Formation and Cleavage of 2-Keto-3-Deoxygluconate by 2-Keto-3-Deoxygluconate Aldolase of Aspergillus niger

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Received for publication 2 May 1975

2-Keto-3-deoxygluconate aldolase of Aspergillus niger, an enzyme that has not been reported previously, was purified 468-fold. Maximal activity was obtained at pH 8.0 and 50 C. The enzyme exhibited relative stereochemical specificity with respect to glyceraldehyde. The $K_m$ values for 2-keto-3-deoxygluconate, glyceraldehyde, and pyruvate were 10, 13.3, and 3.0 mM, respectively. The effects of some compounds and inhibitors on enzyme activity were examined. Stability of the enzyme under different conditions was investigated. The equilibrium constant was about $0.33 \times 10^{-4} M$.

Recent results from our laboratory (3-5) indicated that certain strains of Aspergillus niger utilize and degrade gluconate via a newly discovered nonphosphorylative pathway that can be illustrated as follows:

$$\text{gluconate} \xrightarrow{\text{dehydratase}} \text{KDG} \xleftarrow{\text{aldolase}} \text{pyruvate} + \text{glyceraldehyde}$$

Furthermore, the occurrence of this pathway in 28 different fungi was established (6). To our knowledge, 2-keto-3-deoxygluconate (KDG) aldolase is an enzyme not previously recognized. Dahms and Anderson (2) recently demonstrated a 2-keto-3-deoxyladonic acid aldolase that was unable to cleave KDG in a pseudomonad. This paper deals with the purification and study of some of the properties of KDG aldolase.

MATERIALS AND METHODS

Organism. Aspergillus niger 73 was obtained from the culture collection of the National Research Centre of Egypt.

Media and cultures. A. niger was grown on Cza-pek-Dox medium containing 5% sodium gluconate as the sole source of carbon. Erlenmeyer flasks (250 ml), each containing 50 ml of sterile medium, were inoculated and incubated at 30 C. After 2 days the mycelia were harvested by filtration.

Preparation of KDG. KDG was prepared by the method of Portsmouth (10).

Chemical methods. Determination of KDG, pyruvate, and glyceraldehyde was made as previously described (5). Protein was estimated by ultraviolet absorption or by the method of Sutherland et al. (11).

Aldolase assay. Aldolase was assayed by determining KDG formation from pyruvate and glyceraldehyde or by estimating pyruvate liberated from KDG. The assay contained the substrate(s), sodium phosphate buffer, and a rate-limiting amount of enzyme. One unit is defined as the amount of protein that catalyzes the formation of 1 µmol of KDG per minute at pH 8.0 and 40 C.

RESULTS

Purification of aldolase. Extracts were prepared by grinding blotted-dry mycelia with twice their weight of washed cold sand in a cold mortar and extracting with cold 0.2 M sodium phosphate buffer at pH 8.0. The slurry obtained was centrifuged at 6,000 rpm for 10 min, and the supernatant solution was used for enzyme purification. A summary of the procedure is given in Table 1.

(i) Heat treatment. The crude extract was heated at 60 C for 15 min and then quickly cooled. The precipitate was removed by centrifugation and discarded.

(ii) Acetone fractionation. An equal volume of cold acetone (-15 C) was added to the heat-treated preparation and the resulting precipitate was removed by centrifugation and discarded. Another volume of cold acetone (equal to that used before) was added to the supernatant fluid. The precipitated protein was separated by centrifugation and dissolved in 0.02 M sodium phosphate buffer (pH 8.0).

(iii) Diethylaminoethyl-cellulose chromatography. The diethylaminoethyl-cellulose column (1 by 27 cm) was equilibrated with 0.01 M potassium phosphate buffer (pH 7.7), and the above fraction (13 ml) was placed on the column. Elution was carried out by batchwise addition of 15-ml portions of increasing molarities (0.1 to 0.3 M) of solutions of NaCl in 0.01 M potassium phosphate buffer (pH 7.7). A double volume was used for the 0.15 and 0.175 M solutions. Frac
Table 1. Purification of KDG aldolase

<table>
<thead>
<tr>
<th>Step</th>
<th>Total units</th>
<th>Recovery (%)</th>
<th>Sp act</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>525.3</td>
<td>100</td>
<td>0.188</td>
<td>1</td>
</tr>
<tr>
<td>Heat-treated</td>
<td>490.5</td>
<td>93.7</td>
<td>1.12</td>
<td>5.9</td>
</tr>
<tr>
<td>preparation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetone preparation</td>
<td>288.2</td>
<td>56.7</td>
<td>5.40</td>
<td>28.7</td>
</tr>
<tr>
<td>Diethylaminoethyl-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cellulose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 38</td>
<td>9.0</td>
<td>3.1</td>
<td>88.0</td>
<td>468.0</td>
</tr>
<tr>
<td>Fraction 39</td>
<td>9.0</td>
<td>3.1</td>
<td>88.0</td>
<td>468.0</td>
</tr>
<tr>
<td>Fraction 40</td>
<td>7.0</td>
<td>2.4</td>
<td>85.0</td>
<td>452.0</td>
</tr>
</tbody>
</table>

2.0 ml were collected. The highest specific activity was found in fractions 38, 39, and 40, corresponding to purifications of 468-, 468-, and 452-fold, respectively.

Properties of aldolase. Highly purified fractions of aldolase were used in the following experiments.

(i) pH optimum. Maximal enzyme activity for KDG formation from pyruvate and glyceraldehyde occurred at pH 8.0 (Fig. 1). For the cleavage of KDG, maximal activity took place at the same pH value.

(ii) Relative stereochemical specificity for glyceraldehyde. For KDG formation from pyruvate and glyceraldehyde, the activities with L- and D,L-glyceraldehyde were about 66 and 85%, respectively, of that obtained with the D-isomer. Thus, it appears that both isomers were attacked by the enzyme, although the D-form is the preferred substrate.

(iii) Kinetic constants. $K_m$ values (determined from Lineweaver-Burk plots) for KDG, glyceraldehyde, and pyruvate were 10, 13.3, and 3.0 mM, respectively. When the pyruvate or glyceraldehyde concentration was changed the other was added at saturating levels.

(iv) Effect of temperature. Maximal enzyme activity occurred at 50°C (Fig. 2). The activity at 60°C was about 91% of that obtained at 50°C. This suggested that the enzyme might be of a thermophilic nature, although it belongs to a mesophilic organism. In support of this, it has been found that exposure of the enzyme to a temperature of 60°C for 1 or 2 h resulted only in 25 and 40% loss of the units, respectively.

(v) Effects of various compounds. The enzyme was not inhibited by either ethylenediaminetetraacetate or iodoacetate at concentrations of $10^{-4}$, $5 \times 10^{-3}$, and $10^{-2}$ M. This suggests that no metal cation or sulfhydryl group participates in enzyme activity. This is supported by the finding that the addition of sulfhydryl compounds, such as 2-mercaptoethanol, or metal salts, such as MgCl₂, had no effect on enzyme activity. The enzyme was inhibited completely by HgCl₂ at concentrations as low as $10^{-4}$ M. NaCN at a concentration of $10^{-2}$ M did not produce any inhibition. However, at $5 \times 10^{-2}$ and $10^{-2}$ M it caused 66 and 100% inhibition, respectively.

(vi) Enzyme stability. The enzyme was stable for 3 h when incubated in 0.2 M sodium pyrophosphate buffer at pH 9.5. On the other hand, incubation for 0.5 h in 0.05 M citrate buffer at pH 4.0 resulted in complete loss of activity. It seems that this inactivation is due to the prevailing pH and not to the citrate anion, since addition of potassium citrate at higher concentrations to the reaction mixture did not affect the activity. Reactivation of this inactivated enzyme by incubation for 2 h at pH 8.0, using pyrophosphate buffer, failed. The enzyme was stable for 15 days when kept at 4°C in 0.2 M phosphate buffer (pH 8.0). However, keeping the enzyme at the same temperature but in dilute buffer solutions (0.01 M) led to complete loss of activity in 3 days. Freezing and thawing completely destroyed the activity.
(vii) Equilibrium studies. The equilibrium of the reaction was determined in a reaction mixture containing 4.1 μmol of KDG, 100 μmol of phosphate buffer (pH 8.0), and 16 μg of enzyme protein in a total volume of 1.85 ml. At equilibrium (after 2 h) the composition of the reaction mixture was about 32% pyruvate and 68% KDG. The equilibrium was also determined in the reverse reaction by replacing KDG by 9.0 μmol each of pyruvate and glyceraldehyde and was found to be about 38% pyruvate and 62% KDG. The equilibrium constant was calculated to be about 0.33 × 10⁻¹ M.

(viii) Inducibility of aldolase. Crude extracts of glucose-grown mycelia were devoid of aldolase activity, indicating the inducibility of this enzyme.

**DISCUSSION**

Since KDG aldolase is an enzyme that has not been previously reported, it was of interest to purify it and study some of its properties. The enzyme was purified 468-fold. Maximal activity occurred at pH 8.0, and inhibition studies suggested that no metal ion or sulfhydryl group participates in enzyme activity. Similar properties were reported for 2-keto-3-deoxy-6-phosphogluconate aldolase of *Pseudomonas fluorescens* (7) and *Clostridium formicoacetlicum* (1). On the other hand, the 2-keto-3-deoxyyaladonic acid aldolase of the pseudomonad studied by Dahms and Anderson (2) was shown to require a metal ion for its activity. The results indicated that *A. niger* KDG aldolase has a high thermal stability, since it lost only 25% of its activity when exposed to a temperature of 60 °C for 1 h. In contrast, 2-keto-3-deoxyyaladonic acid aldolase lost about 50% of its activity when exposed to a temperature of 55 °C for 15 min (2). With respect to pH stability, *A. niger* KDG aldolase was stable at pH 9.5 but lost its activity at pH 4.0. Reactivation of the acid-inactivated enzyme by incubation for 2 h at pH 8.0, using pyrophosphate buffer, failed. This is in contrast to the finding reported by Meloche and Wood (8) that 2-keto-3-deoxy-6-phosphogluconate aldolase of *P. fluorescens* lost its activity at pH 9.0 and that this activity could be restored by acidifying the enzyme followed by neutralization. On the other hand, the present study and that carried out by Meloche and Wood (9) on the equilibrium composition of the reactions catalyzed by *A. niger* KDG aldolase and *P. fluorescens* 2-keto-3-deoxy-6-phosphogluconate aldolase indicated that the equilibrium in both reactions is towards C₄ formation. In conclusion, the newly reported *A. niger* KDG aldolase proved to have certain properties that distinguish it from 2-keto-3-deoxy-6-phosphogluconate aldolase of *P. fluorescens* (8) and 2-keto-3-deoxyasonic acid aldolase of the pseudomonad studied by Dahms and Anderson (2).

**ACKNOWLEDGMENT**

This investigation was supported by the National Research Center, Cairo, Egypt.

**LITERATURE CITED**


**Fig. 2. Effect of temperature on aldolase activity. Reaction mixture contained: pyruvate, 20 μmol; D,L-glyceraldehyde, 15 μmol; sodium phosphate buffer (pH 8.0), 160 μmol; an enzyme protein, 13 μg (in a total volume of 1.4 ml). Temperature was as indicated; reaction time was 5 min.**


