Yeast $\alpha$-Isopropylmalate Isomerase

**FACTORS AFFECTING STABILITY AND ENZYME ACTIVITY***

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Yeast $\alpha$-isopropylmalate isomerase was found to be markedly stabilized by high concentrations of glycerol and (NH$_4$)$_2$SO$_4$. Such conditions of high ionic strength inhibited the enzyme, stabilized the enzyme to heat, and affected kinetic parameters. The isomerase was found to exhibit ionic strength-dependent hysteresis when enzyme, totally but reversibly inhibited by storage under conditions of high ionic strength of (NH$_4$)$_2$SO$_4$, was transferred to a lower concentration of (NH$_4$)$_2$SO$_4$. $\alpha$-Isopropylmalate isomerase was found to be sensitive to KCN and certain other chelators. The inactivation by KCN was prevented by high concentrations of (NH$_4$)$_2$SO$_4$. These observations implicated a metal involvement but the nature of the metal was not revealed. The metal involvement and some of the other properties of $\alpha$-isopropylmalate isomerase revealed a similarity to aconitase. The similarities in properties between the isomerase and aconitase are summarized. Studies of yeast $\alpha$-isopropylmalate isomerase indicated that it is a single polypeptide of about $M_t$ = 90,000.

$\alpha$-Isopropylmalate isomerase (2-isopropylmalate dehydratase, EC 4.2.1.33), the second enzyme specific for leucine biosynthesis, catalyzes the conversion of $\alpha$-isopropylmalate to $\beta$-isopropylmalate (2, 3). The enzyme is able to utilize the double-bonded intermediate dimethylcitraconate as substrate, although this compound, unlike $\alpha$- and $\beta$-isopropylmalates, does not accumulate in culture filtrates of the appropriate mutants (2). $\alpha$-Isopropylmalate isomerase has been demonstrated to be highly unstable in extracts from all organisms that have been thus far examined: Neurospora crassa (2, 4), yeast (5, 6), Escherichia coli (7), and Bacillus subtilis (8). However, the enzyme from N. crassa has been reported to be quite stable in vivo (9). The Neurospora enzyme has been partially purified and reported to be stable as an (NH$_4$)$_2$SO$_4$ precipitate (2).

Owing to its instability in all microbial sources examined, $\alpha$-isopropylmalate isomerase had not been examined extensively. Genetic studies suggested that the isomerase would be an interesting enzyme to investigate, for, based on genetic mapping in S. typhimurium (2, 7, 10) and E. coli (11, 12), it seemed to be the only branched chain amino acid biosynthetic enzyme for which two gene products were required, those of the leuC and leuD genes. In a prototrophic derivative of a leuD-deletion mutant of S. typhimurium a mutation in the supQ locus allowed the product of still another gene, newD, to substitute for the normally required leuD activity (13–15). The normal function of supQ or of newD is unknown. In yeast, five complementation groups were involved in determining $\alpha$-isopropylmalate isomerase activity (5).

High ionic strength hydrophobic chromatography (16), which allowed the separation of proteins under conditions of high ionic strength, were employed in order to obtain effectively stabilized purified enzyme for the investigation of some of its properties. Of interest was the investigation of the physical nature of yeast isomerase and the stabilization that resulted under high ionic strength conditions.
Yeast Isopropylmalate Isomerase

6.8, was dialyzed against 100 ml of a supplemented or non-supplemented 0.05 M potassium phosphate, pH 6.8, buffer. In all such experiments, the control was an identical undialyzed preparation stored at 0-5°. Glycerol and other polyol concentrations are expressed in terms of volume to volume.

All heat stability experiments were performed with a 50 to 65% (NH₄)₂SO₄ fraction obtained from extracts of late log cells of wild-type strain S288c as previously described (16). In most experiments the fraction was reconstituted in stabilizing buffer containing 0.05 M potassium phosphate, pH 6.8, 30% glycerol/1.24 M (NH₄)₂SO₄ and adjusted to a concentration of 8 to 9 mg of protein/ml. However, in the experiment shown in Fig. 4, the reconstitution buffer was one containing potassium phosphate only. The volume of the heated samples was 0.40 ml. For Figs. 3 and 4, separate samples were heated for the times indicated and, in addition, the activity in a control extract at 0° was assayed before, after, and sometimes during the heat stability experiment. At no time did the control extract in stabilizing buffer decrease in activity during the course of the experiment. Fumarsase was monitored as an additional control and proved to be more stable to heat than the isomerase.

Experiments testing the sensitivity of the isomerase to heat were performed by slowly raising the temperature of the samples in individual test tubes in a test tube rack with vigorous shaking and monitoring of the temperature. A water bath 8-10° above the temperature desired was used to raise the temperature. When the temperature rose to within 2-3° of that desired, the test tubes were placed in a constant temperature water bath for long term heating experiments. For short term experiments, the temperature was adjusted to the one desired. Extreme care was taken to avoid superheating the preparation. The heat treatment was terminated by placing the test tube in an ice water bath. Assays for enzyme activity were then performed within 30 s. At no time did a precipitate form that would have interfered with the assay.

Determination of Kinetic Parameters—Kinetic parameters were determined by measuring a-isopropylmalate isomerase activity of purified enzyme at 5 or 6 dimethylcitrate concentrations. Computer analysis of the initial velocity data was performed using the program "Hyper" (18, 19) adapted to the Purdue Remote On-line Computing System (PROCSY), a remote terminal support system for the CDC 6500 computer located at the Purdue University Computing Center. The program used the method of least squares in fitting the data to a rectangular hyperbola and to the double reciprocal transformation (20) of the Michaelis-Menten equation (21). Standard deviation values for the Kᵣ and Vᵣ values were also computed.

Disc Gel Electrophoresis—Electrophoretic procedures were performed as previously reported (16). Reagents—Bovine hemoglobin, yeast hexokinase, phenylmethylsulfonyl fluoride, p-phenanthroline, imidazole, Tris buffer, and the tetrasodium salt of ethylenediaminetetraacetic acid were obtained from Sigma Chemical Co. Sulfhydryl reagents were obtained from Calbiochem. The compound 8-hydroxyquinolinesulfonate was purchased from Eastman Organic Chemicals. Ovalbumin, Sephadex G-25, and Sepharose 4B were obtained from Pharmacia Fine Chemicals, Inc. Enzyme grade (NH₄)₂SO₄ from Nutritional Biochemicals Corp. and Ultrapure sucrose from Mann Research Laboratories were employed. Lysozyme was purchased from the latter company. Glycerol was obtained from Mallinkrodt Chemical Works. Bovine serum albumin was obtained from Pentex, Inc., and chromotripsinogen A and β-galactosidase from Worthington Biochemical Corp. RCN was purchased from Fisher Scientific Corp. Sodium dodecyl sulfate was obtained from J T Baker Chemical Co. Dimethylcitraconate and β-isopyruvylmalate were obtained from Reel Laboratories (Lafayette, Ind.). α-Isopropylmalate was a gift of Dr. G. B. Kohlhaw, Biochemistry Department, Purdue University. All other chemicals were reagent grade.

All microbiological media were obtained from Difco Laboratories. Yeast Strains—Bakers' yeast employed was obtained from Anheuser-Busch, Inc. Wild-type strain S288c of Saccharomyces cerevisiae was obtained from the Yeast Genetics Stock Center, Donner Laboratory, University of California, Berkeley. Calif.

RESULTS

Effect of Ionic Strength on Stability—Initial observations on α-isopropylmalate isomerase from bakers' yeast, yeast strain S288c, or Lindegren strain 00615 were similar: the activity in crude extracts was unstable but was stabilized by high protein concentration. As had been previously reported (5), the enzyme appeared sensitive to dilution. Furthermore, the reaction with α-isopropylmalate or β-isopropylmalate as substrate resulted in a constant reaction rate for an appreciable period, while the reaction with dimethylcitraconate produced a time course plot that deviated from linearity after 1 to 2 min. It appeared that the α-isopropylmalate isomerase was stabilized upon introduction into a standard reaction mixture.

In attempts to purify the enzyme it had been observed that an (NH₄)₂SO₄ fraction was destabilized upon passage of a preparation over a Sephadex G-25 column. The possibility that (NH₄)₂SO₄ or some dialyzable component stabilized the enzyme was considered. Indeed, dialysis of a stable (NH₄)₂SO₄ fraction against a buffer containing K₂SO₄ or (NH₄)₂SO₄ markedly decreased the rate of inactivation.

Dialysis of stable 50 to 65% (NH₄)₂SO₄ fractions prepared as described previously (16) against supplemented potassium phosphate buffers containing a range of concentrations of sulphydryl reagents, EDTA, or phenylmethylsulfonyl fluoride yielded half-lives for the enzyme of 2 to 3 hours. β-Isopyruvylmalate, glycerol, and (NH₄)₂SO₄ appeared to be agents that retarded the rate of inactivation (Table I) in such dialysis experiments. Dimethylcitraconate failed to increase the half-life of a dialyzed preparation, just as it failed to stabilize the enzyme in a reaction mixture, as α-isopropylmalate and β-isopyruvylmalate did. Increasing the (NH₄)₂SO₄ and increasing glycerol concentrations rendered the buffer increasingly effective in stabilizing the enzyme. A buffer containing 30% glycerol combined with β-isopyruvylmalate resulted in a half-life greater than that when either agent was used alone.

### Table I

<table>
<thead>
<tr>
<th>Supplement to 0.05 M potassium phosphate, pH 6.8</th>
<th>Half-life</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>2-3</td>
</tr>
<tr>
<td>1 mM dimethylcitraconate</td>
<td>2-3</td>
</tr>
<tr>
<td>1 mM β-isopyruvylmalate</td>
<td>14</td>
</tr>
<tr>
<td>1 mM α-isopropylmalate + 1 mM dimethylcitraconate</td>
<td>12</td>
</tr>
<tr>
<td>0.83 M (NH₄)₂SO₄</td>
<td>7</td>
</tr>
<tr>
<td>1.24 M (NH₄)₂SO₄</td>
<td>27</td>
</tr>
<tr>
<td>1.65 M (NH₄)₂SO₄</td>
<td>30</td>
</tr>
<tr>
<td>2.07 M (NH₄)₂SO₄</td>
<td>57</td>
</tr>
<tr>
<td>10% glycerol</td>
<td>7</td>
</tr>
<tr>
<td>30% glycerol</td>
<td>10</td>
</tr>
<tr>
<td>50% glycerol</td>
<td>50</td>
</tr>
<tr>
<td>30% glycerol + 1 mM β-isopyruvylmalate</td>
<td>46</td>
</tr>
<tr>
<td>5% glycerol + 0.41 M (NH₄)₂SO₄</td>
<td>5</td>
</tr>
<tr>
<td>10% glycerol + 0.41 M (NH₄)₂SO₄</td>
<td>7</td>
</tr>
<tr>
<td>20% glycerol + 0.83 M (NH₄)₂SO₄</td>
<td>24</td>
</tr>
<tr>
<td>30% glycerol + 1.24 M (NH₄)₂SO₄</td>
<td>90</td>
</tr>
<tr>
<td>30% glycerol + 0.83 M (NH₄)₂SO₄</td>
<td>90</td>
</tr>
<tr>
<td>30% glycerol + 1.24 M (NH₄)₂SO₄</td>
<td>-</td>
</tr>
</tbody>
</table>

*Enzyme diluted 1:100 in above buffer, not dialyzed.

Enzyme diluted 1:100 in unsupplemented buffer. not dialyzed

<table>
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<tr>
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<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% propranediol</td>
<td>10</td>
</tr>
<tr>
<td>30% propranediol + 30% saturated (NH₄)₂SO₄</td>
<td>93</td>
</tr>
</tbody>
</table>

* Months.
* Days.
After various combinations of glycerol and (NH₄)₂SO₄ were examined, it was found that a 30% glycerol buffer containing 1.24 m (NH₄)₂SO₄ resulted in stabilization (Table I). Partially purified enzyme diluted 100-fold and stirred in such a buffer displayed a half-life of 14 days in contrast to a half-life of 2 hours in a buffer consisting of potassium phosphate. Propanediol was found to be slightly effective in preventing inactivation, but produced a parallel though less marked stabilization when combined with (NH₄)₂SO₄. These data, along with observations on the Salmonella typhimurium enzyme, have indicated that α-isopropylmalate isomerase is markedly stabilized by polyols and Hofmeister (22) series salts in general.

Attempts to reverse the inactivation have failed. In a dialysis experiment, a rapid change from a buffer of high ionic strength in (NH₄)₂SO₄ to one of low ionic strength resulted in a process of inactivation that could be terminated but not reversed upon return to the original buffer. Furthermore, lyophilized dialysates have failed to influence enzyme activity when supplemented in a reaction mixture or dialysis buffer.

In agreement with the observation that the isomerase produced nonlinear kinetics as a result of enzyme inactivation in standard potassium phosphate/dimethylcitraconate reaction mixtures, such standard reaction mixtures containing enzyme showed decay of activity when initiated with substrate every 5 min (Fig. 1). However, a reaction mixture containing 13.2% glycerol and 0.55 m (NH₄)₂SO₄ resulted in not only inhibition but also reduced decay in activity. The plot of specific activity indicated inhibition by high ionic strength, while the plot of percentage of original activity at time zero indicated the extent of stabilization. When (NH₄)₂SO₄ and glycerol were included in the reaction mixture, isomerase activity was inhibited, but the reaction rate was constant.

Effect of pH on Stability—Since the isomerase was completely inhibited in pH 4.5 or 5.0, 20 m potassium acetate reaction mixtures, but could be reactivated by an adjustment of the pH to neutrality, the predicted correlation of inhibition, precipitation, and stabilization was tested. Partially purified 50 to 65% (NH₄)₂SO₄ fractions containing 5.0 to 6.0 mg of protein/ml were resuspended in 0.20 m potassium acetate, pH 5.0; 0.20 m potassium phosphate, pH 7.0; and 0.20 m Tris-HCl, pH 9.0. Buffers. Isomerase activity with dimethylcitraconate as substrate was monitored as a function of time (Fig. 2). Since the pH 5.0 preparation was a suspension, some sampling error appeared. The method of least squares was therefore used to plot the line representing percentage of original activity versus time in the case of the pH 5.0 treatment. At pH 5.0, the isomerase in suspension form was stabilized. The isomerase was labile at pH 7.0 and even more so at pH 9.0, both being conditions where no precipitation occurred. An exactly identical pattern was observed when the β-isopropylmalate activity of the enzyme was followed. A control activity, fumarase, which was co-purified in (NH₄)₂SO₄ fractions, proved to be completely stable at pH 7.0 and pH 9.0, but was rapidly inactivated at pH 5.0. Thus, as had been predicted and previously observed, the isomerase was more stable in the solid state, as a precipitate, or under conditions where the enzyme was inhibited but in solution.

Stability to Heat—Heat stability experiments were performed with the isomerase from wild-type strain E298c enzyme which was very similar in its properties to that from bakers’ yeast. Under conditions of high ionic strength, in the stabilizing glycerol/(NH₄)₂SO₄ buffer, the isomerase was quite stable to heat. The half-life at 55° was 4½ hours. With 1-, 4-, and 8-min exposure times a rapid transition occurred in the range of 60–65° yielding a temperature of 50% inactivation for a 1 min exposure time at 62° (Fig. 3). Perhaps relevant to this observation was the reported determination that the strength of the hydrophobic bond increases with increasing temperature up to approximately 80° (23). The heat inactivation process in such experiments was terminated upon return of the sample to 0° but was not reversed.

Under conditions of low ionic strength, in phosphate buffer without glycerol and (NH₄)₂SO₄, the isomerase was extremely sensitive to heat (Fig. 4). In this case, however, the untreated extract had a half-life of ⅓ hour. Activity after 1 min of heat treatment was expressed in terms of percentage of control activity, which was undergoing a slower decay during storage at 0°. The temperature for 50% inactivation was 23° compared to 62° in the previous experiment. Furthermore, exposure of enzyme to 55° for 5 s resulted in 100% inactivation, whereas isomerase under conditions of high ionic strength exhibited a half-life of 4½ hours at 55°.

pH Optimum—The pH optimum for purified bakers’ yeast isomerase was found to be 5.0, as shown in Fig. 5. Under these conditions, isomerase activity was 100% in comparison to the control activity. When the pH was lowered to 4.5 or 5.0, activity decreased to 50 and 15%, respectively. Activity in standard potassium phosphate stabilizing enzyme in dialysis buffer fraction containing 5.0 to 6.0 mg of protein/ml was not detectable. The reaction rate was constant at pH 5.0, as shown in Fig. 6. The enzyme was labile at pH 7.0 and even more so at pH 9.0, both being conditions where no precipitation occurred. An exactly identical pattern was observed when the β-isopropylmalate activity of the enzyme was followed. A control activity, fumarase, which was co-purified in (NH₄)₂SO₄ fractions, proved to be completely stable at pH 7.0 and pH 9.0, but was rapidly inactivated at pH 5.0. Thus, as had been predicted and previously observed, the isomerase was more stable in the solid state, as a precipitate, or under conditions where the enzyme was inhibited but in solution.
Yeast Isopropylmalate Isomerase

50 55 60 65
TEMPERATURE (°C)

FIG. 3. Stability to heat of yeast α-isopropylmalate isomerase under conditions of high ionic strength. Enzyme was exposed to each temperature for 1 (○), 4 (△), or 8 (▲) min. Activity was expressed as a percentage of the stable untreated control.

FIG. 4. Stability to heat of yeast α-isopropylmalate isomerase under conditions of low ionic strength. Enzyme was exposed to each temperature for 1 min. Activity was expressed as a percentage of the control which was undergoing a slow decay at 0°.

α-isopropylmalate isomerase was determined over a range of pH 4.0 to pH 9.0 utilizing 0.20 M potassium acetate, potassium phosphate, and Tris-HCl buffers. The pH curves obtained with all three substrates rose sharply at approximately pH 6.0 and displayed generally broad optima over the range of pH 7.0 to pH 9.0.

Effect of Ionic Strength on Kinetic Parameters—The effect of (NH₄)₂SO₄ on purified bakers' yeast isomerase was determined. Dimethylcitraconate concentration was varied in a standard reaction mixture and in a reaction mixture 0.40 M in (NH₄)₂SO₄. A plot of velocity versus substrate concentration suggested an altered Vₘₐₓ. A computer analysis of the data was used to determine Kₐ values for enzyme under the two conditions. In a standard reaction mixture, the Kₐ for dimethylcitraconate was found to be 2.16 ± 0.024 × 10⁻⁴ M (Vₘₐₓ = 0.975 ± 0.032 μmol/min/mg of protein). In a reaction mixture containing 0.40 M (NH₄)₂SO₄, the Kₐ for dimethylcitraconate was 2.37 ± 0.053 × 10⁻⁴ M (Vₘₐₓ = 0.536 ± 0.037 μmol/min/mg of protein). It was concluded that high ionic strength altered the Vₘₐₓ without significantly affecting the Kₐ. This result was reflected in a Lineweaver-Burk double reciprocal plot of the data (Fig. 5).

Inhibition by Salts—Purified isomerase from bakers' yeast was used to examine the inhibition of the enzyme by salts, especially salts high in the Hofmeister series. The salts chosen were KF, (NH₄)₂SO₄, potassium phosphate, and KCl. Their effects on the reaction with dimethylcitraconate as substrate (Fig. 6) and with β-isopropylmalate as substrate (Fig. 7) were examined. The ionic strength, μ, was plotted against the percentage of the activity that was observed with a standard reaction mixture.

The inhibition profiles for the case of dimethylcitraconate were quite similar to those for β-isopropylmalate. The order of effectiveness for the salts in inhibiting the isomerase was: KF > potassium phosphate > (NH₄)₂SO₄ > KCl. The order paralleled somewhat the Hofmeister series. The displacement of (NH₄)₂SO₄ was perhaps a result of the greater chaotropic effect of the NH₄⁺ ion than that of the K⁺ ion. Solubility limitations obviated the use of K₂SO₄.

When introduced into the reaction mixture, KF, (NH₄)₂SO₄, and potassium phosphate were each effective in promoting a linear, constant reaction rate, especially at higher salt concentrations. The indication was that potent inhibitors of the isomerase were potent stabilizers. KCl was ineffective in promoting a constant reaction rate, as it was least effective in inhibiting the enzyme.

An intriguing observation was a lag observed when enzyme was assayed with β-isopropylmalate as substrate under conditions of intermediate ionic strength of (NH₄)₂SO₄. Consequently, since initial velocities were determined, the β-isopropylmalate curve for (NH₄)₂SO₄ intercepted the abscissa at a lower ionic strength than did the dimethylcitraconate plot. This phenomenon, termed ionic strength-dependent hysteresis, was examined further with purified bakers' yeast isomerase.

Ionic Strength-dependent Hysteresis—The hysteretic response of the isomerase to decreased ionic strength could be clearly observed only at higher (NH₄)₂SO₄ concentrations. Consequently, the enzyme, which was routinely stored in the glycerol/(NH₄)₂SO₄ buffer, conditions that totally inhibited the enzyme, could be observed to undergo activation when the change in ionic environment was to one of intermediate ionic strength. A family of curves based on the time course of the enzyme reaction could be generated that illustrated increased inhibition and increased lag with increased (NH₄)₂SO₄ concentration (Fig. 8). The hysteretic response could be observed with β-isopropylmalate and α-isopropylmalate, but not dimethylcitraconate. β-Isopropylmalate was routinely employed in the
FIG. 6. Inhibition by various salts of yeast α-isopropylmalate isomerase with dimethylcitraconate as substrate. The salts that were examined are KF (○), potassium phosphate (△), (NH₄)₂SO₄ (●), and KCl (□).

FIG. 7. Inhibition by various salts of yeast α-isopropylmalate isomerase with β-isopropylmalate as substrate. The salts that were examined are KF (○), potassium phosphate (△), (NH₄)₂SO₄ (●), and KCl (□).

Investigation of the hysteretic response. From a family of curves the time to maximal velocity as a function of ionic strength could be determined (Fig. 9). The maximal lag was approximately 3 min. However, as can be seen from the family of curves in Fig. 8, a difficulty in estimating the lag period for a highly inhibited isomerase reaction mixture arose.

It became apparent that a change in ionic strength allowed slow conformational changes, the activation process. Therefore, based on the steep inhibition profile for the case of KF, one would not expect to observe and in fact could not observe a lag period in a KF reaction mixture. However, for isomerase in a potassium phosphate reaction mixture, ionic strength-dependent hysteresis could not be demonstrated either. A brief delay early in the enzyme reaction appeared within two cuvette dwell-times, approximately 10 s.

Sensitivity of Yeast α-Isopropylmalate Isomerase to Chelators—Purified bakers' yeast α-isopropylmalate isomerase was used to test the effect of the presence of agents that chelate metals in an isomerase reaction mixture. Previous experiments with partially purified isomerase from Neurospora (2, 4) indicated that this enzyme was insensitive to EDTA, o-phenanthroline, and α,α-dipyridyl at a concentration of 0.1 mM. Much higher protein concentrations were used to observe activity than would be necessary with purified enzyme. The stereochemistry of the aconitase and isomerase had been shown to be completely analogous in that the reactions occur by trans elimination, an apparent intramolecular transfer of hydrogen, and an addition of the elements of water. The study of the isomerase mechanism involved the determination of the configuration of α-isopropylmalate and β-isopropylmalate obtained from culture filtrates of Neurospora in addition to the study of label incorporation (24, 25). The study of the mechanism of aconitase involved crystallographic studies of its substrates (26) or was based on experiments that employed purified pig heart aconitase that was activated with Fe(NH₄)₂(SO₄)₃ prior to use (27). Despite identity in reaction mechanisms, unlike the isomerase, the aconitase has been clearly shown to have an Fe⁴⁺ requirement based on study of the substrates (28) and the enzyme (29-32). The enzyme was also found to be strongly inhibited by cyanide (33). Thus, an examination of the sensitivity of purified yeast α-isopropylmalate isomerase to chelating agents was deemed necessary. The yeast isomerase was found to be only slightly sensitive to certain chelators. As is shown in Table II, the chelators KCN, imidazole, and EDTA at a concentration of 1.0 mM in the reaction mixture had no effect on the initial velocity of the enzyme. The agents o-phenanthroline and 8-hydroxyquinoline-sulfonate inhibited the enzyme 16% at a concentration of 0.1 mM. Of great interest was an observation that the KCN reaction mixture produced a time course plot that deviated from the control plot as the reaction progressed.

Standard pH 7.0 reaction mixtures containing 0, 0.1, or 1.0 mM KCN when initiated with bakers' yeast isomerase displayed identical initial velocities, but the reaction rate decreased rapidly with time; the higher the KCN concentration the more rapid the quenching. This quenching was observed...
with all three substrates. The phenomenon with dimethylcitric acid as substrate is illustrated in a plot of optical density decrease versus time in Fig. 10. Enzyme that was inactivated quickly in the presence of 10 mM KCN and dimethylcitric acid could not be reactivated by the addition of the other substrate. Fumarase, which was often used as a control enzyme in partially purified preparations, was never observed to be affected by the highest KCN concentration tested. A structurally related salt, KSCN, had no effect at a concentration of 10 mM. KCN hastened the decay of isomerase activity during storage, but the effect could be retarded by high concentrations of (NH₄)₂SO₄.

Equilibrium of the Isomerase-catalyzed Reaction—In confirmation of the earlier study in the Neurospora enzyme (4), it was found that the equilibrium in vitro favored the reaction progressing in the direction opposite to that occurring during leucine biosynthesis. Interestingly, a similar observation had been made on the aconitase (34).

Molecular Weight Determination by Sucrose Density Centrifugation of Stabilized α-Isopropylmalate Isomerase—Sucrose density gradient centrifugation was performed in a Beckman model L-2 ultracentrifuge using a 25.1 rotor at a speed of 25,000 rpm for 40 hours in order to obtain molecular weight estimates. An S288c 50 to 65% (NH₄)₂SO₄ fraction was resuspended in 0.05 M potassium phosphate, pH 6.8/1 mM β-isopropylmalate/1.2 M (NH₄)₂SO₄/5% sucrose containing 5 mg of hemoglobin/ml and 0.40 ml were applied onto a gradient of total volume of 30 ml ranging in sucrose concentration from 10% to 20%. A similar procedure was used in subjecting isomerase to 25 to 40% glycerol or sucrose gradients with β-isopropylmalate, and (NH₄)₂SO₄. Glycerol gradient centrifugation performed in the absence of 1.2 M (NH₄)₂SO₄ failed to yield any detectable isomerase activity in fractions. The molecular weight was determined by the relationship of Martin and Ames (35). In agreement with the observations with α-isopropylmalate isomerase from Neurospora by Gross et al. (2) a molecular weight estimate based on multiple determinations was approximately 90,000.

Molecular Weight Determination of Sodium Dodecyl Sulfate-Denatured α-Isopropylmalate Isomerase—Molecular weight determinations by sodium dodecyl sulfate disc gel electrophoresis of purified S288c isomerase using standards of known molecular weight were performed. An estimate of approximately 90,000 was obtained from the isomerase. Interestingly, this molecular weight estimate was very similar to that reported for aconitase of 89,000 (30).

DISCUSSION

High concentrations of (NH₄)₂SO₄ and glycerol have marked effects on the properties of α-isopropylmalate isomerase, inhibition of activity (showing a decrease in Vₘₐₓ), protection from decay during storage, protection from heat inactivation, and protection from inactivation by chelating agents. These properties on stability and the opposing effects of glycerol and (NH₄)₂SO₄ on retention of the enzyme on valine-Sepharose and leucine-Sepharose columns were exploited in a purifica-
The effect of sulfate or other Hofmeister series salts on the α-isopropylmalate isomerase may be an effect on intramolecular interactions in the proteins. While such salts do affect intermolecular interaction between protein subunits, there is no evidence that the α-isopropylmalate isomerase of yeast contains more than a single polypeptide chain or that there are any quaternary interactions involved. Since the effects of the ionic strength are exhibited by the purified enzyme, it seems unlikely that ionic strength protects a region of the enzyme that interacts with another protein or a membrane to its detriment. However, the possibility that ionic strength would affect an interaction with other cell components in vivo or in crude extracts cannot be discounted. It should be noted that in yeast all of the enzymes of branched chain amino acid biosynthesis prior to the isomerase step have been shown to be associated with mitochondria (36, 37). No association of α-isopropylmalate isomerase or β-isopropylmalate dehydrogenase (also a labile enzyme in yeast), with mitochondria could be shown, however.

Although yeast α-isopropylmalate isomerase appears to be a single polypeptide of approximately 90,000 daltons, the possibility that more than one gene product is required for its activity in vivo is favored by the findings of several genetic loci necessary for its activity (5). Indeed, it is presently only known that sodium dodecyl sulfate gels reveal only a single size of polypeptide chain and that during centrifugation in stabilizing buffers there is no association to higher molecular weight forms. There is, however, a reversibly inactive form of the enzyme. It cannot yet be eliminated that two (or more) different Mₚ = 90,000 species associate into an enzymatically active form only under low ionic strength conditions. The more likely possibility is, however, that the additional genetic loci in yeast play some as yet unexplained regulatory or organizational role. Indeed, such a role might account for the second genetic locus found to affect isomerase activity in both Escherichia coli (11, 19) and S. typhimurium (9, 7, 10) and could be the basis of suppression of the leuD deletion mutants by the supQ and newD loci (13-15) in S. typhimurium.

Based on several observations, it appears that an environment of high ionic strength influences the intramolecular interactions of α-isopropylmalate isomerase. Since the enzyme when stored in stabilizing buffer is completely inhibited, it seems plausible that the state of the enzyme under such conditions could be compacted or partially "salted out." The stabilized compacted conformation can be activated when introduced into a reaction mixture of low ionic strength that allows unfolding and access to substrate. The enzyme in the unfolded conformation is more labile, and thus undergoes another transition to an inactive state.

Evidence for a conformational change is provided by an ionic strength-dependent hysteresis. The isomerase displays a lag when removed from stabilizing and inhibiting high salt conditions and subjected to conditions of intermediate ionic strength of (NH₄)₂SO₄. The activation period is several minutes and is dependent on α-isopropylmalate or β-isopropylmalate. The observation that dimethylcitraconate is ineffective in stabilizing the isomerase, unlike the hydroxyl-substituted substrates, is correlated with the fact that it does not induce the hysteretic response.

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Hysteretic enzymes are defined by Frieden (38, 39) as those enzymes that respond slowly (in terms of some kinetic characteristic) to a rapid change in ligand, either substrate or modifier concentration. The ligand may be a specific effector, as in the case of the hysteretic response of threonine deaminase to the addition of isoleucine during the reaction (40) or a proton, as in the case of the activation of phosphofructokinase by raising the pH from 6 to 7.5 (41). The former hysteretic response involves an isomerization, while the latter involves a polymerization. Most examples of hysteretic enzymes involve either conformational changes or molecular weight changes. A likely interpretation for the isomerase is that the activation process is one involving a gradual unfolding and deinhibition of the enzyme, and follows a rapid change in ionic strength, from a buffer of 30% glycerol/1.24 M (NH₄)₂SO₄ to one in the range of 0.60 to 1.0 M (NH₄)₂SO₄. Such a decrease in ionic strength results in a lag period of several minutes, the time for the enzyme to attain an active unfolded state that is accessible to α-isopropylmalate or β-isopropylmalate. A more extreme decrease in ionic strength of the reaction mixture presumably results in an activation too rapid to detect in a spectrophotometer.

The isomerase bears a similarity to aconitase with respect to molecular weight (30), stability (33, 34, 42), stabilization by glycerol (33, 42) and (NH₄)₂SO₄ (42), sensitivity to dialysis (43), insensitivity to EDTA (30), sensitivity to Cu²⁺, Hg²⁺, CN⁻, and S⁰ (2, 33), and reaction mechanism and stereochemistry (24-32). Both enzymes are followed by an irreversible step, one catalyzed by a dehydrogenase and involving an oxidative decarboxylation. Especially interesting is the fact that both the β-isopropylmalate dehydrogenase from yeast and the NAD-specific isocitrate dehydrogenase from yeast are cold-sensitive enzymes.

Other enzymes bear similarity to aconitase and the isomerase in some of their features. The homoaconitase of lysine biosynthesis in yeast, another enzyme catalyzing a hydration reaction, is inhibited by o-phenanthroline and n,tu-dipyridyl (44). A suspected ferrous ion requirement has not been demonstrated. Tartaric acid dehydrase from Pseudomonas putida can be stored as a stable (NH₄)₂SO₄ fraction (45). Passage of this preparation over a dithyminoethyl-cellulose column purifies the protein 3-fold but with a yield of less than 10%. However, the enzyme can be reactivated with ferrous ions and glutathione or cysteine. Aconitase isomerase, also from P. putida, catalyzes the interconversion of cis-aconitate and trans-aconitate. In the presence of 20% glycerol the rate of decay of the initial velocity is greatly decreased (46). It is suggested that glycerol stabilizes an undissociated form of the enzyme. E. coli dihydroxyacid dehydrase, an enzyme common to isoleucine and valine biosynthesis, has been found to be unstable in extracts. The enzyme has been reported to require cysteine and ferrous ions for activity (47).
Future studies on yeast α-isopropylmalate isomerase will elucidate the cofactor requirement of the enzyme and attempt to determine the roles of the multiple genes determining isomerase activity.

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