Purification and Characterization of D-Lyxose Isomerase*

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The metabolism of n-lyxose by Aerobacter aerogenes PRL-R3 involves isomerization to d-xylulose followed by phosphorylation to d-xylulose 5-phosphate (1, 2). The enzyme which catalyzes the first reaction in the sequence, n-lyxose isomerase, is of interest because it initiates the degradation of an unnatural pentose in an organism which can utilize as a sole carbon source at least 7 of the 8 aldopentoses (2) and most (and possibly all) of the 16 aldohexoses.1 The utilization of certain of the rarer pentoses and pentitols by this organism has been shown by Mortlock, Fossitt, and Wood (3) not to involve new enzymes, but the selection of derepressed mutants containing higher levels of nonspecific enzymes which also act on and are induced by the more common pentoses or pentitols (4, 5). The present communication, which describes the purification and partial characterization of n-lyxose isomerase from A. aerogenes PRL-R3, indicates that a different situation exists for n-lyxose. Kinetic and specificity studies and the inability to demonstrate n-lyxose isomerase in cells grown on substrates other than d-lyxose indicate that this enzyme is elaborated specifically to metabolize the unnatural pentose n-lyxose.

EXPERIMENTAL PROCEDURE

Bacteriological—A strain of A. aerogenes PRL-R3 selected for the ability to grow on D-lyxose without a lag (see “Growth and Induction Studies”) was grown aerobically at 30° for 18 to 24 hours and harvested by centrifugation. The medium consisted of 1.35% Na2HPO4·7H2O, 0.15% KH2PO4, 0.3% (NH4)2SO4, 0.02% MgSO4·7H2O, 0.0005% FeSO4·7H2O, and 0.5% D-lyxose (autoclaved separately).

Analytical—Ketopentose was measured by the cysteine-carbazole method (6). When hexoses were used as substrates for the isomerase reaction, the ketose products were measured by the method of Roe (7) except in the experiment described in Table V. When absolute values for D-fructose were needed (as in Fig. 8), the values obtained by the Roe procedure (7) were corrected for interference by D-mannose. Protein was determined spectrophotometrically with the aid of a nomograph (courtesy of Calbiochem) based on the data of Warburg and Christian (8).

Chemical—D-Xylose was prepared by refluxing o-xylose with dry pyridine (9), and purified by chromatography on Dowex 1-borate (10) after removing excess o-xylose by crystallization. All other chemicals were commercial preparations.

Enzymic—Yeast alcohol dehydrogenase (twice crystallized), pig heart malic dehydrogenase, and horseradish peroxidase (crystalline) were purchased from the Worthington Biochemicals Corporation. Alcohol dehydrogenase and malic dehydrogenase were assayed by measuring absorbance changes at 340 μM with a Gilford recording spectrophotometer. The assay mixture for alcohol dehydrogenase contained in a volume of 0.15 ml: ethanol, 25 μmoles; NAD, 0.2 μmole; Tris-HCl buffer (pH 8.5), 22.5 μmoles; and enzyme. The assay mixture for malic dehydrogenase contained in a volume of 0.15 ml: oxaloacetic acid (pH 5.5), 0.1 μmole; NADH, 0.05 μmole; Tris-maleate buffer (pH 7.5), 40 μmoles; and enzyme. Controls were minus oxaloacetate to correct for NADH oxidase, and with pyruvate replacing oxaloacetate to check for possible lactic dehydrogenase activity. The assay tubes for peroxidase contained a mixture of 1.50 ml of 0.003% H2O2 in 0.01 M potassium phosphate buffer (pH 6.0) and 0.02 ml of 1.0% o-dianisidine in methanol. After adding enzyme (1 μl), the rate of absorbance increase at 460 μm was read in a colorimeter.

Standard D-Lyxose Isomerase Assay—The 1.0-ml reaction mixture contained 20 μmoles of D-lyxose, 10 μmoles of MnCl2, 60 μmoles of sodium cacodylate buffer (pH 7.0), and enzyme. After incubation at 25° for 10 minutes, the reaction was stopped and ketopentose determined by adding the cysteine-carbazole reagents directly to the reaction mixture. The tubes were then incubated at 35° for exactly 20 minutes and the absorbance at 540 μm was read in a colorimeter. The increase in absorbance above a control without enzyme was a measure of isomerase activity. A unit was defined as the amount of enzyme that gave an absorbance increase of 1.0/10 minutes (equivalent to the isomerization of 1.26 μmoles of D-lyxose per hour) in the standard assay. The linearity of the assay is shown in Fig. 1.

RESULTS

Purification of D-Lyxose Isomerase

All operations were carried out at 0-4°. Extracts were prepared by suspending washed cells in 0.001 M EDTA (pH 7.0) and exposing them to some vibration for 2 to 4 minutes in a Raytheon 10-Kc oscillator circulated with ice water. The supernatant fluid resulting from 15 minute centrifugation at 31,000 × g was the crude extract.

Ammonium Sulfate Fractionation—Ammonium sulfate, 3.7 g, was dissolved in 140 ml of crude extract containing 16 mg of protein per ml with a ratio of light absorbance at 280 to 260 μm equal to 0.60. Then 25 ml of 2% protamine sulfate were added slowly with stirring. The precipitate that formed was removed by centrifugation and discarded. To the supernatant solution (165 ml) were added 19.9 g of ammonium sulfate (60% of satur-
**Table I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>6300</td>
<td>2.8</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>2800</td>
<td>9.4</td>
<td>3</td>
<td>44</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>2390</td>
<td>35</td>
<td>12</td>
<td>39</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>1370</td>
<td>200</td>
<td>71</td>
<td>22</td>
</tr>
<tr>
<td>Alumina C&lt;sub&gt;7&lt;/sub&gt; gel</td>
<td>307</td>
<td>366</td>
<td>130</td>
<td>6</td>
</tr>
</tbody>
</table>

- Corrected for the portion of each fraction not further purified.

**Fig. 2.** Equilibrium of the L-lyxose isomerase-catalyzed reaction. The reaction mixture (1.0 ml) contained 400 µg of L-lyxose or D-xylulose, 10 µmoles of MnCl₂, 60 µmoles of sodium cacodylate buffer (pH 7.0), and excess purified D-lyxose isomerase. The incubation temperature was 25°C. Aliquots were removed at the time intervals shown, and assayed for D-xylulose by the cysteine-carbazole method, correcting for the 540 nm absorbance contribution of D-lyxose. ●●●, D-lyxose as substrate; ○○○, L-lyxose as substrate.

**Fig. 1.** Linearity of the L-lyxose isomerase assay. The conditions are described in the text.
with 0.483 to 1.94 M \( M \) in 0.05 M sodium cacodylate buffer, pH 7.0. These were layered with 0.20 ml of enzyme solution in 0.05 M sodium cacodylate, pH 7.0. The centrifugations were run for 16 to 17 hours at 35,000 rpm in a Spinco model L centrifuge. The temperature in the tubes at the end of the runs was 7°. Three standards were used: alcohol dehydrogenase (mol. wt., 151,000 (13)), malic dehydrogenase (mol. wt., 40,000 (14)), and peroxidase (mol. wt., 40,000 (15)). \( \nu \)-Lytosose isomerase was a Sephadex G-100 fraction. The results depicted in Fig. 5 demonstrate the symmetry of the peaks and the resolution that was obtained. Table II gives a summary of data from three separate determinations. With 7.4S as an average sedimentation coefficient for alcohol dehydrogenase (12), the sedimentation coefficients for malic dehydrogenase and peroxidase were calculated by the relationship \( S_1 \times \text{distance}_2 = S_2 \times \text{distance}_1 \) (12) to be 3.9S and 3.5S, respectively. These values are close to the values of 3.6S and 3.5S, respectively, obtained by other methods (14, 15). \( \nu \)-Lytosose isomerase consistently sedimented between these two 40,000 molecular weight standards, at 3.7S to 3.8S. Therefore, assuming that \( \nu \)-lytose isomerase is roughly spherical, as are most enzymes, its molecular weight is estimated to be 40,000. Thus, it is smaller than the other enzymes involved in pentose metabolism that have been studied in this organism (4).

**Metal Ion Specificity** \( \nu \)-Lytosose isomerase requires a metal ion for activity. Treatment of the enzyme with 0.01 M EDTA resulted in a complete loss of activity when assayed in the absence of added metal ions. After dialysis against distilled water the enzyme could be reactivated by the metal ions shown in Table III. With \( 10^{-4} \) and \( 10^{-3} \) M metal ion concentrations, \( \nu \)-lytose isomerase was activated most effectively by manganese, to some extent by ferrie and ferrous iron, slightly by calcium and cobalt, and not at all by magnesium, zinc, or copper.
D-Lyxose isomerase of A. aerogenes

**Table II**

Summary of NaCl gradient centrifugations

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Molecular weight</th>
<th>Sedimentation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol dehydrogenase*</td>
<td>151,000 (13)</td>
<td>(7.4) (7.4) (7.4)</td>
</tr>
<tr>
<td>Malic dehydrogenase*</td>
<td>40,000 (14)</td>
<td>3.9 3.9 3.9</td>
</tr>
<tr>
<td>Peroxidase*</td>
<td>40,000 (15)</td>
<td>3.5 3.5 3.5</td>
</tr>
<tr>
<td>D-Lyxose isomerase*</td>
<td></td>
<td>3.7 3.8 3.8</td>
</tr>
</tbody>
</table>

a Standards.

b Apparent molecular weight of D-lyxose isomerase is 40,000.

**Table III**

Activation of D-lyxose isomerase by metal ions

The standard assay was used except that 0.01 M MnCl₂ was replaced by the metal salts indicated. The enzyme was a Sephadex G-100 fraction treated with 0.01 M EDTA and dialyzed against distilled water on a Sephadex G-25 column.

<table>
<thead>
<tr>
<th>Metal salt</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁻² M</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>100</td>
</tr>
<tr>
<td>FeCl₂</td>
<td>26</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>15</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>4</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>8</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>0</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>0</td>
</tr>
</tbody>
</table>

Substrate Specificity—Crude extracts of D-lyxose-grown cells contained isomerase activity for D-xylose, D-mannose, D-mannose 6-phosphate, and D-glucose 6-phosphate, as well as D-lyxose. Isomerase activities for each of these sugars were assayed for each fraction during the purification of D-xylose isomerase. Table IV shows that the ratios of activity on D-xylose, D-mannose 6-phosphate, and D-glucose 6-phosphate to D-lyxose varied widely in the different fractions, with activity on the former three being removed completely at some stage of purification. The stage of activities on D-lyxose and D-mannose, however, remained nearly constant in all fractions throughout the 100-fold range of purification, suggesting that isomerization of D-lyxose and D-mannose is catalyzed by the same enzyme. This is supported by the finding that with a mixture of D-lyxose and D-mannose as the substrate, a competitive rather than additive effect was observed (Table V).

Neither crude extracts of D-lyxose-grown cells nor purified D-lyxose isomerase isomerized D-glucose, D-ribose, D- or L-arabinose, or L-xylose at a detection level of 0.5% of the rate of D-lyxose isomerization. Thus, of the aldoses tested, only D-lyxose and D-mannose, which is structurally similar to D-lyxose at the first 4 carbon atoms, were isomerized by the purified enzyme.

**Table IV**

Comparison of isomerase activities on D-lyxose, D-xylose, D-mannose, D-mannose 6-phosphate, and D-glucose 6-phosphate during purification of D-lyxose isomerase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>D-Mannose</th>
<th>D-Mannose-6-P</th>
<th>D-Glucose-6-P</th>
<th>D-Xylose</th>
<th>D-Lyxose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (40–45% saturation)</td>
<td>1.06</td>
<td>0.50</td>
<td>0.31</td>
<td>1.14</td>
<td>1.14</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (46–56% saturation)</td>
<td>0.02</td>
<td>0.22</td>
<td>0.22</td>
<td>0.76</td>
<td>0.76</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (47–50% saturation)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>1.02</td>
<td>1.20</td>
<td>0.10</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>1.02</td>
<td>1.20</td>
<td>0.10</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Alumina C₇ supernatant</td>
<td>0.95</td>
<td>10.5</td>
<td>6.0</td>
<td>0.45</td>
<td>0.45</td>
</tr>
<tr>
<td>Alumina C₇ eluate</td>
<td>1.00</td>
<td>5.0</td>
<td>2.0</td>
<td>0.45</td>
<td>0.45</td>
</tr>
</tbody>
</table>

c Ratios of activity in the crude extract have been arbitrarily set at 1.00.
d No activity on D-mannose 6-phosphate.
e No activity on D-xylose.
f No activity on D-glucose 6-phosphate.

**Table V**

Effect of D-mannose on isomerization of D-lyxose

The standard assay was used except that 0.02 M D-lyxose was replaced by the substrates indicated. The enzyme was a DEAE-cellulose fraction. Ketose products were measured by the cysteine-carbazole reaction; controls indicated that mannose did not interfere with color development.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amount</th>
<th>Absorbance at 540 mλ</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Lyxose</td>
<td>0.02</td>
<td>0.61, 0.61</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>0.06</td>
<td>0.15, 0.16</td>
</tr>
<tr>
<td>D-Lyxose plus D-mannose</td>
<td>0.02 + 0.06</td>
<td>0.45, 0.43</td>
</tr>
</tbody>
</table>
**Table VI**

**Inhibition of d-lyxose isomerase by p-chloromercuribenzoate**

The standard assay was used with the addition of p-chloromercuribenzoate as indicated. The enzyme was a DEAE-cellulose fraction.

<table>
<thead>
<tr>
<th>p-Chloromercuribenzoate</th>
<th>Absorbance at 540 mp</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.61</td>
</tr>
<tr>
<td>10^-4</td>
<td>0</td>
</tr>
<tr>
<td>10^-5</td>
<td>0</td>
</tr>
<tr>
<td>10^-6</td>
<td>0.13</td>
</tr>
<tr>
<td>10^-7</td>
<td>0.61</td>
</tr>
</tbody>
</table>

The activity could not be restored with the further addition of GSH, and GSH was never found to be protective or stimulatory during fractionation or assay. Iodoacetate, GSSG, 2,6-dichlorophenolindophenol, or K$_3$Fe(CN)$_6$ at 10^-3 M and pH 7.0 showed no inhibition of d-lyxose isomerase in the standard assay.

**Growth and Induction Studies**

Growth of *A. aerogenes* PRL-R3 on d-lyxose normally occurred only after a 4-day lag, as noted by Mortlock and Wood (2). After repeated transfer on mineral medium containing n-lyxose, however, we obtained a strain which would grow on d-lyxose without a noticeable lag even after serial transfer on a d-glucose medium. By all other criteria the selected strain was identical with the parent strain. Both strains, however, formed d-lyxose isomerase only when grown on d-lyxose; cells grown with d-mannose, d-fructose, or d-glucose as the sole carbon source contained no detectable activity.

**Discussion**

Two enzymes, d-lyxose isomerase and d-xylulokinase, are instrumental in converting d-lyxose to d-xylulose 5-phosphate, the common intermediate in pentose metabolism. The kinase is also induced by and involved in the metabolism of d-xylose, d-arabitol, and xylitol in *A. aerogenes* PRL-R3 (2). d-Xylulokinase induced by d-xylose has been purified from this organism by Bhuyan and Simpson and shown to be specific for d-xylulose (17). It would be of interest to determine if the d-xylulokinases induced by each of these four substrates are identical.

In contrast to d-xylulokinase, d-lyxose isomerase is induced only by d-lyxose. The lack of induction by growth on d-glucose, pentitols, and pentoses other than d-lyxose was shown by Mortlock and Wood (2). This report extends their findings by demonstrating a lack of induction by growth on d-fructose and d-mannose, even in a strain selected for growth on d-lyxose. This is of interest because d-mannose and d-fructose are substrates for the enzyme. In contrast, a mutant of *Pseudomonas saccharophila* selected for growth on d-fructose contains a d-mannose isomerase which also acts on d-lyxose at 11% of the rate on d-mannose (18). The *Pseudomonas* enzyme has no metal ion requirement, unlike the d-lyxose isomerase of *A. aerogenes*. Although d-mannose isomerase invariably is present in d-fructose-positive mutants of *P. saccharophila* and cannot be induced in the parent strain, its function is not known because the mutants apparently also contain d-fructokinase (19). In *A. aerogenes*, d-lyxose isomerase clearly is essential for growth on d-lyxose.
D-Lyroxose isomerase has a higher affinity (lower \( K_m \)) for D-
lyxose than for D-mannose and achieves a higher maximum
velocity with D-lyxose than with D-mannose. These facts
are similar to the observation that D-lyxose isomerase was not
elaborated during growth on D-mannose or D-fructose indicate
that D-lyxose, usually considered to be an unnatural pentose, is
the natural substrate for the enzyme. The mechanism of the
acquisition of this new specific protein is not yet understood. A
similar situation may exist for D-allokinase, which is induced
in this organism by growth on D-allose (20).

The inability of D-mannose to induce D-lyxose isomerase
(D-mannose isomerase) in this organism, the demonstration that
its constitutive hexokinase is specific for D-glucose (21), and the
inability to demonstrate a kinase for D-mannose and D-fructose
even though these hexoses can be metabolized constitutively (21),
indicate that previously unrecognized mechanisms exist for the
metabolism of common hexoses (22).

SUMMARY

D-Lyroxose isomerase was purified 130-fold from extracts of
Aerobacter aerogenes PRL-R3. The enzyme was characterized
with respect to substrate specificity, metal ion requirement, pH
optimum, molecular weight, and kinetic constants. D-Mannose
was isomerized in addition to D-lyxose, but the kinetic constants
and induction studies indicated that the unnatural pentose
D-lyxose is the natural substrate for the enzyme.

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