassium phosphate (pH 7.5), 10⁻³ M MgCl₂, 3 × 10⁻⁴ M mercaptoethanol, and decreasing concentrations of (NH₄)₂SO₄ over the range of 68 to 46% saturation (4°). After standing at room temperature for several hours, crystals appeared in the samples extracted with 50 to 54% saturated (NH₄)₂SO₄.

Recrystallization—The crystals from the preceding step were collected by centrifugation at 25° and redissolved in 54% (NH₄)₂SO₄ in the cold and stored at 4°. In 1 to 2 days recrystallization was complete. The recrystallized enzyme had a specific activity about 60-fold greater than crude extracts of derepressed S. typhimurium. The final specific activity was 400 to 500 times greater than the specific activity of the enzyme in crude extracts of wild type S. typhimurium. The over-all yield was reproducibly between 25 and 35%. At pH values above 5.3, the enzyme was quite stable when kept at 4° and in the presence of MgCl₂ and mercaptoethanol, so that the procedure could be interrupted at any point.

Criteria of Purity

The recrystallized enzyme appears to be a homogeneous protein. No increase in specific activity was obtained by chromatography on Sephadex G-200 or DEAE-cellulose.

Sedimentation Velocity—Fig. 1 is a series of photographs of the sedimentation pattern of the recrystallized isomeroreductase. The enzyme sedimented as a single component with a symmetrical schlieren peak at all concentrations tested. An s₂₀,ₚ of 9.0 S was obtained.

Immunological Criteria—When examined by the Ouchterlony immunodiffusion technique, antiserum prepared against recrystallized isomeroreductase formed only one precipitin band and showed a reaction of identity with crude and partially purified enzyme preparations (Fig. 2), suggesting that the purified isomeroreductase was immunologically homogeneous.
Previous work with a partially purified isomeroreductase from *S. typhimurium* (4) had established that the activity with \(\alpha\)-acetol-\(\alpha\)-hydroxybutyrate was about 5 times greater than that with \(\alpha\)-acetolactate. Our results confirm this finding (Table I). Furthermore, we have found that the recrystallized enzyme had an absolute requirement for added \(\text{Mg}^{2+}\) at an optimal concentration of \(3 \times 10^{-3}\) M and was specific for NADPH as coenzyme. The optimal pH for activity in the forward direction was 7.5 (Fig. 4).

### Stoichiometry

\(\alpha\)-Acetolactate, 25 \(\mu\)moles, and 15 \(\mu\)moles of NADPH were incubated with an excess of enzyme until 9.7 \(\mu\)moles of NADPH had been oxidized. The reaction was terminated by the addition of 0.1 volume of 50% \(\text{H}_{2}\text{SO}_4\) and the amounts of \(\alpha\)-acetolactate remaining and dihydroxyisovalerate formed were determined. Data for the stoichiometry of the reaction are shown in Table II.

### Reversibility

In agreement with the results of Armstrong and Wagner (4), attempts to show a dihydroxy acid-dependent reduction of NADP\(^+\) at pH 7.5 were unsuccessful. However, it was found that the reaction was readily reversible at higher pH values. The optimal pH for the reverse reaction was found to be 9.4 (Fig. 5). Table III shows the stoichiometry between the amount of NADP\(^+\) reduced and \(\alpha\)-acetolactate formed when \(\alpha,\beta\)-dihydroxyisovalerate was the substrate for the reverse reaction. At saturating levels of dihydroxy acid, the activity with \(\alpha,\beta\)-dihydroxy \(\beta\)-methylvalerate as substrate was 4 to 5 times greater than the activity with \(\alpha,\beta\)-dihydroxyisovalerate as substrate. Just as NADH could not be substituted for NADPH in the forward direction NAD did not substitute for NADP in the reverse reaction.

### Inhibitors

A number of structurally related compounds were tested for inhibition of the conversion of \(\alpha\)-acetolactate to \(\alpha,\beta\)-dihydroxyisovalerate. Table IV lists those compounds which were inhibitory. Compounds which inhibited less than 10% when tested at 0.01 M concentration included \(\alpha\)-hydroxybutyrate, isovalerate, \(\alpha\)-ketovalerate, acetoin, and \(3\)-hydroxy-3-methyl-2-butanoate. A plot of reciprocal velocity against reciprocal \(\alpha\)-acetolactate concentration indicated the inhibition by \(\alpha\)-methylvalerate to be competitive (Fig. 6).

### Table II

**Stoichiometry between \(\alpha\)-acetolactate and NADPH utilization and dihydroxyisovalerate formation**

<table>
<thead>
<tr>
<th>(\alpha)-Acetolactate utilized</th>
<th>NADPH oxidized</th>
<th>Dihydroxyisovalerate formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\mu)moles</td>
<td>(\mu)moles</td>
<td>(\mu)moles</td>
</tr>
<tr>
<td>10.0</td>
<td>9.7</td>
<td>9.8</td>
</tr>
</tbody>
</table>

Fig. 3. Sedimentation equilibrium data for the purified isomeroreductase at an initial protein concentration of 0.06%. The buffer was 0.01 M potassium phosphate, pH 7.5. Centrifugation was at 15,220 rpm and 25°. Equilibrium was attained after 24 hours. Log c represents the log of the fringe displacement in arbitrary units and z represents the distance, in centimeters, from the center of rotation.

Fig. 4. Effect of pH on isomeroreductase activity. The substrate is \(\alpha\)-acetolactate; the buffer is potassium phosphate. Specific activity equals units per mg of protein.

Molecular Weight

The enzyme, at several concentrations, was subjected to equilibrium centrifugation by the meniscus depletion method of Yphantis (17). Fig. 3 illustrates a plot of the log of the fringe displacement against the distance from the center of the rotor for one such experiment. These plots are linear at protein concentrations of 0.03% and 0.06%. Assuming a partial specific volume \((\tilde{v})\) of 0.74 ml per g, the apparent weight average molecular weight of the isomeroreductase was found to be approximately 220,000.
Fig. 5. Effect of pH on reversibility of the isomeroreductase reaction. The substrate is \( \alpha,\beta \)-dihydroxy-\( \beta \)-methylvalerate; the buffer is carbonate-bicarbonate. Specific activity equals micromoles of NADP reduced per min per mg of protein.

TABLE III

Stoichiometry between amount of NADP\(^+\) reduced and \( \alpha \)-acetolactate formed

The incubation mixtures contained 20 \( \mu \)moles of \( \alpha,\beta \)-dihydroxy-isovalerate, 1 \( \mu \)mole of NADP\(^+\), 1.5 \( \mu \)moles of MgCl\(_2\), 100 \( \mu \)moles of Na\(_2\)CO\(_3\)-NaHCO\(_3\) buffer (pH 9.4), and purified enzyme in a total volume of 0.5 ml. The time of incubation was 25 min.

<table>
<thead>
<tr>
<th>Experiment and isomeroreductase added</th>
<th>NADP reduced</th>
<th>( \alpha )-Acetolactate formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu )l</td>
<td>( \mu )moles</td>
<td>( \mu )moles</td>
</tr>
<tr>
<td>1.</td>
<td>20</td>
<td>0.023</td>
</tr>
<tr>
<td>2.</td>
<td>50</td>
<td>0.058</td>
</tr>
</tbody>
</table>

TABLE IV

Inhibitors of isomeroreductase activity

The conditions of incubation are as described in "Experimental Procedure."

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )-Methylactate</td>
<td>0.001</td>
<td>61</td>
</tr>
<tr>
<td>( \alpha )-Keto-( \beta )-hydroxyisovalerate</td>
<td>0.001</td>
<td>58</td>
</tr>
<tr>
<td>( \alpha )-Hydroxybutyrate</td>
<td>0.01</td>
<td>48</td>
</tr>
<tr>
<td>( \alpha )-Hydroxy-( \alpha )-methylbutyrate</td>
<td>0.01</td>
<td>45</td>
</tr>
<tr>
<td>( \alpha )-Hydroxyisovalerate</td>
<td>0.01</td>
<td>38</td>
</tr>
<tr>
<td>( \alpha )-Ketoisovalerate</td>
<td>0.01</td>
<td>21</td>
</tr>
</tbody>
</table>

Stereospecificity for NADPH

The stereospecificity of the isomeroreductase with respect to the hydrogen atom at position 4 of NADPH was determined by incubating the \( \alpha \)-acetoxyhydroxy acids with stereospecifically labeled NADPH as described under "Experimental Procedure."

The results of the experiments are shown in Table V. These results established that the hydrogen atom transferred to the dihydroxy acid came from position 4B of NADPH. Since only 50 to 60% of the tritium in the NADPH-4B-\(^{3}H\) was transferred to the dihydroxy acid, but none of the tritium was transferred from the NADPH-4A-\(^{3}H\), the possibility that the commercially obtained NADP\(^+\)-4-\(^{3}H\) was not labeled solely in position 4 was tested by reoxidizing the NADPH-4B-\(^{3}H\) with glutamate dehydrogenase, an enzyme of known B stereospecificity (19). The results show that glutamate dehydrogenase removed about the same percentage of tritium as did the isomeroreductase and suggest that the remainder of the radioactivity was most probably in positions 2 and 6 of the pyridine ring.

\( \alpha \)-Keto-\( \beta \)-Hydroxy Acid Reductase Activity

Crude extracts of \( S. \) typhimurium catalyzed an NADPH-dependent reduction of \( \alpha \)-keto-\( \beta \)-hydroxyisovalerate at pH 7.5. The rate of reduction was not affected by added Mg\(^{++}\) or by the addition of EDTA. These results confirm the observations of Radhakrishnan et al. (3) for the reductase of \( E. \) coli and \( N. \) crassa. Crude extracts of \( S. \) typhimurium were...
TABLE VI
Enzymatic specific activities during purification

The assay procedures are described under "Experimental Procedure." The purification steps are as in Table I. Specific activities are expressed as micromoles of NADPH oxidized per min per mg of protein.

<table>
<thead>
<tr>
<th>Step</th>
<th>Isoamoreductase, pH 7.5</th>
<th>Reductase, pH 7.5</th>
<th>Reductase, pH 9.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.081</td>
<td>0.028</td>
<td>0.003</td>
</tr>
<tr>
<td>2</td>
<td>0.064</td>
<td>0.037</td>
<td>0.009</td>
</tr>
<tr>
<td>3</td>
<td>0.118</td>
<td>0.073</td>
<td>0.011</td>
</tr>
<tr>
<td>4</td>
<td>0.220</td>
<td>0.060</td>
<td>0.022</td>
</tr>
<tr>
<td>5</td>
<td>0.707</td>
<td>0.040</td>
<td>0.076</td>
</tr>
<tr>
<td>6</td>
<td>1.623</td>
<td>0.0</td>
<td>0.106</td>
</tr>
<tr>
<td>7</td>
<td>1.875</td>
<td>0.0</td>
<td>0.188</td>
</tr>
</tbody>
</table>

FIG. 7. The effect of pH on the a-keto-b-hydroxyisovalerate reductase activity of the purified isomeroreductase. The assay was performed as described under "Experimental Procedure" with 3 µg of enzyme. Δ, Tris-HCl buffer; O, carbonate-bicarbonate buffer.

TABLE VII
Requirements for a-keto-b-hydroxyisovalerate isomerase activity

The complete system contained 10 µmoles of a-keto-b-hydroxyisovalerate, 1.5 µmoles of MgCl₂, 0.5 µmole of NADP, 75 µg of enzyme, and either 100 µmoles of potassium phosphate buffer (pH 7.5) or 100 µmoles of carbonate-bicarbonate buffer (pH 9.4) in a final volume of 0.4 ml. Incubation was for 20 min at 37°.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>pH 7.5</th>
<th>pH 9.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>66.0</td>
<td>0.7</td>
</tr>
<tr>
<td>Minus Mg²⁺</td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>Minus NADP, plus NADPH</td>
<td>11.8</td>
<td>42.0</td>
</tr>
<tr>
<td>Minus NADP, plus NAD</td>
<td>2.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Minus NADP, plus AP NADP</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Minus NADP, plus AP NADP</td>
<td>71.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Also observed to catalyze an NADPH-dependent reduction of a-keto-b-hydroxyisovalerate at pH 9.4. This activity was completely dependent upon added Mg²⁺ and was specific for NADPH as hydrogen donor. The purified enzyme also exhibited this pH 9.4 reductase activity, but had no pH 7.5 reductase activity. Table VI compares these two activities and the over-all isomeroreductase activity during the course of the enzyme purification. The ratio of isomeroreductase activity to the pH 9.4 reductase activity is constant throughout purification. The effect of pH on the activity of the purified enzyme in the reductase assay is shown in Fig. 7. The curve shows a broad range for optimal activity between pH 8.8 and 10.2.

α-Keto-b-Hydroxy Acid Isomerase Activity

The finding that the purified isomeroreductase could act as an α-keto-b-hydroxyisovalerate reductase suggested that it might also be able to catalyze the isomerization of α-keto-b-hydroxyisovalerate to α-acetolactate. Table VII shows that this was indeed the case. The isomerization required Mg²⁺ and a triphosphopyridine nucleotide. Of particular interest is the effect of pH on the nucleotide specificity. At pH 7.5, NADP was about 6 times as effective as NADPH at equimolar concentrations, whereas at pH 9.4 NADPH was essential for the isomerization reaction. At pH 9.4 in the presence of NADPH, some of the α-keto-b-hydroxyisovalerate was also reduced to the dihydroxy acid. Neither oxidized nor reduced NAD could be substituted at either pH, but the acetylpyridine analogue of NADP was as effective as NADP at pH 7.5. The specificity for the triphosphopyridine nucleotide coenzymes and the requirement for Mg²⁺ strongly suggest that the isomerase activity was, in fact, a property of the purified isomeroreductase. Figs. 8 and 9 show that the rate of isomerization bore a linear relationship to enzyme concentration and to the time of incubation for at least 30 min, when the reaction was conducted at pH 7.5.

Role of Ascorbic Acid

Recently, Allaudeen and Ramakrishnan (25) reported that ascorbic acid stimulated the conversion of the acetoxyacid to the dihydroxy acids when crude extracts of Mycobacterium tuberculosis were used as the source of enzyme. They further showed that, in the absence of NADP, the crude extracts would convert the acetoxyacid to the α-keto-b-hydroxy acids and that this activity, termed acetoxyacid activity.

FIG. 8. α-Keto-b-hydroxyisovalerate isomerase activity as a function of enzyme concentration. The reaction mixture contained 100 µmoles of phosphate buffer (pH 7.5), 10 µmoles of α-keto-b-hydroxyisovalerate, 1.5 µmoles of MgCl₂, 0.2 µmole of NADP, and enzyme, as indicated, in a final volume of 0.4 ml. The reaction mixtures were incubated at 37° for 20 min.

FIG. 9. Rate of formation of α-acetolactate from α-keto-b-hydroxyisovalerate. The reaction conditions were the same as described in the legend to Fig. 8, except that 6 µg of enzyme were used.
isomerases, was dependent upon added ascorbic acid. Isomerase activity was optimal at pH 7.5. No Mg\(^{++}\) was added to the assay system. The crude extracts also exhibited \(\alpha\)-keto-\(\beta\)-hydroxy acid reductase activity when assayed at pH 7.5 in the absence of Mg\(^{++}\). Earlier, Satyanarayana and Radhakrishnan (6) had observed that ascorbic acid stimulated the conversion of \(\alpha\)-acetohydroxybutyrate to \(\alpha,\beta\)-dihydroxy-\(\beta\)-methylvalerate but inhibited the conversion of \(\alpha\)-acetolactate to \(\alpha,\beta\)-dihydroxyisovalerate by a partially purified isomeroreductase from \(P.\) radiatus. The effect of ascorbic acid on the isomeroreductase from other sources has not been reported. Since the studies with the \(M.\) \(tuberculosis\) system were performed with crude extracts, it is not possible to tell whether the activities measured represented the catalytic properties of a single enzyme or the activities of three distinct enzymes. In view of their findings, however, it was of interest to test the effect of ascorbic acid on the purified isomeroreductase from \(S.\) \(tphimurium\). At concentrations of 1 to 20 \(\mu\)M, ascorbic acid had no effect on the rate of conversion of either \(\alpha\)-acetolactate or \(\alpha\)-acetohydroxybutyrate to the corresponding dihydroxy acids. The effect of ascorbic acid on the \(\alpha\)-keto-\(\beta\)-hydroxy acid isomerase activity was tested at both pH 7.5 and pH 9.4. Since ascorbic acid interfered with the determination of \(\alpha\)-acetolactate as acetoin, the reaction mixtures (after decarboxylation of \(\alpha\)-acetolactate to acetoin) were applied to columns \((0.6 \times 7\) cm\)) of Dowex 1-formate to adsorb the ascorbic acid. The columns were washed with sufficient water to yield a total effluent volume of 10 ml. Portions of the effluent were then assayed for acetoin. The results, given in Table VIII, show that ascorbic acid could not substitute for the required pyridine nucleotides and did not stimulate the isomerase activity of the purified enzyme when present in addition to the pyridine nucleotides.

Is \(\alpha\)-Keto-\(\beta\)-Hydroxyisovalerate a Free Intermediate?

Since the purified enzyme exhibited a number of catalytic properties that are consistent with \(\alpha\)-keto-\(\beta\)-hydroxyisovalerate being an intermediate in the reversible over-all conversion of \(\alpha\)-acetolactate to \(\alpha,\beta\)-dihydroxyisovalerate, attempts were made to show the accumulation of \(\alpha\)-keto-\(\beta\)-hydroxyisovalerate from \(\alpha\)-acetolactate or \(\alpha\)-acetohydroxybutyrate. Experiments were performed over the pH range 7.0 to 10.0 with many combinations and concentrations of the reaction components. Repeated efforts to find conditions that would lead to the formation of \(\alpha\)-keto-\(\beta\)-hydroxyisovalerate were unsuccessful.

A further attempt to show the existence of free \(\alpha\)-keto-\(\beta\)-hydroxyisovalerate was made by performing the enzymatic rearrangement and reduction of radioactive \(\alpha\)-acetolactate in the presence of a pool of unlabeled \(\alpha\)-keto-\(\beta\)-hydroxyisovalerate. If \(\alpha\)-keto-\(\beta\)-hydroxyisovalerate were a free intermediate, the specific radioactivity of the dihydroxyisovalerate formed should be considerably reduced, owing to dilution by the pool of unlabeled \(\alpha\)-keto-\(\beta\)-hydroxyisovalerate. Furthermore, incorporation of radioactivity into the reisolated \(\alpha\)-keto-\(\beta\)-hydroxyisovalerate pool should be observed. The composition of the reaction mixture in one such experiment is given in Table IX. The reaction was stopped by the addition of 0.1 volume of 30\% \(H_2SO_4\) and the reaction mixture was incubated for an additional 30 min to decarboxylate the residual \(\alpha\)-acetolactate to acetoin. The mixture was then neutralized and applied to a Dowex 1-formate column, \(1 \times 20\) cm. The acetoin was eluted with distilled water, the \(\alpha,\beta\)-dihydroxyisovalerate with 1 \(N\) formic acid and the \(\alpha\)-keto-\(\beta\)-hydroxyisovalerate with 1 \(M\) sodium formate. The results are given in Table IX. The enzymatically formed dihydroxyisovalerate had essentially the same specific radioactivity as the \(\alpha\)-acetolactate used. No radioactive \(\alpha\)-keto-\(\beta\)-hydroxyisovalerate was detected. These experimental results thus indicate that, during the enzymatic conversion of \(\alpha\)-acetolactate to dihydroxyisovalerate, \(\alpha\)-keto-\(\beta\)-hydroxyisovalerate did not accumulate as a free intermediate, nor did it exchange with an enzyme bound intermediate.

DISCUSSION

The studies reported here with a highly purified preparation of \(S.\) \(tphimurium\) isomeroreductase provide evidence that this protein also exhibits \(\alpha\)-keto-\(\beta\)-hydroxyisovalerate isomerase and reductase activities. The absolute dependence of the reductase activity upon Mg\(^{++}\) and the absence of activity at pH 7.5 serve to distinguish it clearly from the Mg\(^{++}\)-independent reductase activity recognized by Radhakrishnan et al. (3). The various interconversions catalyzed by the purified enzyme are shown in Fig. 10. Although experiments were not performed with \(\alpha\)-keto-\(\beta\)-hydroxy-\(\beta\)-methylvalerate, the isomer of \(\alpha\)-acetolactate hydroxybutyrate, which is also an isomeroreductase substrate, it seems probable that the purified enzyme would also utilize it as a substrate for both isomerization and reduction. The isomeroreductase therefore resembles malic enzyme (26) and the NADP-linked isocitric dehydrogenase (27-29) in possessing the ability to catalyze several separate reactions. The requirement for NADP or NADPH in the isomerase reaction even though no change in oxidation state occurs suggests that the nucleotide is required for substrate binding. In contrast, it has been possible
requires both Mg++ and coenzyme for the inactivation to occur. Furthermore, an analogue of the substrate which irreversibly inhibits the enzyme requires both Mg++ and coenzyme for the inactivation to occur. This requirement for the nucleotide has its parallels in other studies. Thus, Rutter and Lardy (26) have shown that the malic enzyme-catalyzed decarboxylation of oxaloacetate is markedly stimulated by NADP, and Rose (30) has shown that NADPH is required for the isocitric dehydrogenase-catalyzed exchange of hydrogen at the β-methylene group of α-ketoisovalerate. Also, NAD has been shown to be required for the arsenolysis and phosphate-exchange reactions catalyzed by glyceraldehyde phosphate dehydrogenase (31, 32). It has been postulated that for these enzymes the pyridine nucleotides provide a steric or an electronic environment in the active site that is necessary for activity (33). Similarly, in the case of the isomerase activity described here, it may be that the coenzyme is necessary for the enzyme to assume the particular conformation at pH 7.5 and with the reduced form at pH 9.4 is not readily apparent.

The demonstration of both isomerase and reductase activities in the purified protein suggests that the over-all reaction involves an enzyme-substrate complex in which rearrangement and reduction occur almost simultaneously. If such were the mechanism, the α-keto-β-hydroxy acids may not occur even as enzyme-bound intermediates, and the isomerase and reductase activities described here would simply represent interesting, but mechanistically unimportant, side reactions.

Acknowledgments—We wish to express our thanks to Dr. David Filmer who provided instruction and valuable assistance in the ultrafiltration studies.

REFERENCES

![Diagram](http://www.jbc.org/)

**Fig. 10.** Reactions catalyzed by acetohydroxy acid isomerase to show by direct binding experiments that the coenzyme binds in the absence of either substrate or Mg++. Furthermore, an analogue of the substrate which irreversibly inhibits the enzyme requires both Mg++ and coenzyme for the inactivation to occur. This requirement for the nucleotide has its parallels in other studies. Thus, Rutter and Lardy (26) have shown that the malic enzyme-catalyzed decarboxylation of oxaloacetate is markedly stimulated by NADP, and Rose (30) has shown that NADPH is required for the isocitric dehydrogenase-catalyzed exchange of hydrogen at the β-methylene group of α-ketoisovalerate. Also, NAD has been shown to be required for the arsenolysis and phosphate-exchange reactions catalyzed by glyceraldehyde phosphate dehydrogenase (31, 32). It has been postulated that for these enzymes the pyridine nucleotides provide a steric or an electronic environment in the active site that is necessary for activity (33). Similarly, in the case of the isomerase activity described here, it may be that the coenzyme is necessary for the enzyme to assume the particular conformation needed for binding α-keto-β-hydroxyisovalerate. The reason for the enhanced activity with the oxidized form of the coenzyme at pH 7.5 and with the reduced form at pH 9.4 is not readily apparent.

The demonstration of both isomerase and reductase activities in the purified protein suggests that the over-all conversion of the α-acetohydroxy acids to the α,β-dihydroxy acids involves an initial isomerization to the α-keto-β-hydroxy acid followed by an NADPH-dependent reduction to the dihydroxy acid. However, the failure to show the formation of α-keto-β-hydroxyisovalerate from either α-acetolactate or α,β-dihydroxyisovalerate under a variety of conditions and the failure to incorporate radioactive into an α-keto-β-hydroxyisovalerate pool from labeled α-acetolactate suggest that free α-keto-β-hydroxyisovalerate does not participate in the over-all reaction.

It seems possible, in view of the demonstrated catalytic properties of the enzyme, that the over-all reaction involves an enzyme-bound form of the α-keto-β-hydroxy acid as an intermediate and that this intermediate is much more rapidly converted to either the α-aceto-α-hydroxy acid or the α,β-dihydroxy acid than it equilibrates with free α-keto-β-hydroxy acid. However, it is not possible, at this time, to eliminate a concerted mechanism in which rearrangement and reduction occur almost simultaneously. If such were the mechanism, the α-keto-β-hydroxy acids may not occur even as enzyme-bound intermediates, and the isomerase and reductase activities described here would simply represent interesting, but mechanistically unimportant, side reactions.

The demonstration of both isomerase and reductase activities in the purified protein suggests that the over-all conversion of the α-acetohydroxy acids to the α,β-dihydroxy acids involves an initial isomerization to the α-keto-β-hydroxy acid followed by an NADPH-dependent reduction to the dihydroxy acid. However, the failure to show the formation of α-keto-β-hydroxyisovalerate from either α-acetolactate or α,β-dihydroxyisovalerate under a variety of conditions and the failure to incorporate radioactive into an α-keto-β-hydroxyisovalerate pool from labeled α-acetolactate suggest that free α-keto-β-hydroxyisovalerate does not participate in the over-all reaction.
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Stuart M. Arfin and H. Edwin Umbarger

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