Catabolism of Gentisic Acid by Two Strains of *Bacillus stearothermophilus*

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(Received 17 November 1978)

INTRODUCTION

Certain members of the genus *Bacillus* catabolize *p*-hydroxybenzoate to gentisic acid (2,5-dihydroxybenzoic acid) followed by oxidative ring-cleavage to yield maleylpyruvate. The ring-cleavage is catalysed by gentisate 1,2-dioxygenase which, together with enzymes catalysing the further dissimilation of maleylpyruvate, is induced in these bacteria by growth with *p*-hydroxybenzoate or benzoate (Buswell & Clark, 1976; Crawford, 1976). Some species of the genus *Pseudomonas* degrade maleylpyruvate by a reduced glutathione (GSH)-dependent *cis*, *trans* isomerization reaction to fumarylpyruvate which, in turn, is hydrolysed to fumaric and pyruvic acids (pathway B, Fig. 1; Lack, 1959, 1961; Tanaka *et al.*, 1957; Hareland *et al.*, 1975). Other species of *Pseudomonas* hydrolyse maleylpyruvate directly to maleate and pyruvate, without prior *cis*, *trans* isomerization, using a GSH-independent maleylpyruvate hydrolase (pathway A, Fig. 1; Hopper *et al.*, 1968, 1971). In one species, *Pseudomonas* 2,5X, both GSH-independent and GSH-dependent enzymes are reported to exist (Crawford & Frick, 1977).

In the gentisate-degrading bacilli so far described, catabolism of maleylpyruvate is not stimulated by GSH (Crawford, 1975, 1976); in some cases, maleate was identified as the end-product, thus providing direct evidence that maleylpyruvate can be hydrolysed by a GSH-independent maleylpyruvate hydrolase. These observations have been confirmed using *N*-ethylmaleimide (NEM) which inhibits the GSH-dependent *cis*, *trans* isomerization of maleylpyruvate to fumarylpyruvate but not the GSH-independent maleylpyruvate hydrolase (Crawford & Frick, 1977). Buswell & Clark (1976) also reported that GSH did not stimulate maleylpyruvate conversion by extracts of a facultative thermophilic *Bacillus*, implying that direct hydrolysis to pyruvic and maleic acids occurred, although maleate was not identified as a reaction product. The results of further examination of gentisate degradation in this and in a second strain of *Bacillus stearothermophilus* are now described. These results show that in one strain *cis*, *trans* isomerization of maleylpyruvate to fumarylpyruvate is catalysed by a NEM-sensitive enzyme, but in the other, gentisate is converted to fumarate and pyruvate in a GSH-independent reaction (pathway C, Fig. 1).

METHODS

The isolation and cultivation of one organism used in this study, a facultative thermophilic *Bacillus* species, was described previously (Buswell & Clark, 1976). This bacterium has been tentatively identified as an atypical strain of *Bacillus stearothermophilus*, strain PM41. *Bacillus stearothermophilus* strain C1 was isolated from soil by elective culture with *p*-hydroxybenzoate at 55 °C and cultured under the same conditions as PM41. Cell extracts were prepared and clarified as described previously (Buswell & Clark, 1976).

Maleylpyruvate was prepared using a partially purified extract of PM41 cells grown with *p*-hydroxybenzoate. Crude extract was brought to 70 % saturation with (NH₄)₂SO₄ solution, pH 7.6. Precipitated protein was collected by centrifugation, redissolved in 0.1 M-Tris/HCl buffer, pH 7.6, and applied to a Sephadex G-150 column (2.5 × 90 cm). The column was eluted with similar buffer and 5 ml fractions were collected. Fractions

Fig. 1. Catabolism of gentisate by *Bacillus* spp.: pathway A, *Bacillus* spp. (Crawford, 1975, 1976); pathway B, *B. stearothersophilus* C1; pathway C, *B. stearothersophilus* PM41.

Compounds: I, gentisate; II, malelpyruvate; III, fumarylpyruvate; IV, pyruvate; V, maleate; VI, fumarate.

Enzymes: A, gentisate 1,2-dioxygenase; B, malelpyruvate hydrolase; C, GSH-dependent malelpyruvate isomerase (NEM-sensitive); D, GSH-independent malelpyruvate isomerase; E, fumarylpyruvate hydrolase.

38 to 65, containing gentisate 1,2-dioxygenase but no malelpyruvate-metabolizing enzyme activity, were pooled and brought to 70 % saturation with $(\text{NH}_4)_2\text{SO}_4$. The precipitate was collected by centrifugation and redissolved in 0.1 M- $\text{KH}_2\text{PO}_4/\text{KOH}$ buffer, pH 7.6. Malelpyruvate was prepared by incubating purified extract (approx. 10 mg protein), 150 μmol ferrous ammonium sulphate and 300 μmol gentisate (adjusted to pH 7.0) in 12 ml of 0.1 M- $\text{KH}_2\text{PO}_4/\text{KOH}$ buffer, pH 7.6, at 30 °C for 1.75 h in an orbital incubator shaker set at 80 rev. min^{-1} . After this period the solution was diluted with an equal volume of water and the pH was adjusted to 7.2. Absolute ethanol (5 vol.) at -18 °C was added, the precipitated protein was removed by centrifugation, and the product was extracted by the procedure described by Lack (1961). Fumarylpyruvate was prepared by the procedure of Lack (1959).

Gentisate 1,2-dioxygenase [EC 1.13.11.4; gentisate: oxygen 1,2-oxidoreductase (decyclizing)] was assayed by measuring the rate of oxygen consumption at 50 °C with a Clark oxygen electrode. Assay mixtures (3.0 ml) contained 250 μmol Tris/HCl buffer, pH 7.5, 1.0 μmol gentisate and enzyme (0.6 to 1.2 mg protein). Enzymic conversion of malelpyruvate was determined at 28 °C from the rate of decrease in absorbance at 330 nm. Assay mixtures (3.0 ml) contained 250 μmol Tris/HCl buffer, pH 7.5, enzyme (0.2 to 0.5 mg protein) and, to initiate the reaction, 100 nmol malelpyruvate (estimated by assuming that $\epsilon = 13000$ at 330 nm). Fumarylpyruvate hydrolase activity was measured at 28 °C from the rate of decrease in absorbance at 340 nm resulting from hydrolysis of fumarylpyruvate to fumaric and pyruvic acids (Lack, 1959). Assay mixtures (3.0 ml) contained 250 μmol Tris/HCl buffer, pH 7.5, 100 μmol fumarylpyruvate ($\epsilon = 9400$ at 340 nm; Tanaka *et al.*, 1957) and enzyme (40 to 95 μg protein). Fumarate hydratase (EC 4.2.1.2; L-maleate hydro-lyase) and maleate isomerase (EC 5.2.1.1; maleate *cis-trans*-isomerase) activities were assayed essentially as described by Scher & Jakoby (1969). Freshly prepared extracts were used at all times. Assays were performed at 28 and 50 °C, the same patterns of activity and inhibition being observed at both temperatures. Dicarboxylic acids were identified as their dimethyl esters by gas-liquid chromatography (g.l.c.). Mass spectroscopy was performed on a VG model 16F linked to a VG 2020 data system. Protein was measured by the method of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

The metabolism of benzoate and *p*-hydroxybenzoate was examined in 19 strains of thermophilic bacilli isolated from different sources. When bacteria were grown on either of these compounds, extracts of all isolates contained gentisate 1,2-dioxygenase activity. Strains C1

Table 1. *Effect of N-ethylmaleimide (NEM) on conversion of maleylpyruvate by extracts of B. stearothermophilus strains PM41 and C1*

Conversion of maleylpyruvate was assayed as described in Methods. Where indicated, 1.0 mM-NEM was added to the reaction mixture 2 min before addition of substrate. When no NEM was added, the reaction was initiated 2 min after addition of extract to the reaction mixture. Specific activities are expressed as $\text{nmol min}^{-1} (\text{mg protein})^{-1}$.

Strain	Growth substrate	Specific activity		Activity remaining (%)
		Without NEM	With NEM	
PM41	Benzoate	56.8	55.8	98
PM41	<i>p</i> -Hydroxybenzoate	592	592	100
C1	Benzoate	242	13.3*	5.5
C1	<i>p</i> -Hydroxybenzoate	252	15.0*	6.0

* Non-linear rate: specific activity calculated on the basis of maleylpyruvate conversion during the first 3 min.

and PM41, tentatively identified as strains of *Bacillus stearothermophilus* (Gordon *et al.*, 1973), were selected for further study.

Extracts of *B. stearothermophilus* C1 grown with benzoate or *p*-hydroxybenzoate oxidized gentisate but not catechol or protocatechuate. Direct evidence that gentisate is an intermediate in *p*-hydroxybenzoate catabolism by this bacterium was obtained using 2,2'-bipyridyl to inhibit the ring-cleavage enzyme (Hopper & Chapman, 1971; Buswell & Clark, 1976). The product which accumulated from *p*-hydroxybenzoate was identified as gentisate by thin-layer chromatography and mass spectrometry. The activities of gentisate 1,2-dioxygenase [8.7 and $1.4 \mu\text{l}$ oxygen consumed $\text{min}^{-1} (\text{mg protein})^{-1}$ after growth with benzoate or *p*-hydroxybenzoate, respectively] were increased 30-fold and 5-fold compared with the activity in extracts of non-induced bacteria grown with fumarate. This is in contrast to induction patterns previously reported for strain PM41 in which much higher activities of the ring-fission enzyme were induced by *p*-hydroxybenzoate (Buswell & Clark, 1976).

When GSH ($0.3 \mu\text{mol}$) was included in reaction mixtures, the rate of maleylpyruvate dissimilation by crude extracts of C1 grown with benzoate increased by 10 to 15 %. Short periods of dialysis did not enhance the GSH-dependence of this enzyme. However, the rate of maleylpyruvate conversion by unsupplemented extracts was inhibited approximately 95 % by 1 mM-NEM (Table 1). Excess GSH ($0.3 \mu\text{mol}$) restored only about 10 % of the original activity. Fumarate was identified by g.l.c. as a reaction product. Fumarylpyruvate hydrolase was active in extracts of bacteria grown with benzoate or *p*-hydroxybenzoate [specific activities were 0.51 and $0.52 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$, respectively] but fumarate hydratase and maleate isomerase were not detected. Although a non-specific inhibition of maleylpyruvate metabolism by NEM cannot be excluded, these results strongly indicate that strain C1 catalyses a GSH-dependent *cis*, *trans* isomerization of a type not previously reported in members of this genus.

NEM (1 mM) did not inhibit maleylpyruvate catabolism by extracts of strain PM41 (Table 1). This is consistent with an earlier report that the rate of maleylpyruvate conversion was not enhanced by GSH (Buswell & Clark, 1976). Although these results suggested that maleylpyruvate was hydrolysed directly to pyruvate and maleate (pathway A, Fig. 1), fumaric acid has now been identified as a reaction product. Therefore, in strain PM41, maleylpyruvate is isomerized to fumarylpyruvate by a GSH-independent enzyme. Cell extracts did not contain maleate isomerase.

Hagedorn *et al.* (1977) demonstrated a transient accumulation of fumarylpyruvate by extracts of *Bacillus megaterium* which had been grown with benzoate or *m*-hydroxybenzoate and they too suggested that a GSH-independent *cis*, *trans* isomerization had occurred. No

fumarylpyruvate accumulated during maleylpyruvate metabolism by strain PM41, but this was probably because it was degraded as rapidly as it was formed; the specific activities of fumarylpyruvate hydrolase were 0.074 and 0.72 $\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$ after growth with benzoate or *p*-hydroxybenzoate, respectively. Attempts to separate maleylpyruvate isomerase from fumarylpyruvate hydrolase have so far been unsuccessful because these enzymes are unstable during purification.

Maleylpyruvate isomerase activity in crude extracts of strains PM41 and C1 was proportional to protein concentration (0.1 to 0.9 mg) and no substrate inhibition was evident (see Lack, 1961). However, the isomerase of both organisms is unstable and the normal linear rate of activity observed spectrophotometrically progressively decreased during the assay, a response seen within hours of extract preparation in the case of strain C1. Extracts prepared in buffer containing 10 mM-dithiothreitol did not exhibit this loss of activity even after several weeks at -20°C . Similarly, when assays were initiated with substrate, prior dilution of non-stabilized extracts in the reaction cuvette greatly reduced the extent of the linear rate compared with that observed when the reaction was initiated by addition of extract. Products of isomerase and hydrolase activities were not inhibitory and the reasons for this response are under investigation. Fumarylpyruvate hydrolase activity in extracts of strains C1 and PM41 was not significantly affected by NEM (1 mM) and was stable for at least a month at -20°C .

The reactions by which benzoate and *p*-hydroxybenzoate are converted to gentisate remain obscure because, as Crawford (1976) has reported for *Bacillus laterosporus*, cell-free extracts are not active towards the growth substrates. Two possible alternatives for *p*-hydroxybenzoate degradation – dehydroxylation to benzoate followed by hydroxylation at C-2 and C-5 of the ring, or hydroxylation at C-1 with concomitant migration of the carboxyl group to an *ortho* position – have been documented (Crawford, 1976). Extracts of strains C1 and PM41 grown with benzoate or *p*-hydroxybenzoate contain high activities of an inducible NADH (or NADPH)-dependent *m*-hydroxybenzoate-6-hydroxylase activity. The possible role of *m*-hydroxybenzoate as an intermediate in the degradation pathways is the subject of further study.

We thank Dr G. R. Jamieson and Elizabeth Reid of the Chemistry Department, Paisley College, for the g.l.c. analyses, and Dr D. B. Harper, Queens University, Belfast, for mass spectra. A grant-in-aid for equipment from the Royal Society to J.A.B. is gratefully acknowledged.

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