Crystalline d-Serine Dehydrase*

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SUMMARY

A procedure is described for the preparation of crystalline D-serine dehydrase from mutant C6 of Escherichia coli K-12, a constitutive mutant for this enzyme. The molecular weight is 37,300, and 1 mole of pyridoxal 5-phosphate is bound per mole of enzyme. D-Threonine is dehydrated at one-fifth the rate of D-serine, and slight activity is also observed with L-serine and DL-allothreonine.

The enzymatic deamination of serine has been known for almost 30 years (1). Oxidative deamination of D-serine (2) and transamination (3) of L-serine lead to the formation of hydroxy pyruvate, while dehydration of both isomers yields pyruvate (4). Following the demonstration by Wood and Gunsalus (5) that extracts of Escherichia coli contain an enzyme system which catalyzes the dehydration of L-serine, Yanofsky (6) and Metzler and Snell (7) showed that Neurospora crassa and E. coli also contain a serine dehydrase, specific for the D-isomer, which requires pyridoxal 5-phosphate. From experiments on the induction of serine dehydrase, Pardee and Prestidge (8) concluded that D-serine and D-threonine are dehydrated by the same enzyme.

The isolation by McFall (9) of a group of E. coli mutants which are constitutive for D-serine dehydrase (D-serine hydrolyase (deaminating), EC 4.2.1.14) made available a rich source of the enzyme. In the present paper we present a method for the preparation of crystalline D-serine dehydrase and report some of the properties of the pure enzyme.

EXPERIMENTAL PROCEDURE

Materials and Methods

A culture of the constitutive mutant, C6, of E. coli K-12 was a generous gift of Dr. Elizabeth McFall. D-Serine, L-serine, D- and L-threonine, and DL-allothreonine were purchased from Calbiochem. DEAE-cellulose was obtained from Carl Schleichert and Schuell. D-α-Deuteroserine was prepared by racemizing L-serine (11) in deuterium oxide. L-Serine (2.1 g) yielded 376 mg of D-α-deuteroserine (α, +0.8° in water; 7.60 atom % excess deuterium). Infrared and nuclear magnetic resonance spectra confirmed the identity of the product. The amount of pyruvate formed was determined by the method of Friedemann and Haugen (12). Substrate was omitted from the control. Since crude extracts of E. coli contain an active enzyme system for the oxidation of pyruvate, specific activities obtained by this assay procedure are low. Higher values are obtained if pyruvate oxidase is inhibited by adding 1 µmole of cupric chloride to the reaction mixture; e.g., an extract of repressed cells with a specific activity of 0.75 µmole per hour per mg in the presence of cupric chloride had a specific activity of 0.3 µmole per hour per mg when it was omitted. A spectrophotometric assay, based on the measurement of the rate of DPNH oxidation in the presence of lactic dehydrogenase, can be used after Step 4 of the purification procedure. DPNH oxidase, which is present as an impurity at earlier stages in the procedure, seriously interferes with the optical method. For the spectrophotometric assay the following components in a final volume of 0.88 ml were added to a cuvette having a 1-cm light path: 140 µmoles of potassium phosphate buffer (pH 7.4), 0.28 µmole of DPNH, 0.1 mg of lactic dehydrogenase, 25 µmoles of D-serine, and enzyme. Absorbance readings were taken at 30-second intervals.

One unit of D-serine dehydrase is the amount of enzyme that catalyzes the formation of 1.0 µmole of pyruvate per min. Specific activity is units per mg of protein.

Growth of Bacteria

The bacterial growth medium was prepared by dissolving 200 g of Bacto-peptone, 148 g of K2HPO4, 52 g of KH2PO4; and 4 g of MgCl2·6H2O in 40 liters of water. A solution of 400 g of glucose in 800 ml of water was autoclaved separately and added to the sterilized Bacto-peptone-salt solution. The medium was inocu-
Isolation of Enzyme

Table I is a summary of the results obtained in the purification procedure. All operations were carried out at 0–5°C. 

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Volume</th>
<th>Protein</th>
<th>Units</th>
<th>Specific activity*</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>/min</td>
<td>/min/mg</td>
<td>%</td>
</tr>
<tr>
<td>1. E. coli K-12, mutant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₅₇ extract</td>
<td>600</td>
<td>17,580*</td>
<td>6,300</td>
<td>0.358</td>
<td>100</td>
</tr>
<tr>
<td>2. DEAE-cellulose column eluate</td>
<td>1,200</td>
<td>1,080</td>
<td>4,320</td>
<td>4.0</td>
<td>70</td>
</tr>
<tr>
<td>3. (NH₄)₂SO₄ fraction</td>
<td>63</td>
<td>108.5, 508</td>
<td>16.7</td>
<td>52.5</td>
<td></td>
</tr>
<tr>
<td>4. Cs₃(P0₄)₆ gel-cellulose column eluate</td>
<td>0.43</td>
<td>7.91, 541</td>
<td>195</td>
<td>24.4</td>
<td></td>
</tr>
<tr>
<td>5. Crystals</td>
<td>1.0</td>
<td>4.8</td>
<td>968</td>
<td>195 (280)</td>
<td>14.8</td>
</tr>
<tr>
<td>5A. Mother liquor</td>
<td>1.0</td>
<td>3.1</td>
<td>605</td>
<td>195 (280)</td>
<td>9.6</td>
</tr>
</tbody>
</table>

*From 100 g of cells.

Step 1: Extraction—Packed cells (100 g) were suspended in 500 ml of 0.01 M potassium phosphate buffer, pH 7.2. The suspension was divided into 100-ml portions and each portion was treated for 3 min with the probe-type Branson LS-75 sonifier at full power. During sonic disruption the cell suspension (in a min. The supernatant solution was retained. The precipitate was resuspended in 100 ml of buffer and treated again by the same procedure. The combined extracts were used in the next step.

Step 2: Chromatography on DEAE-cellulose—DEAE-cellulose (standard type; capacity, 0.9 meq per g) was washed successively with 0.5 M KOH, water, and 0.1 M potassium phosphate, pH 7.2. The washed cellulose was suspended in 0.01 M potassium phosphate, pH 7.2, and packed into a column (4 × 37 cm) under a pressure of 5 p.s.i. The eluate from the column was at pH 7.2. The E. coli extract was applied to the column and impurities were eluted with 0.001 M potassium phosphate, pH 7.2. Buffer flows through the column at the rate of approximately 100 ml per hour. The dialyzed enzyme solution was allowed to soak into a DEAE-cellulose (Step 4A) is required prior to crystallization.

Step 3: Fractionation with Ammonium Sulfate—The concentrated enzyme solution was allowed to soak into a DEAE-cellulose column (2.5 × 38 cm) which had been prepared as described in Step 2. After the column had been washed with about 100 ml of 0.01 M potassium phosphate, pH 7.2, the enzyme was eluted with approximately 800 ml of 0.005 M potassium phosphate, pH 7.2. The fractions containing the enzyme were combined (400 ml) and concentrated to 0.5 to 1 ml by ultrafiltration to give a yellow solution (5 to 15 mg of protein per ml; specific activity, 195) of homogeneous enzyme which can be crystallized as described in Step 5.

Preparations of calcium phosphate gel with a low adsorptive capacity have yielded d-serine dehydrase with a specific activity as low as 64. In this case, further purification on DEAE-cellulose (Step 4A) is required prior to crystallization.

Step 4: Chromatography on Calcium Phosphate—Calcium phosphate gel (300 ml; 30 mg per ml), prepared by the method of Swingle and Tiselius (15, 16), was poured as a thin layer of cellulose powder and equilibrated with a 0.001 M potassium phosphate, pH 7.2. Buffer flows through the column at the rate of approximately 100 ml per hour. The dialyzed enzyme preparation from Step 3 was applied to the column, which was then washed with 200 ml of 0.001 M potassium phosphate, pH 7.2, and the enzyme was eluted with approximately 800 ml of 0.005 M potassium phosphate, pH 7.2. The fractions containing the enzyme were combined (400 ml) and concentrated to 0.5 to 1 ml by ultrafiltration to give a yellow solution (5 to 10 mg of protein per ml).

Step 5: Crystallization—Solid ammonium sulfate (0.50 saturation) was added to the enzyme solution from the preceding step. Yellow crystals (Fig. 1A) were first visible after the solution (in a small test tube covered with filter paper) was maintained at 4°C for 1 day. Maximum yield of crystalline enzyme was obtained after 12 days. The specific activity did not change following a second crystallization.

RESULTS

Stability of Enzyme

When the enzyme (1.0 mg per ml in 0.2 M potassium phosphate, pH 7.4) is allowed to remain at room temperature for 6 hours, it loses 10% of its activity. At 0°C or in the frozen state, it appears to be stable almost indefinitely. Repeated freezing and thawing over a period of 1 or 2 months causes a 20 to 30% loss in activity. Heating a dilute solution of the enzyme at 54°C for 1 min almost completely destroys enzymatic activity.

Purity and Molecular Weight

Starch gel electrophoresis in sodium borate buffer, pH 8.6, of a solution of crystallized enzyme showed the presence of only one protein. The schlieren patterns (Fig. 1B) obtained in the ultracentrifuge in a sedimentation velocity experiment with the
FIG. 1. A, d-serine dehydrase crystals (X 500). The crystals are approximately 8 X 16 μ. B, sedimentation pattern of crystalline d-serine dehydrase at 56,100 rpm in 0.05 M potassium phosphate buffer, pH 7.4; 3.51 mg per ml.
crystalline enzyme also indicate the presence of one component ($e_{280}^{	ext{nm}} = 3.4$ S at a protein concentration of 1.75 mg per ml). The molecular weight of d-serine dehydrase, as determined by the method of La Bar and Baldwin (17) on a solution containing 3.51 mg of protein per ml in 0.05 M potassium phosphate (pH 7.4), is 37,300. The frictional ratio ($f/f_0$) is 1.07, which indicates that the enzyme molecules are nearly spherical. In an ultracentrifuge experiment with a synthetic boundary cell, a solution of enzyme with an absorbance of 5.0 at 280 nm was found by fringe count to have a protein concentration on a refractive index basis (18) of 3.51 mg per ml. The concentration of a d-serine dehydrase solution can therefore be obtained by multiplying the absorbance at 280 nm by the factor 0.702. The molar extinction coefficients at 280 and 415 nm are 53,300 and 5,330, respectively.

The specific activity values listed in Table I were calculated with a protein concentration based on the assumption that a solution containing 1 mg of protein per ml has an absorbance of 1.0 at 280 nm. When the protein concentration is corrected as indicated above, the specific activity of the crystalline enzyme is 280 µmoles per min per mg or 10,400 moles of substrate reacting per min per mole of enzyme.

**Pyridoxal 5-Phosphate Content**

The spectrum of the enzyme is shown in Fig. 2. In addition to the absorption maximum at 380 nm, there is a second peak at 415 nm, which is in the region expected for enzymes containing pyridoxal 5-phosphate. Resolution of the enzyme and coenzyme by treatment with cysteine (19) causes both a disappearance of the 415 nm peak and a complete loss of enzymatic activity. Addition of an excess of pyridoxal 5-phosphate to the resolved enzyme restores 100% of the activity.

The amount of pyridoxal 5-phosphate present in the enzyme was determined from the absorbance at 330 nm of the thiazolidine formed after reaction with cysteine (20). Since $1.88 \times 10^{-3}$ mole of enzyme contained $1.86 \times 10^{-4}$ mole of pyridoxal 5-phosphate, 1 mole of pyridoxal 5-phosphate is bound to 1 mole of enzyme.

**Substrate Specificity**

Maximum activity is obtained with d-serine as substrate. At all stages throughout the purification procedure, d-threonine is dehydrated at one-fifth the rate of d-serine (8). Attempts to alter the ratio of d-serine to d-threonine activities by chemical modification of the enzyme have so far been unsuccessful. When 25 µmoles of dl-allothreonine are incubated for 30 min with a 50-fold excess of enzyme in the standard colorimetric assay procedure, the amount of keto acid formed is 1% of the amount that would be formed, under the same conditions, from d-serine. Similarly, L-serine shows less than 0.1% of the activity of d-serine; and L-threonine, DL-O-methylserine, d-cysteine, D-tryptophan, m-homoserine, and m-homocysteine are not converted to keto acids. Overnight incubation of 5 µmoles of L-serine with a large excess of enzyme resulted in the formation of 5 µmoles of keto acid, while 5 µmoles of DL-alloethreonine yielded 2.5 µmoles of product. This latter result suggests that only one isomer of allothreonine is dehydrated.

Inhibition of d-serine dehydrase by L-serine, dl-allothreonine, and DL-O-methylserine was determined in the following manner. Three mixtures, containing 2.5 µmoles of d-serine plus 25 µmoles of L-serine, 1.0 µmole of L-serine plus 25 µmoles of DL-allothreonine, and 1.0 µmole of d-serine plus 12.5 µmoles of DL-O-methylserine, respectively, were incubated in the standard assay procedure. In the mixture containing L-serine, 75% less keto acid was formed than if the L isomer had not been present. The inhibition caused by DL-alloethreonine was 70%, and by DL-O-methylserine, 40%.

**pH and pD Optima**

The $K_m$ values for d-serine and d-threonine determined graphically by the method of Lineweaver and Burk (21) are $0.7 \times 10^{-4}$ M and $0.91 \times 10^{-3}$ M, respectively. The specific activity of the enzyme as a function of pH and pD is shown in Fig. 3. As can be seen from the figure, the pH optimum is 8.0.
and the pH optimum is 9.2. The shift of 1.2 pH units in changing from a H₂O to a D₂O medium may possibly be related to a change in the ionization constant of a functional group participating in the reaction. The specific activity of the enzyme at the pH optimum is 1.25 times greater than at the pH optimum.

DISCUSSION

Although data are available on only a few highly purified or crystalline preparations of enzymes containing pyridoxal 5-phosphate, these enzymes are proteins with molecular weights of about 100,000 to 300,000 or greater and contain several (usually 4) molecules of pyridoxal 5-phosphate. D-Serine dehydrase is an exception to this generalization in that its molecular weight is 37,300, and it contains only 1 molecule of bound pyridoxal 5-phosphate. The absorption spectrum of D-serine dehydrase indicates that the coenzyme may be bound to the apoenzyme as a Schiff base.

The identity of the groups to which the coenzyme is bound was investigated further. Crystalline holoenzyme (2.8 mg) in 1.0 ml of water was dialyzed for 10 min against 50 ml of 0.01 M sodium borohydride, and for 2½ hours against water. The specific activity of the enzyme dropped from 195 to 10 as a result of the borohydride reduction and was not restored by the addition of pyridoxal 5-phosphate. The reduced enzyme was hydrolyzed in a sealed tube for 21 hours in 6 N HCl. The hydrolysate was evaporated to dryness and dissolved in 0.1 ml of water, and 0.05 ml of the solution was submitted to paper chromatography (22). A blue-white fluorescent spot with an RF of 0.29 was detected. Since the RF of authentic D-pyridoxyllysine is 0.31, the coenzyme is probably bound to the ε-amino group of a lysine residue in conformity with other pyridoxal 5-phosphate enzymes (23).

All of our attempts to reverse the over-all reaction have been unsuccessful. Serine was not formed in reaction mixtures containing pyruvate, ammonia, and enzyme, and incubation of enzyme (or apoenzyme), pyruvate, and pyridoxamine 5-phosphate did not liberate ammonia. Evidence for the formation of an intermediate which has an exchangeable hydrogen atom was obtained in an experiment in which 75 mg of D-serine and 0.22 mg of crystalline enzyme were incubated for 65 min at 25° (pD 9.2) in a solution (final volume, 5.0 ml) containing 99.8 % D₂O. The reaction reached 40% completion. Crystalline D-serine (20 mg) isolated from the reaction mixture after chromatography on Dowex 50-H⁺ (24) contained 1.28 atom % excess deuterium (0.09 atom of deuterium incorporated per molecule of serine). When L-serine, which is a very poor substrate for the enzyme, was substituted for D-serine and incubated for 65 min in a reaction mixture identical with the one described above, no incorporation of deuterium could be detected.

The ratios of the rates of dehydration of serine and α-deuteroserine at pH values of 0.1, 7.4, 6.7, and 5.7 are 1.03, 1.05, 1.78, and 2.10, respectively. This finding is consistent with the reasonable postulation that, at lower pH values, the extraction of a proton from the α position of serine is rate-limiting.

Four enzymes from E. coli are known to have L-serine dehydrase activity (25-27), but there appears to be only one enzyme which catalyzes the dehydration of d-serine (8). Starch gel electrophoresis at each step of the purification procedure described in this paper also indicated that there is only one d-serine dehydrase. The d-serine dehydrase prepared from repressed and derepressed E. coli K-12 and from mutant C4 of E. coli K-12 fractionated identically throughout the purification procedure.

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REFERENCES

Crystalline d-Serine Dehydrase
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