Absolute Configuration of a Metabolite in the
$m$-Fission Pathway of Protocatechuate

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An aldolase, which is induced in Pseudomonas testosteroni during growth with $p$-hydroxybenzoate, preferentially attacks the $R$ form of 4-hydroxy-4-methyl-2-oxoglutarate, a metabolite of protocatechuic catabolism.

4-Hydroxy-4-methyl-2-oxoglutarate (HMG) possesses an asymmetric carbon (C-4) and is a catabolite in the degradation of protocatechuic by meta-fission (4). The present investigation shows that one enantiomer, (R)-HMG (Fig. 1), is preferentially degraded by the aldolase of this pathway. Another metabolic route has recently been described in which the ring-fission product from protocatechuic is oxidized rather than hydrolyzed (4). However, this pathway does not require a second aldolase since we find that HMG aldolase, partially purified from Pseudomonas testosteroni, readily cleaves the corresponding metabolite of the alternative route, namely 4-carboxy-4-hydroxy-2-oxoadipate (CHA). Tack et al. (8) have also shown that CHA is a substrate for HMG aldolase purified to homogeneity from Pseudomonas putida. This enzyme likewise attacked one enantiomer of HMG, but the configuration was not established (8).

The chemical synthesis of HMG (3) and assay of HMG aldolase (8) were as previously described. We are indebted to D. J. Hopper for the following procedure used to purify HMG aldolase. Extracts of P. testosteroni were prepared in the 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8, which was used throughout, and were treated with protamine sulfate (2). Protein in the supernatant which precipitated between 40 and 60% saturation with (NH$_4$)$_2$SO$_4$ was then taken up in buffer and applied to a column (30 by 2.5 cm) of diethylaminoethylcellulose. When eluted with a linear gradient of 0.1 to 0.3 M NaCl in buffer, HMG aldolase activity appeared in tubes 49 to 65 of the 10-ml fractions collected. These were pooled, precipitated with (NH$_4$)$_2$SO$_4$ at 70% saturation, dissolved in buffer, and applied to a column (90 by 5.5 cm) of Sephadex G-150. Elution with buffer (fractions of 13 ml) gave aldolase activity in tubes 76 to 84 which were pooled and concentrated by pressure dialysis (Table 1).

The amounts of pyruvate formed by the Co$^{2+}$-catalyzed fission of racemic HMG were compared with those formed enzymatically by measuring the total decreases in absorbance at 340 nm for 3-ml reaction mixtures (pH 8) containing 0.3 mg of reduced nicotinamide adenine dinucleotide (NADH), 5 units of lactate dehydrogenase, and 0.14 $\mu$ mole of HMG. Aldolase (0.012 mg of protein) and CoSO$_4$ (2.4 $\mu$ moles, added without enzyme) then catalyzed the release of amounts of pyruvate that oxidized 0.15 and 0.26 $\mu$ mole of NADH, respectively. When CoSO$_4$ was added to an enzymatic reaction as it neared completion, a total of 0.27 $\mu$ mole of NADH reacted. These calculations take into account a small increase in absorbance, equivalent to 0.01 $\mu$ mole of NADH, which occurred when additions of CoSO$_4$ were made in the absence of substrate or enzyme. The stoichiometry agrees with the scheme of Fig. 1; namely, one enantiomer was attacked by the aldolase, and both were decomposed on adding Co$^{2+}$.

The enantiomer serving as substrate for HMG aldolase was identified by oxidizing to citramalate (Fig. 1) that portion of the racemate which remained after partial enzymatic degradation. The isolation and purification of citramalate as described by Hopper et al. (5) give rather low recoveries, and crude extract (5 ml) was therefore used with larger amounts of HMG (60 mg in 150 ml of buffer). After incubation for 3 min, the HMG which remained was oxidized during 15 min with 2 ml of 30% hydrogen peroxide. Excess of reagent was removed by catalase, and a sample of citramalate was isolated, purified, and taken up in 5 ml of water. Fluorometric analysis (7) of this
Fig. 1. Degradation of racemic 4-hydroxy-4-methyl-2-oxoglutarate (HMG). An aldolase from Pseudomonas testosteroni cleaved (R)-HMG in a racemic mixture to give pyruvate; this was assayed by means of lactate dehydrogenase. (S)-HMG which accumulated in the reaction was converted by treatment with hydrogen peroxide into L-citramalate. Cobalt sulfate catalyzed a nonenzymic fission of both enantiomers of HMG.

Table 1. Purification of HMG aldolase

<table>
<thead>
<tr>
<th>Protein (mg)</th>
<th>Specific activity (units/mg of protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>6,400</td>
<td>0.15</td>
</tr>
<tr>
<td>Protamine sulfate treatment</td>
<td>3,720</td>
<td>0.18</td>
</tr>
<tr>
<td>Ammonium sulfate, 40–60% saturation</td>
<td>1,140</td>
<td>0.55</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>98</td>
<td>2.35</td>
</tr>
<tr>
<td>Sephadex G-150 chromatography</td>
<td>18</td>
<td>8.86</td>
</tr>
</tbody>
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solution showed that it contained 6.5 μmoles of citramalate/ml. The optical rotation, when enhanced with sodium citrate and ammonium molybdate (5, 6), was +23 ± 2 millidegrees, corresponding to an excess of about 2.4 μmoles of L(+) over D(−)-citramalate in 1 ml. A determination of L-citramalate using a cladtral extract (1) gave, for 1 ml, 4.6 μmoles, that is, 2.7 μmoles of L in excess of D-citramalate. These separate determinations, therefore, indicated that (S)-HMG was less readily destroyed by the aldolase, and that (R)-HMG was preferentially attacked (Fig. 1).

When HMG and extract were used at the concentrations of the last experiment, half of the HMG disappeared within 5 min and the remainder in about 1 hr. While this may indicate that the aldolase is not absolutely specific for (R)-HMG, it is perhaps more probable that accumulating (S)-HMG undergoes nonenzymic racemization to its enantiomer which is immediately removed. This may explain why yields of pyruvate have been reported (4) in excess of those expected from (R)-HMG exclusively.

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Literature Cited


