Occurrence and Some Properties of Two Threonine Dehydratases in Euglena gracilis

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(Received 23 June 1982)

Two threonine dehydratases, I and II, were found to occur in Euglena gracilis and each enzyme was purified partially. Like the biosynthetic threonine dehydratases of other organisms, dehydratase I was feedback-inhibited by isoleucine and not affected by adenylates. Dehydratase II was not influenced by branched-chain amino acids and adenylates. Occurrence of dehydratase II, with a much higher activity than dehydratase I, suggests that dehydration of threonine is not the key point in the regulation of isoleucine biosynthesis in E. gracilis.

INTRODUCTION

Threonine dehydratase (EC 4.2.1.16) is an enzyme which catalyses the deamination of threonine to 2-oxobutyrate. Since 2-oxobutyrate is both the substrate for isoleucine synthesis and the inhibitor of valine synthesis, balanced synthesis of those amino acids is based on the carbon flow from threonine (Squires et al., 1981).

In previous papers we reported that the growth of Euglena gracilis was depressed by exogenous threonine (Oda et al., 1982a) and that this inhibition is due to the inhibitory effect of 2-oxobutyrate on valine synthesis (Oda et al., 1982b, c). From preliminary experiments threonine dehydratase in the crude extract was found to be free from end-product inhibition by isoleucine so that this enzyme caused excessive formation of toxic 2-oxobutyrate (Oda et al., 1982b). To investigate the regulation of isoleucine-valine biosynthesis in E. gracilis we purified this enzyme and discovered it to occur as two isoenzymes. The present paper reports partial characterization of the two threonine dehydratases in E. gracilis.

METHODS

Organism and culture. Euglena gracilis SM-ZK (a streptomycin-bleached mutant derived from strain z) was cultured in a medium for 5 d as reported previously (Oda et al., 1982a).

Preparation of crude enzyme. Cells (11 g, wet weight) were washed and resuspended in 42 ml 50 mM-potassium phosphate buffer (pH 7.5). Buffers employed for the purification procedure were supplemented with 0-1 mM-2-mercaptoethanol, 0-1 mM-EDTA, 10 μM-pyridoxal 5-phosphate and 20% (v/v) ethylene glycol unless otherwise stated. The cells were disrupted by sonication (10 kHz for 5 min) at 4°C. The supernatant obtained by centrifugation of the sonicate at 10000 g for 30 min was used as crude enzyme.

Enzyme assay. The reaction mixture contained in a total volume of 0-5 ml 50 mM-L-threonine, 10 μM-pyridoxal 5-phosphate, 50 mM-glycine/NaOH buffer (pH 9-5) and 0-05 ml enzyme suspended in 50 mM-potassium phosphate buffer (pH 7-5). After incubation at 37°C for 10 min, the reaction was stopped by the addition of 0-5 ml 1 M-HCl; the 2-oxobutyrate formed was determined by the method of Shizuta & Tokushige (1971). One unit of enzyme activity was defined as the amount of enzyme required to produce 1-0 μmol 2-oxobutyrate min⁻¹ at 37°C.

Protein was determined by the method of Bradford (1976).

Molecular weight determination by gel filtration. Molecular weight of the enzyme was determined by the method of Andrew (1964) using a column (30 x 75 cm) of Sephacyr S-300. Chromatography was carried out at 4°C at a flow rate of 30 ml h⁻¹ using 50 mM-potassium phosphate buffer (pH 7-5) as eluant. The column was calibrated with glutamate dehydrogenase (yeast, mol. wt 320000), catalase (bovine liver, 232000), alcohol dehydrogenase

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(yeast, 150000), lactate dehydrogenase (pig heart, 135000), malate dehydrogenase (yeast, 67000) and cytochrome c (horse heart, 12400).

RESULTS

Separation and partial purification of threonine dehydratases

Typical purification steps of threonine dehydratases from E. gracilis are summarized in Table 1. The crude enzyme was centrifuged at 100000 g for 1 h in a Hitachi 55P-72 ultracentrifuge and the supernatant was applied to a DEAE-cellulose column (2.5 x 20 cm). After washing with 40 ml 50 mM-potassium phosphate buffer (pH 7.5) the column was eluted with a linear concentration gradient (50-400 mM) of potassium phosphate. Dehydratase activity was separated into two peaks, one eluted by 50 mM and the other by 200 mM-potassium phosphate, and they were named threonine dehydratase I and II, respectively. Dehydratase I was inhibited by isoleucine while dehydratase II was not, and the ratio of dehydratase I activity against that of dehydratase II was about 0.15. The active fractions of dehydratase I were diluted with 20% ethyleneglycol containing 0.1 mM-2-mercaptoethanol, 0.1 mM-EDTA, 10 mM-pyridoxal 5-phosphate to 20 mM-potassium phosphate (final concentration) and applied to a column (1.7 x 18 cm) of DEAE-Bio-Gel A. The column was eluted with a gradient (50-400 mM) of potassium phosphate. The fractions of dehydratase II from DEAE-cellulose were brought to 60% saturation of ammonium sulphate, and the precipitate was dissolved in 50 mM-potassium phosphate buffer (pH 7.5) and chromatographed on Sephacryl S-300 (3.0 x 75 cm). The active fractions were applied to a column of DEAE-Bio-Gel A, and the column was eluted with a gradient (50-400 mM) of potassium phosphate. Both active fractions of dehydratase I and II obtained from DEAE-Bio-Gel A were dialysed for 12 h against 50 mM-potassium phosphate buffer (pH 7.5) and used as the partially purified enzyme preparations.

The molecular weight of dehydratase II was determined to be about 250000 by chromatography on Sephacryl S-300.

Stability

Storing the crude enzyme prepared in the absence of ethyleneglycol at 4 °C caused a decrease in enzyme activity to 55% of the original activity in 24 h, while loss of the activity was hardly observed in the presence of 20% ethyleneglycol or 1 mM-potassium phosphate. On incubation of the enzyme preparation at 37 °C for 10 min the activity of dehydratase I decreased by 16% and that of dehydratase II by 36%. Loss of dehydratase I was prevented by 1 mM-isoleucine but that of dehydratase II was not. Although isoleucine improved the heat stability of dehydratase I, loss of specific activity and recovery of this enzyme by ammonium sulphate fractionation or gel filtration were not prevented by the addition of isoleucine.

pH optima

Maximum activity of dehydratase I was obtained at pH 9.5 to 11.0 (Fig. 1a). In the presence of isoleucine the activity was decreased and the pH optimum was shifted to higher values. Dehydratase II exhibited a broad maximum activity at pH above 8.5 (Fig. 1b). The two enzymes were activated at pH 7.5 (Tris/HCl buffer) by four salts (Fig. 2a, b); the activation was much more extensive with dehydratase I than with II.

Kinetic constant and specificity

Dehydratase I and II followed normal Michaelis-Menten kinetics, giving $K_m$ values of 34 and 13 mM, respectively. Isoleucine inhibited dehydratase I competitively, the $K_i$ was 0.22 mM. Both enzymes dehydrated L-serine but not the D-isomers of threonine and serine. The relative rates of pyruvate formation from L-serine by dehydratase I and II were 26 and 16% of threonine dehydration, respectively.
Threonine dehydratases in Euglena gracilis

Fig. 1. Effects of pH on threonine dehydratase I (a) and II (b). Enzyme activity was determined without L-isoleucine (○) and with 0.4 mM (●) or 1.0 mM (△) L-isoleucine. Buffers: pH 7.5–8.5, 50 mM-Tris/HCl; pH 9.0–12.0, 50 mM-glycine/NaOH.

Fig. 2. Effects of salts on threonine dehydratase I (a) and II (b). Both enzymes were dialysed against 20 mM-Tris/HCl buffer (pH 7.5), and those activities were determined at pH 7.5 (50 mM-Tris/HCl) in the presence of potassium phosphate (○), sodium phosphate (▲), potassium chloride (●) or sodium chloride (△).

Table 1. Partial purification of two threonine dehydratases from E. gracilis

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity [units (mg protein)^{-1}]</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>42.0</td>
<td>1060</td>
<td>117</td>
<td>0.110</td>
<td>100</td>
</tr>
<tr>
<td>2. Supernatant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. DEAE cellulose</td>
<td>31.5</td>
<td>233</td>
<td>8.70</td>
<td>0.0373</td>
<td>7.4</td>
</tr>
<tr>
<td>4. DEAE-Bio-Gel A</td>
<td>23.0</td>
<td>50.6</td>
<td>4.58</td>
<td>0.0905</td>
<td>3.9</td>
</tr>
<tr>
<td>3. DEAE-cellulose</td>
<td>70.0</td>
<td>85.4</td>
<td>75.0</td>
<td>0.878</td>
<td>64.1</td>
</tr>
<tr>
<td>4. Ammonium sulphate 60% pellet</td>
<td>4.54</td>
<td>54.6</td>
<td>53.0</td>
<td>0.971</td>
<td>45.3</td>
</tr>
<tr>
<td>5. Sephacryl S-300</td>
<td>48.2</td>
<td>8.03</td>
<td>30.6</td>
<td>3.81</td>
<td>26.2</td>
</tr>
<tr>
<td>6. DEAE-Bio-Gel A</td>
<td>27.2</td>
<td>2.20</td>
<td>20.8</td>
<td>9.45</td>
<td>17.8</td>
</tr>
</tbody>
</table>

Effects of branched-chain amino acids and adenylates

Dehydratase I was strongly inhibited by isoleucine, and, to a lesser extent, by leucine; 50% inhibition was observed with 0.35 mM-isoleucine or 6 mM-leucine. Valine showed neither activation nor inhibition of dehydratase I. Table 2 shows the effects of combinations of these three amino acids. The inhibition by isoleucine was additive to that by leucine. Valine slightly antagonized the inhibitions by isoleucine and leucine.
Table 2. Effect of L-isoleucine and related amino acids on threonine dehydratase I

L-Isoleucine (0.5 mM), L-leucine (4.0 mM) and L-valine (10.0 mM) were added to the reaction mixture.

<table>
<thead>
<tr>
<th>Addition to reaction mixture</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>64</td>
</tr>
<tr>
<td>Leucine</td>
<td>39</td>
</tr>
<tr>
<td>Valine</td>
<td>0</td>
</tr>
<tr>
<td>Isoleucine + leucine</td>
<td>71</td>
</tr>
<tr>
<td>Isoleucine + valine</td>
<td>45</td>
</tr>
<tr>
<td>Leucine + valine</td>
<td>30</td>
</tr>
<tr>
<td>Isoleucine + leucine + valine</td>
<td>51</td>
</tr>
</tbody>
</table>

Both dehydratases I and II were neither activated nor inhibited by adenylates such as AMP, ADP or ATP at 1 mM.

**DISCUSSION**

Two threonine dehydratases of *E. gracilis* were partially purified and distinguished from each other in some properties.

Threonine dehydratases are known to be classified into two groups, biosynthetic and biodegradative enzymes, by their functions (Umbarger, 1973). The former serves to supply 2-oxobutyrate required for isoleucine biosynthesis and the latter appears to be involved only in the breakdown of threonine presumably to supply energy. Biosynthetic dehydratase have been isolated in a number of micro-organisms (Umbarger, 1973) and plants (Bryan, 1980), and a few organisms have been shown to produce both types of threonine dehydratase. The biosynthetic enzyme of *Escherichia coli* which is inhibited or repressed by isoleucine and insensitive to AMP is synthesized in aerated minimal medium containing glucose, whereas the biodegradative enzyme of this organism which is activated by AMP and not inhibited by isoleucine is synthesized in a complex medium only in the absence of glucose under anaerobic conditions (Wood, 1969). Two similar enzymes have been found in pea seedlings (Tomova et al., 1968). Recently Yoshida et al. (1982) have reported that *Proteus morganii* produced both enzymes simultaneously under aerobic culture conditions.

Threonine dehydratase I of *E. gracilis* was feedback-inhibited by isoleucine and not activated by adenylates. Valine, which is known as a positive effector of biosynthetic dehydratase (Umbarger, 1973), did not activate dehydratase I but antagonized the inhibition by isoleucine or leucine. A slight shift of the optimum pH of *E. gracilis* dehydratase I is also found in other biosynthetic enzymes (Yoshida et al., 1982). Effects of salts on dehydratase I are quite similar to those on the biosynthetic enzyme of *Brevibacterium flavum* (Miyajima & Shiio, 1972). These properties indicate that *E. gracilis* dehydratase I is a biosynthetic enzyme. However, a remarkable difference in the properties between *E. gracilis* dehydratase I and biosynthetic threonine dehydratases of other organisms is that the *E. gracilis* enzyme shows no homotropic cooperativity for substrate, which is known to be a typical property of the biosynthetic enzymes (Umbarger, 1973), in the presence or absence of isoleucine.

Threonine dehydratase II of *E. gracilis* was not susceptible to inhibition or activation by branched-chain amino acids and adenylates. Molecular weight of this enzyme was larger than the dehydratase of some other organisms (150000–200000) (Umbarger, 1973) but smaller than the biosynthetic enzyme of brewers’ yeast (300000) (Kovaleva et al., 1981). In the preliminary experiments dehydratase activity in the crude extract of *E. gracilis* was not inhibited by isoleucine and the production of this enzyme was neither induced nor repressed by threonine or branched-chain amino acids (Oda et al., 1982b). Under the previous assay condition, 50 mM-Tris/HCl (pH 8.5) in the absence of potassium phosphate, the dehydratase I activity was less than 20% of that determined under the present assay condition, 50 mM-glycine/NaOH (pH 9.5) in the presence of 5 mM-potassium phosphate. Accordingly, the dehydratase activity of the
crude enzyme as assayed previously was largely due to isoleucine-insensitive dehydratase II. From the above results it is also noted that dehydratase II is produced constitutively.

Dehydratase I might possibly be converted into dehydratase II by desensitization during extraction from E. gracilis cells like in homoserine O-acetyltransferase (Shiio & Ozaki, 1981), but this is not the case. We found that unusual accumulation of isoleucine during the growth inhibition by threonine was due to an insufficiently controlled formation of 2-oxobutyrate from exogenous threonine (Oda et al., 1982b). This observation supports the existence of isoleucine-insensitive threonine dehydratase in E. gracilis. Since dehydration of threonine is not the key point in the regulation of isoleucine biosynthesis as discussed above and an excess amount of endogenous 2-oxobutyrate is highly toxic in E. gracilis (Oda et al., 1982b), synthesis of threonine should be regulated strictly in vivo and overproduction of 2-oxobutyrate seems to be prevented under normal conditions.

REFERENCES


