HYDROXYLATION OF SALICYLIC ACID TO GENTISATE BY A BACTERIAL ENZYME

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1. Introduction

Several initial reactions involved in the microbial catabolism of salicylate have been described. Oxidative decarboxylation to produce catechol is well documented [1] and salicylate hydroxylase (salicylate, NADH: oxygen oxidoreductase (1-hydroxylating, 1-decarboxylating) EC 1.14.13.1) has been purified and its properties have been investigated [2,3]. Non-oxidative decarboxylation of salicylate to phenol [4], hydroxylation to 2,3-dihydroxybenzoate [5,6], to 2,4-dihydroxybenzoate [7] or hydroxylation at the C-5 position to yield gentisate [8,9] have also been observed in various bacteria and fungi. Enzymes reducing salicylate to o-hydroxybenzaldehyde and o-hydroxybenzyl alcohol have been reported in Neurospora crassa [10,11].

In this report we describe an inducible bacterial enzyme which catalyses the hydroxylation of salicylate to gentisate according to the scheme:

\[
\text{COOH} \quad \text{NAD(P)}^+ \text{H} \quad \text{O}_2 \quad \text{OH} \quad \text{COOH}
\]

2. Materials and Methods

The bacterium, strain K17 [12], was described as KS 1741 by Sundman [13] and strain 33 by de

Smedt and de Ley [14]. Although originally described as an Agrobacterium, recent evidence [12,14] has suggested that it is not a member of any recognized genus. A manuscript is in preparation proposing its designation as the type species of a novel genus, Lignobacter.

The bacterium was grown at 28°C in 10–121 batches in a New Brunswick Model 19 Fermenter Assembly with aeration set at 1500 ml/min and agitation at 250 rev./min. The growth medium contained per 1: 2.0 g NH4Cl; 3.8 g K2HPO4 · H2O; 2.1 g KH2PO4; 0.3 g MgSO4 · 7H2O; 0.1 g NaCl; 10 mg CaCl2 and 0.01 mg FeSO4. After adjusting the pH to 6.7 with HCl the medium was supplemented with the vitamin solution described in [13] and 0.01% (w/v) yeast extract (Difco). Salicylate (5 mM) was added as a filter-sterilized solution of the sodium salt. Cells were harvested by centrifugation at 4°C, washed twice with the salt solution described above and stored frozen until required.

For the preparation of extracts, bacteria were suspended in 3 vol. 0.1 M KH2PO4 buffer (pH 7.0) containing 1 mM dithiothreitol and broken by 4 passages through the X-press (AB Bax, Nacka, Sweden) previously cooled to −20°C. After treatment with bovine pancreas deoxyribonuclease (10 μg/ml) for 15 min at room temperature, unbroken cells and cell debris were removed by centrifugation at 30 000 x g for 30 min. The supernatant material was further centrifuged at 100 000 x g for 30 min and the soluble fraction retained for enzyme assays.

Enzymic activity was assayed at 30°C by measuring oxygen consumption with a Clark oxygen electrode. Gentisate was identified (a) on TLC plates (Eastman Chromagram sheets, type 13181) developed
in iso-propanol: methanol: chloroform (65:25:10), and visualized by spraying with Gibb’s reagent (2% w/v 2,6-dichloroquinone-4-chlorimide in ethanol); (b) by gas chromatography of the trimethylsilyl derivative and comparison with the retention time of authentic gentisate. Protein was determined by the biuret method [15].

3. Results

In strain K17, catabolism of salicylic acid proceeds through an initial hydroxylation of the C5 position. Washed suspensions of salicylate-grown K17 cells incubated with salicylate in the presence of 1 mM \( \alpha,\alpha' \)-bipyridyl accumulated gentisate. \( \alpha,\alpha' \)-Bipyridyl has previously been shown to inhibit the further degradation of the dihydroxy compound [16]. Furthermore, high induced levels of gentisate 1,2-dioxygenase, but not those ring-cleaving enzymes attacking 2,3-dihydroxybenzoate or protocatechuate, were present in bacterial extracts (Table 1). Only low rates of oxygen consumption were observed in the presence of catechol.

In reaction mixtures containing excess reduced pyridine nucleotide, extracts of salicylate-grown bacteria consumed 2.1 mol of \( O_2 \) per mol of salicylate oxidized. Maximum rates of oxygen uptake were observed with NADPH although NADH also served as coenzyme (Table 2); addition of FAD or FMN had no further stimulatory effect. Since the extracts contained high gentisate 1,2-dioxygenase activity, the hydroxylation step consumed approx. 1 mol \( O_2 \)/mol salicylate and appears to involve a typical mono-oxygenase reaction. NADPH (or NADH) consumption could not be established with the bacterial extracts used here due to reduction of the oxidized co-enzyme by an enzyme-catalyzed reaction involving the products of gentisate catabolism. The hydroxylase is inducible since no activity was detected in extracts of bacteria grown on glutamate. Attempts to isolate gentisate from reaction mixtures containing bacterial extract, salicylate, NADPH and \( \alpha,\alpha' \)-bipyridyl were inconclusive and only trace amounts of gentisate were detectable by gas chromatography. When several analogues were tested as substrates for the enzyme results indicated the enzymes to possess a high degree of specificity for salicylate (Table 3).

### TABLE 1
Activity of ring-cleavage enzymes in extracts of K17 grown on salicylate and on glutamate

The reaction mixture contained in 3 ml: 250 \( \mu \)mol Tris–HCl buffer (pH 7.0), 1.61 mg (salicylate) or 5.3 mg (glutamate) cell extract protein. The reaction was initiated by addition of 1.0 \( \mu \)mol substrate. Temp. 30°C. Values are corrected for \( O_2 \) consumption in the absence of substrate.

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>( O_2 ) consumption (nmol/min/mg protein) on</th>
<th>Catechol</th>
<th>Protocatechuate</th>
<th>Gentisate (^a)</th>
<th>2,3-Dihydroxybenzoate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td></td>
<td>N.D.</td>
<td>2.2</td>
<td>0.5</td>
<td>N.D.</td>
</tr>
<tr>
<td>Salicylate</td>
<td></td>
<td>8.2</td>
<td>3.3</td>
<td>163.5</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

\(^a\) 0.1 \( \mu \)mol FeSO\(_4\) was added to assays involving gentisate.
N.D., not detectable.

### TABLE 2
Effect of cofactor supplementation on salicylate-5 hydroxylase in extracts of K17 grown on salicylate

The reaction mixture contained in 3 ml: 250 \( \mu \)mol K-phosphate buffer (pH 7.0); 5.1 mg cell extract protein; 1.0 \( \mu \)mol NADH or NADPH as indicated; 0.5 \( \mu \)mol salicylate. Temp. 30°C. Values are corrected for \( O_2 \) consumption in the absence of substrate.

<table>
<thead>
<tr>
<th>Cofactor supplement</th>
<th>Specific activity (nmol ( O_2 )/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.9</td>
</tr>
<tr>
<td>NADH</td>
<td>12.5</td>
</tr>
<tr>
<td>NADPH</td>
<td>14.7</td>
</tr>
</tbody>
</table>

[^1]: 0.1 \( \mu \)mol FeSO\(_4\) was added to assays involving gentisate.
[^2]: N.D., not detectable.
TABLE 3
Specificity of salicylate-5-hydroxylase activity in extracts of K17 strain on salicylate

The reaction mixture contained in 3 ml: 250 mol K-phosphate buffer pH 7.0; 5.1 mg cell extract protein; 1.0 mol NADPH; reaction was initiated by addition of 0.5 mol substrate. Temp. 30°C. Values are corrected for O₂ consumption in the absence of substrate.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (nmol O₂/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicylate</td>
<td>9.0</td>
</tr>
<tr>
<td>Benzoate</td>
<td>N.D.</td>
</tr>
<tr>
<td>m-Hydroxybenzoate</td>
<td>N.D.</td>
</tr>
<tr>
<td>p-Hydroxybenzoate</td>
<td>0.9</td>
</tr>
<tr>
<td>Phenol</td>
<td>N.D.</td>
</tr>
<tr>
<td>Catechol</td>
<td>&lt;0.2 ᵉ</td>
</tr>
<tr>
<td>2,3-Dihydroxybenzoate</td>
<td>2.0</td>
</tr>
<tr>
<td>2,4-Dihydroxybenzoate</td>
<td>0.3</td>
</tr>
<tr>
<td>2,6-Dihydroxybenzoate</td>
<td>0.7</td>
</tr>
<tr>
<td>o-Hydroxyphenylacetate</td>
<td>N.D.</td>
</tr>
<tr>
<td>o-Hydroxyphenylpropionate</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

⁹ Oxygen consumption observed in excess of that due to low levels of catechol dioxygenase.
N.D., not detectable.

4. Discussion

Although gentisate has been reported as an intermediate in salicylate metabolism by mammalian, plant and microbial systems the enzymology of the initial hydroxylation step has not been studied in detail [17]. Genetic studies of K17 indicate that the gene coding for the hydroxylase is borne on a plasmid [17]. Degradation of salicylate through catechol has been shown to be plasmid-coded in Pseudomonas putida [18] but no catabolism via catechol has been found in K17.

Gentisate catabolism in K17 proceeds via ring cleavage to maleylpyruvate followed by a GSH-dependent cis, trans isomerization to fumarylpyruvate [19,20]. This pathway seems to be chromosomally coded since mutants which are unable to grow on salicylate can still grow on 3-hydroxybenzoate which is also dissimilated through gentisate.

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References