THE META-CLEAVAGE OF CATECHOL BY A THERMOPHILIC BACILLUS SPECIES.

J.A.BUSWELL
Department of Biology,
Paisley College of Technology, Paisley, United Kingdom.

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SUMMARY. Cell extracts of a thermophilic strain of Bacillus grown on phenol metabolise catechol by the meta-cleavage route to yield 2-hydroxymuconic semialdehyde. The product of ring-fission may be further converted by a NAD^+-dependent semialdehyde dehydrogenase or by a non-NAD^+-dependent enzyme reaction. Both enzymes are induced in phenol-grown cells and high levels of activity are detectable at 50°.

The dissimilation of many aromatic compounds through the diphenol, catechol, and the further oxygenative ring-fission of this intermediate by the ortho- or the meta-cleavage routes, is well documented in mesophilic micro-organisms, (1),(reviewed by Dagley,(2). It has been shown also that the product of meta-cleavage, 2-hydroxymuconic semialdehyde (2-HMS), is further metabolised by two distinct enzymes. Nishizuka et al (3), reported the conversion of 2-HMS to γ-oxalcrotonate by a NAD^+-dependent dehydrogenase present in extracts of a pseudomonad grown on o-cresol, whereas Dagley and Gibson(4) and Bayly and Dagley(5) demonstrated that with their strain a hydrolytic fission of the ring cleavage product occurs, yielding formate and 2-oxopent-4-enoic acid. More recently both NAD^+-dependent dehydrogenase and non NAD^+-dependent hydrolase activities have been shown to coexist in benzoate-grown Azotobacter cells (6,7) and in a napthalene-grown pseudomonad(8). In this report evidence is presented for the induction of catechol 2,3-oxygenase in an obligate thermophilic Bacillus species by growth on phenol and for the existence of the enzymes responsible for the initial reactions in the subsequent conversion of 2-HMS via the two meta-cleavage pathways.

MATERIALS AND METHODS: A thermophilic Bacillus species (strain PH24), isolated by elective culture from industrial sediment, was grown at 55° in a mineral salts medium containing (g/l); K_2HPO_4, 0·5; NH_4Cl, 1·0;

$MgSO_4 \cdot 7H_2O$, 0·02; yeast extract (Oxoid), 0·2; casamino acids (Difco), 0·1; trace elements solution of Barnett and Ingram(9), 1ml/l, and phenol, 0·5; the pH was adjusted to 7·2. Succinate (2g/l) was substituted in place of phenol for the growth of non-induced cells. Cells were harvested towards the end of the exponential phase, washed in phosphate buffer, pH 7·5, suspended in 4 vols. of 100mM phosphate buffer, pH 7·5, and broken by ultrasonic treatment for 5 min with a 100W ultrasonic disintegrator. The cell extracts were clarified by centrifugation at 35,000g for 20 min and the supernatant further centrifuged at 150,000g for 60 min to eliminate formate dehydrogenase activity. Cell extracts used in the assay of ring-fission activity were supplemented with acetone (10% $^{v}/v$) immediately after the initial clarification. Partial purification of the catechol 2,3-oxygenase activity was effected as follows: the fraction precipitating between 50 and 65% with acetone was redissolved in 100mM potassium phosphate buffer, pH 7·5, containing 10% $^{v}/v$ acetone and applied to a column (17cmx3cm) of DEAE cellulose. The column was eluted with the same buffer and 9ml fractions collected. Fractions 58-80, which contained very low 2-HMS dehydrogenase and hydrolase activities, were pooled and used to determine the stoicheiometry of ring-cleavage.

The enzyme assays were the same as those described by Sala-Trepat and Evans(7). Oxygen uptakes were measured by using a Clark oxygen electrode. On cessation of oxygen uptake on catechol the amount of 2-hydroxymuconic semialdehyde formed was determined from the absorbance at 375 nm in a suitably diluted sample, on the basis of a molar absorption coefficient of $3{\cdot}3 \times 10^4$ (10). Solutions of the ring-fission products of catechol and methylcatechols were prepared using a heat-treated extract (55°C for 10 min) of Pseudomonas putida NCIB 10015 (kindly provided by Dr. P.A. Williams) grown on phenol (7). Formate was determined by the acetylacetone method of Nash(11) after reduction to formaldehyde(12). NADase (NAD glycohydrolase EC

3•2•2•6) was purchased from Sigma Chemical Company. Protein concentrations were measured by the biuret method (13).

RESULTS AND DISCUSSION. Sonically prepared extracts of phenol-induced cells oxidised catechol, 3-methylcatechol and 4-methylcatechol at high rates with the transient accumulation of yellow intermediates. The yellow compounds produced showed identical spectral characteristics in alkaline, acidic and neutral conditions with those of the respective meta-cleavage products, namely, 2-hydroxymuconic semialdehyde (2-HMS), 2-hydroxy-6-oxohepta-2,4-dienoic acid, and 2-hydroxy-5-methylmuconic semialdehyde (2-hydroxy-5-MMS). Ring-fission activity towards catechol was detectable only at very low levels in extracts of succinate-grown cells and increased about 400-fold by growth on phenol.

TABLE 1 Substrate specificity of catechol 2,3-oxygenase preparations from Bacillus PH24 grown on phenol.

Substrate	Specific activity (μmoles/min/mg protein)	Relative rates of O_2 uptake (Catechol = 100%)
Catechol	1260	100
3-Methylcatechol	1160	92
4-Methylcatechol	2212	175
Pyrogallol	1378	109
Protocatechuate	N•D	-
2,3-Dihydroxybenzoate	N•D	-
Phloroglucinol	N•D	-
Resorcinol	N•D	-
Quinol	N•D	-
1,2,4-Benzentriol	368	29
4-Fluorocatechol	1191	95

Oxygen consumption was measured using the Clark oxygen electrode. The reaction mixture contained in a total volume of 3ml, 250μmoles phosphate buffer, pH 8•0; 0•2ml extract (2•8mg protein), and 1•0μmole substrate. The temperature was 55°C. Values are corrected for endogenous oxygen uptake and for spontaneous oxidation of the substrate N•D - not detected.

As reported for catechol 2,3-oxygenase preparations obtained from Pseudomonas spp. (14-17) and Azotobacter vinelandii (7), crude extracts of Bacillus PH24 catalysed the oxygenative cleavage of other catechol derivatives. Pyrogallol, 1,2,4-benzenetriol and 4-fluorocatechol were readily metabolised, the latter two substrates to yellow intermediates, but oxidation of protocatechuate,2,3-dihydroxybenzoate, quinol, resorcinol and phloroglucinol was not detected under similar conditions (Table 1). The results presented here do not distinguish non-specific catalysis by a single catechol 2,3-oxygenase or the presence of several oxygenases but partially purified preparations appear to exhibit corresponding activities towards the same range of substrates (unpublished results).

Partial purification of catechol 2,3-oxygenase was effected by acetone precipitation and DEAE-cellulose column chromatography and fractions obtained which contained only very low 2-HMS dehydrogenase activity. These catalysed the stoicheiometric conversion of catechol to 2-HMS and a simultaneous uptake of an equimolar amount of oxygen (Table 2). On the other hand crude extracts supplemented with NAD^+ consumed an additional 0.5 mole of oxygen/mole of substrate to that required by the ring-fission reaction.

Catechol 2,3-oxygenase activity in crude extracts of Bacillus PH24

TABLE 2 Stoicheiometry of catechol oxidation by partially purified extracts from Bacillus PH24 grown on phenol.

Catechol supplied nmoles	O_2 consumed nmoles	2-hydroxymuconic semialdehyde formed nmoles
200	194	212
400	382	410

Oxygen uptake was measured using the Clark oxygen electrode and 2-HMS determined at 378nm. The reaction mixture contained in a total volume of 3ml, 250μmoles of phosphate buffer, pH8·0; 0·1ml extract (0·9mg protein) and 200 (400) nmoles of catechol. The temperature was 55° and O_2 uptakes are corrected for endogenous uptake.

Table 3 Specific activities* of 2-HMS, 2-hydroxy -5-MMS and 2-hydroxy -6- oxohepta -2,4-dienoic acid metabolising enzymes in cell extracts of Bacillus PH24

Assay substrate	No NAD added to the reaction mixture	NAD (0·4µmole) added to the reaction mixture	Ratio of activities NAD/non NAD	Cell extracts treated with NADase**	Remaining activity after NADase treatment %
PHENOL-GROWN CELLS					
2-HMS	82·4	274·2	3·3	65·4	79
2-hydroxy-5-MMS	19·8	271·4	13·7	11·6	58
2-hydroxy-6-oxohepta-2,4-dienoic acid.	317·6	316·4	1·0	310·4	98
SUCCINATE-GROWN CELLS					
2-HMS	1·7	4·2	2·5	0·81	48
2-hydroxy-5-MMS	0·60	3·1	5·2	0·22	37
2-hydroxy-6-oxohepta 2,4-dienoic acid.	5·2	5·1	1·0	5·1	98

* Expressed as nmoles/min/mg protein.

** 1 ml of cell extract was incubated with 1 ml of NADase (containing 1 unit of NADase activity) for 2hr at 37°

The reaction mixture contained in a total volume of 3ml: 250 µmoles phosphate buffer, pH7·5; 0·1ml of phenol-grown extract (0·65mg protein) and 0·3ml (2·55mg protein) of succinate-grown cell extract; 100 nmoles substrate; 0·4 µmole NAD^+ where indicated. The temperature was 50°C. Activities were measured by the decrease in absorbance at 375,382 and 388 nm for 2-HMS, 2-hydroxy -5-MMS and 2-hydroxy-6-oxohepta-2, 4-dienoic acid, respectively.

was very unstable in the presence of air, a feature shared with preparations of the enzyme derived from mesophilic bacteria (7,10). Similarly, reducing agents such as dithiothreitol, mercaptoethanol and glutathione had no protective effect but the addition of 10% v/v acetone maintained enzyme stability for long periods even when stored at $4^{o}C$.

Cell-free extracts from phenol-grown Bacillus PH24 metabolised 2-HMS and the ring-fission products of 3- and 4-methylcatechols. The results shown in Table 3 indicate the presence of two different and non-specific 2-HMS metabolising enzymes, one NAD^{+}-dependent and the other non NAD^{+}-dependent, in extracts of this strain. The activity of the NAD^{+}-dependent enzyme, which is increased about 70-fold by growth on phenol, attacked 2-HMS and 2-hydroxy-5-MMS at approximately the same rate in NAD^{+}-supplemented reaction mixtures but does not attack 2-hydroxy-6-oxohepta-2, 4-dienoic acid, where the aldehyde group is absent.

The reaction products of 2-HMS dehydrogenase activity were determined spectrophotometrically as NADH and γ-oxalocrotonate. The stoicheiometry of NADH formation in NAD^{+}-supplemented reaction mixtures is shown in Table 4. The formation of γ-oxalocrotonate was detected from the appearance of the characteristic spectrum in the 300-400nm region (peak at 350nm) in reaction mixtures made alkaline to pH 12.0.EDTA was included

TABLE 4 The Stoicheiometry of NADH formation from 2-HMS in NAD^{+}-supplemented reaction mixtures by cell-free extracts of Bacillus PH24.

2-HMS metabolised nmoles	NADH formed nmoles	Formate formed nmoles
100	102	N•D
200	186	N•D

The reaction mixture contained in a total volume of 3.0ml; 250μmoles phosphate buffer, pH7.5; 1.0μmole NAD^{+}; 0.05ml extract, 150,000xg supernatant, (1.3mg protein); 100(200) nmoles 2-HMS.
The amount of NADH formed was determined from the absorbance at 340nm when all the semialdehyde had disappeared (followed spectrophotometrically at 375nm) and corrected for NADH oxidase activity in the system. Formate was assayed by the acetylacetone method.
N•D. Not detectable.

TABLE 5 The stoicheiometry of γ-oxalocrotonate formation from 2-HMS by cell-free extracts of Bacillus PH24.

2-HMS metabolised nmoles	γ-Oxalocrotonate formed nmoles
100	104
200	158

The reaction mixture contained in a total volume of 3.0ml; 250μmoles phosphate buffer, pH7.5; 50μmoles Na_2-EDTA; 1.0μmole NAD^+; 0.05ml extract, 35,000xg supernatant (2.4mg protein) and 100(200)nmoles 2-HMS. When all of the semialdehyde had disappeared the γ-oxalocrotonate formed was determined by the absorbance at 350nm after adding one drop of 10M NaOH (pH 12.0) to the reaction mixture and reading off against a standard curve plotted using authentic γ-oxalocrotonate.

in the assay to inhibit product conversion due to γ-oxalocrotonate decarboxylase activity. Clarified extracts (35,000 x g), which contained high levels of NADH oxidase activity, were used in experiments to determine the stoicheiometry of γ-oxalocrotonate production to avoid interference due to the formation of NADH (Table 5).

The non NAD^+- dependent enzyme is also inducible and exhibits high levels of activity against the ring-fission products of 3-methylcatechol and catechol but relatively low activity against 2-hydroxy-5-MMS. Extracts treated with NADase showed a non NAD^+-dependent activity with 2-HMS as substrate about six times that observed against 2-hydroxy-5-MMS. These levels represent about 80% and 60% respectively of the total activity against 2-HMS and 2-hydroxy-5-MMS as measured in crude cell-free extracts in the absence of added NAD^+. This is very much higher than that detected in Pseudomonas(8), and in Azotobacter vinelandii where non NAD^+-dependent activity is negligible (6). Furthermore, the level of induction in the presence of phenol (80-fold against 2-HMS) is considerably greater than reported in Pseudomonas(8).

Of considerable interest is the temperature stability of the 2-HMS-metabolising enzymes. Temperature inactivation studies indicate that the non NAD^+-dependent activity is the more stable (unpublished results) although high activities of both enzymes are readily detectable at 50°C.

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