Crystalline L-Ribulose 5-Phosphate 4-Epimerase from Escherichia coli*

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SUMMARY

L-Ribulose 5-phosphate 4-epimerase has been crystallized from L-arabinose-induced cells of Escherichia coli B/r strain F' araB^-24/araB^-24 after a 40-fold purification. The enzyme is homogeneous in the ultracentrifuge and 98% pure by acrylamide gel disc electrophoresis. The molecular weight determined by sedimentation equilibrium is 1.03 ± 0.01 × 10^5. The enzyme is free of d-ribulose 5-phosphate 3-epimerase activity, does not exhibit any cofactor requirement, and shows maximal activity from pH 7 to 10. An amino acid analysis is presented.

In Escherichia coli B/r, L-ribulose 5-phosphate 4-epimerase (EC 5.1.3.3), which catalyzes the conversion of L-ribulose 5-phosphate to d-xylulose 5-phosphate, is the third enzyme in the pathway of L-arabinose metabolism (1).

\[
\text{L-Arabinose} \xrightarrow{\text{isomerase}} \xrightarrow{\text{kinase}} \text{L-ribulose} + \text{ATP} \rightarrow \text{L-ribulose 5-phosphate} + \text{ADP}
\]

\[
\text{L-Ribulose 5-phosphate} \xrightarrow{\text{4-epimerase}} \text{d-xylulose 5-phosphate}
\]

Genetic studies have shown that the structural genes for these enzymes are contiguous in the order B (L-ribulokinase), A (L-arabinose isomerase), and D (L-ribulose 5-phosphate 4-epimerase) (1). Located next to the B gene are the initiator region I and a regulatory gene C; the latter produces a protein which, in the presence of L-arabinose, causes the expression of genes B, A, D, and the L-arabinose transport system (2-4). The synthesis of L-arabinose isomerase, L-ribulokinase, and L-ribulose 5-phosphate 4-epimerase have been shown to be coordinate (3, 5). In order to understand the basis of this coordination we have undertaken an investigation of the structures of purified arabinose enzymes. This paper will describe a method for the purification and crystallization of L-ribulose 5-phosphate 4-epimerase and some of its physical and chemical properties. The purification and characterization of L-ribulokinase and L-arabinose isomerase have been previously reported (6, 7).

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions—L-Ribulose 5-phosphate 4-epimerase was isolated from cells of E. coli B/r F' araB^-24/araB^-24 (4) grown in casein hydrolysate L-arabinose medium. This mutant, selected for its increased enzyme production, has been cultured under similar conditions to serve as a source of L-arabinose isomerase (7). The growth medium had the following composition: K_2HPO_4-KOH (pH 7.0), 0.1% MgSO_4·7H_2O, 0.01%; (NH_4)_2SO_4, 0.1%; Casamino acids (Difco), 1%; and L-arabinose, 0.15%.

Thirty-six liters of the above medium were sterilized, cooled to 37°C, and inoculated with 4 liters of a log phase culture grown at 37°C in the same medium without L-arabinose. The culture was maintained at 37°C with aeration at 2.5 cu ft per min for 8 hours. The final cell density was 2 to 2.5 × 10^9 cells per ml. Cells were harvested in a refrigerated Sharples centrifuge, and the pellet was suspended in sufficient 10 mM potassium glycylglycine (pH 7.6) to yield 77 mg of protein, dry weight, per ml. The cell suspension was frozen in a Dry ice-acetone bath and stored at -20°C until needed.

L-Ribulose 5-Phosphate 4-Epimerase Assay—The spectrophotometric assay was essentially as previously described (1). The reaction mixture (0.3 ml) contained: 15 μmoles of sodium glycylglycine (pH 7.5), 3 μmoles of reduced sodium glutathione, 0.4 μmole of NAD^+, 1 μmole of MgCl_2, 1 μmole of ATP, 3 μmoles of NADH, 0.1 μmole of thiamine pyrophosphate HCl, 0.2 μmole of L-ribulose 5-phosphate, and excess glyceraldehyde phosphate dehydrogenase and phosphoketolase. After allowing the mixture to warm to 37°C in a cuvette, the enzyme (in 1 to 5 μl) was added to start the reaction, and the reaction was followed by the increase in absorbance at 340 μm and measured with a Gilford model 2000 recording spectrophotometer. The temperature of the reaction was maintained at 37°C by circulating water through the cuvette housing. The reaction rate was linear and proportional to enzyme concentration up to 1 unit of activity. A unit of activity was defined as the formation of 1 μmole of NADPH per hour. Except for the assays done on column eluents, all activities reported were the averages of duplicate assays with...
two enzyme concentrations. In some experiments, where stated, a two-step assay was adopted. Enzyme in buffer was incubated at 37° with L-ribulose 5-phosphate for 5 min, heated to 100° for 30 sec, cooled, and an aliquot of the mixture was added to the remainder of the assay system. The optical density was recorded until no further increase in absorbance was observed. A blank containing no enzyme, but treated in the same manner, served as a control. Results of this two-step assay agree with that of the single step assay within experimental error.

Protein Determinations—During purification, protein was measured by either the method of Lowry et al. (8) with bovine plasma albumin (crystallized bovine plasma albumin, Pentex) as a standard, or, in the case of column eluents, by applying the formula of Layne (9) to optical density readings taken at 260 and 280 mp. The concentrations of pure 4-epimerase solutions were determined with either the Layne method that had been calibrated on a dry weight basis as previously described (7) or by their absorbances at 280 mp, with a specific absorbance value of 1.57 absorbance units per mg of protein, dry weight, per ml (determined in 10 mM NH₄HCO₃, pH 7.75).

Acrylamide Gel Disc Electrophoresis—Electrophoresis in acrylamide gel was performed at 4° according to the method of Davis (10), with a standard gel bed (0.5 × 5.1 cm) of 7.5% acrylamide, Tris glycine electrode vessel buffer (pH 8.3) (10), and a constant current of 3 ma per gel column. After sufficient time to allow the indicator dye (bromphenol blue) to migrate nearly to the full length of the gel column, the gels were removed, stained overnight with aniline blue-black, and destained by electrophoresis in 7.5% acetic acid at room temperature.

Ultracentrifugal Studies—Sedimentation studies were carried out in a Spinco model E analytical ultracentrifuge equipped with phase plate, RTIC, and open end camera. The instrument was critically aligned for interference optics as described previously (7). Rayleigh fringe patterns were analyzed with a Nikon two-dimensional microcomparator, model 6C.

Amino Acid Composition—Analysis of the amino acid composition was performed with a Spinco model 120C automatic analyzer according to the method of Moore and Stein (11). Separate determinations were made for cysteine and cystine as cysteic acid, and methionine as methionine sulfone (12). Tryptophan was determined after basic hydrolysis in Ba(OH)₂ according to the method of Noltmann, Mahowald, and Kuby (13).

Phosphoketolase—During the initial phase of this work, phosphoketolase preparations were gifts of Drs. R. L. Anderson of Michigan State University and R. W. Hogg of University of California at Santa Barbara. Subsequently, phosphoketolase was isolated from a strain of Leuconostoc mesenteroides (obtained from Dr. R. Y. Stanier’s collection) according to the procedure of Heath et al. (14). The phosphoketolase, stored frozen in sodium succinate buffer (10 mM, pH 5.0) at a concentration of 10 mg per ml, was stable for months.

L-Ribulose 5-Phosphate—L-Ribulose 5-phosphate was enzymatically prepared from L-arabinose (Sigma) and ATP (Calbiochem), with the use of purified L-arabinose isomerase (7) and L-ribulokinase (6). The conversion to L-ribulose 5-phosphate was quantitative, and the ester was isolated as the barium salt according to Anderson’s procedure (15).

Other Reagents—L-Xylulose 5-phosphate was a gift from Dr. R. W. Hogg. Crystalline glyceraldehyde phosphate dehydrogenase (from muscle) and thiamine pyrophosphate HCl were obtained from Sigma. Reduced glutathione and NAD⁺ were from Calbiochem. All other reagents were the best obtainable grade from commercial sources.

RESULTS

Several lots of L-ribulose 5-phosphate 4-epimerase have been prepared. Results of the most recent purification are described below and summarized in Table I. All operations were carried out at 0–4°.

Step 1. Extraction of Enzyme—A frozen cell suspension (920 ml) was thawed and treated by sonic oscillation at −5° in 100-ml quantities for 6 to 8 min at level 8 with a Branson 6-kc sonifier. The sonically disrupted suspension was centrifuged for 1 hour at 50,000 × g in a Spinco model L ultracentrifuge. The supernatant fluid (crude extract) was collected.

Step 2. MnCl₂ Treatment—A 1.0 M aqueous solution of MnCl₂

<table>
<thead>
<tr>
<th>TABLE I</th>
</tr>
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<tbody>
<tr>
<td>Purification summary</td>
</tr>
<tr>
<td>Fraction</td>
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<td>---------</td>
</tr>
<tr>
<td>Crude extract</td>
</tr>
<tr>
<td>Ammonium sulfate fraction</td>
</tr>
<tr>
<td>DEAE-cellulose column chromatography</td>
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<tr>
<td>First Sephadex G-200 column</td>
</tr>
<tr>
<td>Second Sephadex G-200 column</td>
</tr>
</tbody>
</table>

![FIG. 1](http://www.jbc.org/) Purification Step 4. Column chromatography on DEAE-cellulose at pH 7.8. For details see the text.
FIG. 2. Purification Step 5. Column chromatography on Sephadex G-200. For details see the text.

was added slowly to the crude extract with stirring to yield a final concentration of 0.05 M MnCl₂. Fifteen minutes after the addition of MnCl₂, the solution was centrifuged at 66,000 × g for 1 hour and the precipitate was discarded.

Step 3. Ammonium Sulfate Fractionation—The supernatant fluid from the previous step was immediately brought to pH 7.6 by the addition of NH₄OH (3% solution). Solid ammonium sulfate was added slowly over a period of 30 min to bring the solution to 40% saturation, and the solution was allowed to equilibrate with stirring for an additional 30 min. The precipitate was discarded.

FIG. 3. Purification Step 6. Rechromatography on Sephadex G-200. For details see the text.

FIG. 4. Sedimentation pattern of pooled fractions from Step 6. A sample of the pooled Sephadex G-200 fraction that had been lyophilized in 1 mM potassium phosphate buffer (pH 7.6) was dissolved in distilled water, dialyzed against 1,000 times its volume of 0.1 ionic strength potassium phosphate buffer (pH 7.6), and sedimented in the analytical ultracentrifuge at 59,780 rpm at 20°C. The protein concentration was 5.77 mg per ml. The photograph was taken with a phase plate angle of 70° at about 24 min after reaching speed.

FIG. 5. Electrophoresis pattern in acrylamide gel of pooled fractions from Step 6. Enzyme from Step 6, 20 μg, was subjected to disc electrophoresis in acrylamide gel and stained as described under "Materials and Methods."
Fig. 6. Photomicrograph of l-ribulose 5-phosphate 4-epimerase crystallized from ammonium sulfate. Phase contrast. Left, about 800 X; right, about 1200 X.

Fig. 7. Effect of pH on l-ribulose 5-phosphate 4-epimerase activity. Enzyme activity was determined by the two-step assay method (“Materials and Methods”). The buffer used in the first step was varied as follows. For pH 2, 3, and 4, glycylglycine-HCl (10 mM glycylglycine adjusted to pH with HCl); for pH 5 and 6, succinate-NaOH (10 mM succinic acid adjusted to pH with NaOH); for pH 6.5 to pH 8.5, glycylglycine-NaOH (10 mM glycylglycine adjusted to pH with NaOH); for pH 9 to pH 10, glycine-NaOH (10 mM glycine adjusted to pH with NaOH). The effect of the test buffer was eliminated in the second step where the concentration of the sodium glycylglycine buffer, pH 7.6, was 30 times that of the test buffer introduced from the first step. Half a unit of 4-epimerase was used in all cases.

Fig. 8. The effect of l-ribulose 5-phosphate concentration on reaction velocity. Reaction velocities were measured by the one-step assay method described under “Materials and Methods,” except that a constant amount of enzyme (0.26 unit) was added to reaction mixtures containing varying amounts of l-ribulose 5-phosphate.

Collected by centrifugation at 35,000 × g for 15 min, resuspended in a minimum amount of 10 mM potassium phosphate buffer (pH 7.6), and dialyzed against 2 liters of the same buffer overnight. The next day the dialysis was continued for 3 more hours with fresh buffer.

Step 4. pH 7.6 DEAE-cellulose Column Chromatography—The dialyzed material was clarified by centrifugation and chromatographed on a column of DEAE-cellulose (4.9 X 135 cm) previously equilibrated with 10 mM potassium phosphate buffer (pH 7.6). The column was developed at a flow rate of 150 ml per
hour, by using a linear NaCl gradient from 0 to 0.5 M (in the above phosphate buffer) and fractions of 16.8 ml were collected. A portion of the elution profile is shown in Fig. 1. Fractions with specific enzyme activities greater than 200 were pooled and concentrated by the addition of solid ammonium sulfate as described above.

**Step 5. Sephadex G-200 Column Chromatography**—The dialyzed material from the previous step (13 ml) was clarified by centrifugation and chromatographed on a column of Sephadex G-200 (375 ml bed volume) at a flow rate of 9 ml per hour collecting 3.3 ml per fraction (Fig. 2). Fractions with specific enzyme activity greater than 1000 were pooled and concentrated by the addition of solid ammonium sulfate as described above.

**Step 6. Sephadex G-200 Rechromatography**—The pooled material from the previous step appeared faintly yellow. Rechromatography on the same column of Sephadex G-200 removed the colored material. The fractions from this step were pooled on the basis of homogeneity in the ultracentrifuge and acrylamide gel electrophoresis (Fig. 3). The pooled material sedimented as a single peak in the ultracentrifuge (Fig. 4) and migrated as a single band in electrophoresis (Fig. 5). A trace of contaminating material which was detected in electrophoresis, constituted less than 2% of the total protein by area integration of a densitometer tracing of the stained gel.

**Crystallization**—Two milliliters of the pooled Sephadex G-200 eluent (4 mg of protein per ml) was brought to 35% saturation with ammonium sulfate by the slow addition of a neutral solution saturated with ammonium sulfate at 4°C. The solution was left in the refrigerator. Crystals appeared in 2 days, and the crystallization process was complete after 3 weeks (Fig. 6). No increase in specific activity was observed.

**pH Optimum**—The pH optimum for 4-epimerase was tested.

**Table III**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>16 hr</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
<th>96 hr</th>
<th>Corrected&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amount&lt;sup&gt;b&lt;/sup&gt;</th>
<th>moles/103,000 g</th>
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<td>Lysine</td>
<td>0.58</td>
<td>0.58</td>
<td>0.58</td>
<td>0.58</td>
<td>0.58</td>
<td>0.58</td>
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<tr>
<td>Histidine</td>
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<td>2.42</td>
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<td>Arginine</td>
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<td>0.40</td>
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<td>Aspartic acid</td>
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<td>0.77</td>
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<td>Proline</td>
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<td>0.60</td>
<td>0.62</td>
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<td>Glycine</td>
<td>0.97</td>
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<td>1.02</td>
<td>1.01</td>
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<tr>
<td>Alanine</td>
<td>1.22</td>
<td>1.20</td>
<td>1.19</td>
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<td>Half-cystine&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Valine</td>
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<tr>
<td>Tyrosine</td>
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<td>0.45</td>
<td>0.43</td>
<td>0.43</td>
<td>0.43</td>
<td>0.45</td>
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<tr>
<td>Phenylyalanine</td>
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<td>0.34</td>
<td>0.32</td>
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<tr>
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<td><strong>Yield</strong></td>
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<td></td>
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<td>93%</td>
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</table>

<sup>a</sup> Obtained by extrapolating serine, threonine, tyrosine, and ammonia to zero time, and valine, isoleucine, and leucine to infinite time of hydrolysis. Values for all other amino acids were averaged over all hydrolysis times.

<sup>b</sup> All values were normalized with respect to average lysine value for the short column and to average glutamic acid value for the long column, to correct for minor fluctuations in flow rates.

<sup>c</sup> Obtained as cysteic acid and methionine sulfone in 24-hour hydrolysis of performic acid oxidized protein.

<sup>d</sup> Obtained after 72-hour basic hydrolysis.

<sup>e</sup> Computed from milligrams of each amino acid present in hydrolysate minus 1 mole of water per mole of amino acid. Total amount in hydrolysate equals 1.50 mg, dry weight.
from pH 2 to 10 with the two-step assay method as described under "Materials and Methods"; the results are shown in Fig. 7. The activity was absent at pH 5 or less, and maximal at pH 7 or above.

$K_m$ for Ribulose 5-Phosphate—The $K_m$ for ribulose 5-phosphate was determined and was found to be $9.5 \times 10^{-4} M$ (Fig. 8).

Substrate Specificity—The enzyme catalyzes the backward reaction at a slightly higher velocity. Equilibrium is established at approximately equal concentrations of ribulose 5-phosphate and n-xylulose 5-phosphate, as determined with the 2-step assay. n-Ribulose 5-phosphate was tested as a substrate by adding n-ribulose, ATP, and excess pure ribulokinase (6) to the reaction mixture. The enzyme shows no detectable n-ribulose 5-phosphate 3-epimerase activity.

Cofactor Requirement—None of the components present in the complete reaction mixture, when preincubated with the enzyme, singly or together, showed any stimulation of 4-epimerase activity over that found when the enzyme was preincubated with buffer alone. The enzyme preparation used had been extensively dialyzed and recycled through Sephadex G-200 columns in 10 mM potassium phosphate buffer. Apparently the 4-epimerase contains no readily dissociable small molecule. The existence of tightly bound small molecules cannot be ruled out.

Molecular Weight—The molecular weight was determined by sedimentation equilibrium at high speed (16) with three different samples representing enzyme from two different purifications. Results are shown in Table II. A plot of the In of the concentration as a function of the square of the distance from the center of rotation for one of the experiments is shown in Fig. 9. From these results an apparent weight average molecular weight of 103,000 ± 1,000 was calculated.

Amino Acid Composition—Results of amino acid analyses are presented in Table III.

**DISCUSSION**

L-Ribulose 5-phosphate 4-epimerase has been purified from *Aerobacter aerogenes* (17) and *Lactobacillus plantarum* (18). The $K_m$ for ribulose 5-phosphate of the *E. coli* enzyme is identical with that of *A. aerogenes*. There is no demonstrable cofactor requirement. Activity of the *E. coli* enzyme is maximal from pH 7 to 10, and about 50% maximal at pH 6, thus resembling the L-ribulokinase of subunits. A better understanding of the significance of their coordinated synthesis can be arrived at only after elucidation of the number and identity of their component polypeptide chains.

REFERENCES

Crystalline l-Ribulose 5-Phosphate 4-Epimerase from *Escherichia coli*
Nancy Lee, James W. Patrick and Michel Masson


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