Isomerase and Decarboxylase Activities in the 4-Hydroxyphenylacetate Catabolic Pathway of *Pseudomonas putida*

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5-Carboxymethyl-2-hydroxymuconate isomerase and 5-carboxymethyl-2-oxo-hex-3-ene-1,6-dioate decarboxylase in the 4-hydroxyphenylacetate *meta*-cleavage pathway have been purified to over 98% homogeneity. The native enzymes, which appear to be monomers, have apparent molecular weights of 37,000. The isomerase shows a pH optimum 8.0 and does not require ions for its catalytic activity; Mg^{2+} is required for the decarboxylase reaction. Apparent K_m values for their respective substrates are 2.8×10^{-4} M for the isomerase and 1.4×10^{-4} M for the decarboxylase. The highly purified enzymes were used to study the spectral characteristics of the decarboxylase substrate.

Introduction

We are contributing to the build up our knowledge of the various factors that regulate the degradation of aromatic compounds by soil bacteria because many man-made chemicals used as detergents and pesticides often contain a benzene nucleus. This knowledge should be useful in the design of compounds sufficiently persistent to perform their desired function but not so stable that they last indefinitely in the environment. The studies on the processes of aromatic degradation involve the use of the *Pseudomonas* because of their profusion in the soil and their ability to grow on a wide range of aromatic compounds [1].

An isomerase and a decarboxylase involved in the 4-hydroxyphenylacetate catabolic pathway of *Escherichia coli* have been characterized [2]. Barbour and Bayly [3, 4] have described experiments with *Pseudomonas putida* mutants which they interpret as evidence for both enzymes being involved in this pathway.

In the present work data on isolation and purification of 5-carboxymethyl-2-hydroxymuconate isomerase and 5-carboxymethyl-2-oxo-hex-3-ene-1,6-dioate decarboxylase from *Pseudomonas putida* are shown as well as a study of some of their physicochemical properties. The purified enzymes have been used to determine the spectral characteristics of their substrates which are different from those ascribed to them from *Pseudomonas putida* mutants extracts [3].

Enzymes: 5-Carboxymethyl-2-hydroxymuconate isomerase (EC 5.3.3.–)
5-Carboxymethyl-2-oxo-hex-3-ene-1,6-dioate decarboxylase (EC 4.1.1.–)

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Materials and Methods

Bacterial and culture methods

The organism used was *Pseudomonas* U (British NCIB 10015), a strain of *Pseudomonas putida* that was isolated from a mud in a creek in Urbana, USA, in 1963 [5]. Cells were cultured on 20 mM 4-hydroxyphenylacetate as carbon source. Medium and growth conditions were as described previously [2].

Preparation of extracts

Extracts were prepared by ultrasonication of cells suspended in 0.1 M Na_2HPO_4-KH_2PO_4 buffer pH 7.0 containing 1 mM dithiotreitol, as described previously [2].

Protein estimation

Protein was measured either colorimetrically with crystalline bovine serum albumen as standard by the modified Folin method [6] or spectrophotometrically [7].

Enzyme assays

5-Carboxymethyl-2-hydroxymuconate isomerase activity was assayed by measuring the decrease in extinction at 300 nm consequent upon the formation of 5-carboxymethyl-2-oxo-hex-3-ene-1,6-dioate. The reaction mixture contained 0.05 μmol 5-carboxy-

methyl-2-hydroxymuconate in 1.0 ml of 0.1 M Na_2HPO_4-KH_2PO_4 buffer pH 8.0 at 30 °C. The rate of spontaneous isomerisation was measured and the reaction was then initiated by addition of enzyme. The molar extinction coefficient for 5-carboxymethyl-2-hydroxymuconate was calculated from Sparnins *et al.* (1974) to be 2×10^4 M^{-1} cm^{-1}. To assay 5-carboxymethyl-2-oxo-hex-3-ene-1,6-dioate decarboxylase activity, an equilibrium mixture of 5-carboxymethyl-2-hydroxymuconate and 5-carboxymethyl-2-oxo-hex-3-ene-1,6-dioate was produced in a cuvette by addition of excess purified 5-carboxymethyl-2-hydroxymuconate isomerase to a reaction mixture containing 0.05 µmol 5-carboxymethyl-2-hydroxymuconate and 5 µmol $MgSO_4$ in 1.0 ml 0.1 M Na_2HPO_4-KH_2PO_4 buffer pH 8.0 at 30 °C. When the decrease in extinction at 300 nm had ceased the reaction was started by addition of the decarboxylase. Under these conditions the action of the 5-carboxymethyl-2-oxo-hex-1,6-dioate decarboxylase could be measured by following the decrease in extinction at 300 nm consequent upon the action of the 5-carboxymethyl-2-hydroxymuconate isomerase operating to maintain the 5-carboxymethyl-2-oxo-hex-3-ene-1,6-dioate/5-carboxymethyl-2-hydroxymuconate equilibrium displaced by the action of the 5-carboxymethyl-2-oxo-hex-3-ene-1,6-dioate decarboxylase. One unit of enzyme activity was the amount catalysing the conversion of 1 µmol of substrate/min.

Molecular weight determination

For estimation of molecular weights, a 40×2.6 cm column of Sephadex G 100 (superfine grade) equilibrated with 0.1 M Na_2HPO_4-KH_2PO_4 buffer pH 7.0 containing 1 mM dithiotreitol was calibrated with ovalbumin, myoglobin and cytochrome *c*. The column was eluted at a flow rate of 24 ml/h under constant pressure and fractions each of 3.2 ml were collected. It was assumed that all the proteins concerned were of the same shape and solvation.

Enzyme purification

5-Carboxymethyl-2-hydroxymuconate isomerase and 5-carboxymethyl-2-oxo-hex-3-ene-1,6-dioate decarboxylase were purified from 4-hydroxyphenylacetate-grown cells. All operations were carried out at 0–4 °C. Approximately 4 g wet weight of cells were suspended in 80 ml Na_2HPO_4-KH_2PO_4 buffer pH 7.0 containing 1 mM dithiotreitol (extracting buffer) and ultrasonicated in a Braun Labsonic sonifier (70–80 watts) for ten 45 s periods with 1 min cooling between each period. The mixture was centrifuged at $20{,}000 \times g$ for 15 min and the supernatant fractionated with solid ammonium sulphate. The protein precipitating between 50–80% saturation was dissolved in 8 ml of the extracting buffer and applied to a 40×2.6 cm Sephadex G 100 (superfine grade) column equilibrated against the eluting buffer. The column was eluted at a flow rate of 24 ml/h at constant pressure and fractions each of 3.2 ml were collected. The six fractions containing the peak activities for both enzymes were pooled and dialysed against 0.01 M Na_2HPO_4-KH_2PO_4 buffer pH 7.0 containing 1 mM dithiotreitol. This sample was applied to a 30×2.6 cm column of DEAE-Sephacel that had been equilibrated against 0.01 M Na_2HPO_4-KH_2PO_4 buffer containing 1 mM dithiotreitol. Both enzymes were bound under these conditions. The elution was carried out by stepwise of 0.04 M NaCl concentration dissolved in 100 ml 0.01 M Na_2HPO_4-KH_2PO_4 buffer pH 7.0 containing 1 mM dithiotreitol and starting from a solution 0.1 M NaCl dissolved in the same buffer.

Electrophoresis

The enzyme purity was tested by means of electrophoresis in 7.5 per cent polyacrylamide gel, according to Davis [8]. The molecular weight of the subunit was estimated by sodium dodecylsulphate-polyacrylamide gel electrophoresis [9] using a calibration kit protein markers ranging between 330,000 and 36,000 daltons of molar mass.

Chemicals

Chromatographic material (columns, collector, etc.) was purchased from LKB. Sephadex G 100, DEAE-Sephacel and electrophoresis calibration protein were from Pharmacia. 5-Carboxymethyl-2-hydroxymuconic acid was prepared as described by Sparnins *et al.* [5]. 4-Hydroxyphenylacetate was obtained from Sigma. All other chemicals were the highest purity commercially available.

Results

Purification and characterization of the enzymes

5-Carboxymethyl-2-hydroxymuconate isomerase and 5-carboxymethyl-2-oxo-hex-3-ene-1,6-dioate de-

Table I. A summary of the purification of 5-carboxymethyl-2-hydroxymuconate isomerase and 5-carboxymethyl-2-oxo-hex-3-ene-1,6-dioate decarboxylase.
(Data for a purification starting with 4 g wet weight of cells)

Purification step	Volume	Protein	[Isomerase] Activity	[Isomerase] Specific activity	Decarboxylase Activity	Decarboxylase Specific activity
	[ml]	[mg]	[units]	[units/mg protein]	[units]	[units/mg protein]
I. Crude extracts	80	1,400	208	0.15	226	0.16
II. $(NH_4)_2SO_4$ fractionation	8.0	168	171	1.01	107	0.64
III. Sephadex G 100 pooled fractions	19.2	24	82	3.4	70	2.9
IV. DEAE-Sephacel fractions 58, 59	7.8	0.22	–	–	26	118.2
fraction 75	3.9	0.19	23	122.1	–	–

carboxylase have been purified by gel filtration and anion-exchange chromatography more than 800-fold and 700-fold, respectively. From the DEAE-Sephacel column the decarboxylase was eluted to 0.14 M NaCl and the isomerase to 0.22 M NaCl dissolved in 0.01 M Na_2HPO_4-KH_2PO_4 buffer pH 7.0 containing 1 mM dithiotreitol (Table I). Polyacrylamide disc gel electrophoresis on aliquots of the fractions containing the peak activity for each enzyme were shown to contain only one band (Fig. 1). The bands were cut out from the corresponding unstained gel and extracted by means of a small glass homogeniser. Such extracts were shown to correspond to the appropriate enzyme activity. Densitometric scanning of the gel in a Kontron Unicon 820 spectrophotometer showed the band to account for 99% of the total absorbance in the case of the isomerase and 98% of the total absorbance in the case of the decarboxylase.

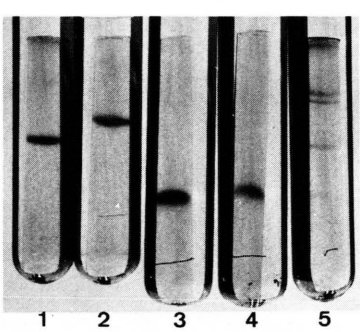

Fig. 1. Polyacrylamide gel electrophoresis of 5-carboxymethyl-2-hydroxymuconate isomerase and 5-carboxymethyl-2-oxo-hex-3-ene-1,6-dioate decarboxylase natives (1) (2) and heat-treated enzymes in presence of SDS (3) (4) as compared with reference proteins: Thyroglobulin (330,000), Ferritin (220,000), Albumin (67,000), Catalase (60,000) and Lactate dehydrogenase (36,000).

As described in Materials and Methods, the molecular weights of the enzymes were estimated to be 38,000 ± 4,000 for the isomerase and 37,000 ± 4,000 for the decarboxylase by gel filtration chromatography. When the subunit molecular weights of the purified enzymes were determined by sodium dodecylsulphate disc-gel electrophoresis the values of 37,000 ± 1,000 were similar to the apparent molecular weight values native enzymes.

Conditions for activity and affinity of the enzymes

Analysis of the pH-dependence of the purified isomerase enzyme with different buffers (Fig. 2) showed that the enzyme activity increase from pH 6.5 to 8.0 with a maximum at this pH and then declined at higher pH values. The slow spontaneous rate of isomerisation was independent of pH within the range 5.5 to 10.0. While the isomerase was active in the absence of Mg^{2+}, decarboxylase was active only in presence of this cation. Both enzymes were stable for a period of at least one month stored at 5 °C in 0.01 M Na_2HPO_4-KH_2PO_4 buffer pH 7.0 containing 1 mM dithiotreitol. The isomerase activity was measured at various concentrations of 5-carboxymethyl-2-hydroxymuconate; the data were plotted according to Lineweaver-Burk obtaining a straight line giving an apparent K_m value of 2.8×10^{-4} M. As the equilibrium between the 5-carboxymethyl-2-hydroxymuconate and 5-carboxymethyl-2-oxo-hex-ene-1,6-dioate was independent of

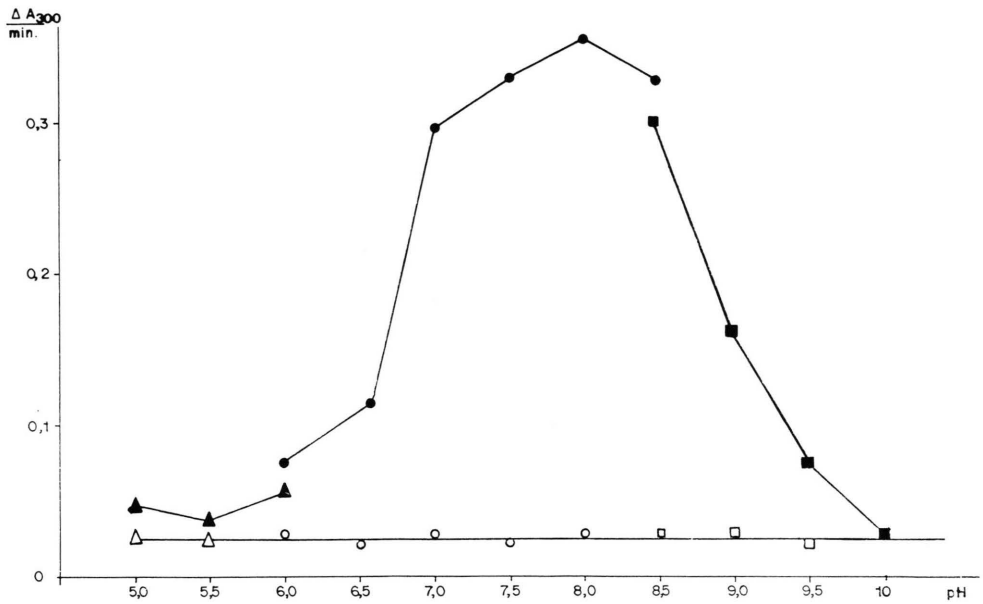

Fig. 2. Variation of the rate of enzymic and non-enzymic isomerisation of 5-carboxymethyl-2-hydroxymuconate with pH. The rate of isomerisation was measured under standard conditions in absence and presence of highly purified 5-carboxymethyl-2-hydroxymuconate isomerase (0.25 μg of protein). The buffer ions were: □ − □, 0.1 M citrate; ○ − ○, 0.1 M phosphate and △ − △, glycinate. The filled symbols represent the enzymic rates uncorrected for the non-enzymic rates, which are indicated by unfilled symbols.

the concentration of the former compound, the decarboxylase activity was measured at different concentrations of 5-carboxymethyl-2-hydroxymuconate, after the addition of an excess of isomerase and waiting until the decrease in extinction at 300 nm had ceased; the data were plotted according to Lineweaver-Burk obtaining an apparent K_m value of 1.4×10^{-4} M.

Spectral characteristics of the substrates

Highly purified enzymes were to investigate the spectral characteristics of the decarboxylase substrate. Fig. 3 shows the difference spectrum of the

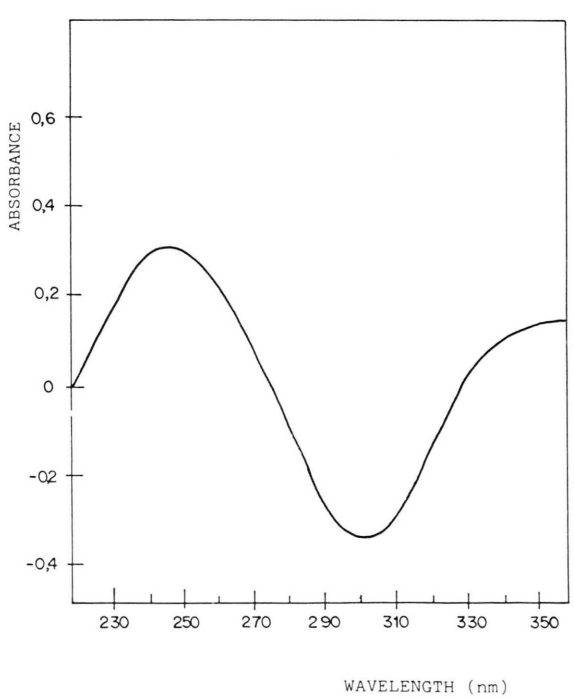

Fig. 3. The ultraviolet absorption spectrum associated with 5-carboxymethyl-2-oxo-hex-3-ene-1,6-dioate formation.

A prepared solution of 0.05 nM 5-carboxymethyl-2-hydroxymuconate and 0.25 μg of highly purified isomerase in 0.1 M Na phosphate buffer pH 8.0 was placed in the reference cuvette. When the rate of enzymic isomerisation had ceased, a similar freshly prepared solution was placed in the experimental cuvette and the difference spectrum measured inmediately.

product of the isomerase reaction, after the equilibrium was formed, against a freshly prepared standard reaction mixture at 30 °C. This spectrum corresponds, presumably, to the compound, 5-carboxymethyl-2-oxo-hex-3-ene-1,6-dionate, the same product of the spontaneous isomerization [2]. This view was further stregthened by an experiment in which a freshly prepared sample of 5-carboxymethyl-2-hydroxymuconate in 0.1 M Na phosphate buffer pH 8.0 was placed into two identical cuvettes and the difference spectrum measured before and after the addition of purified isomerase to the experimental cuvette. Fig. 4 shows that the addition of isomerase very rapidly leads to an increase in absorbance at 246 nm and a corresponding decrease in absorbance at 300 nm. With time, both of the changes decrease such that after 30–40 min the spectrum was almost identical to that seen before addition of the isomerase. In this experiment the isomerase added to the experimental cuvette caused a rapid increase in the rate of the non-enzymic reaction until the equilibrium position was reached. In the reference cuvette the non-enzymic reaction proceeded more slowly but with time the same equilibrium position was reached, resulting in no spectral difference between the two samples.

The identification of the 246 nm absorbing compound as the decarboxylase substrate is further supported by the rapid formation of a maximun at 276 nm (Fig. 5) that is seen when the decarboxylase is allowed to act in concert with the isomerase on 5-carboxymethyl-2-hydroxymuconate. The spectrum at 50 min seems to correspond to the final product of the reaction: 2-hydroxyhepta-2,4-diene-1,7-dioate [2, 5, 10].

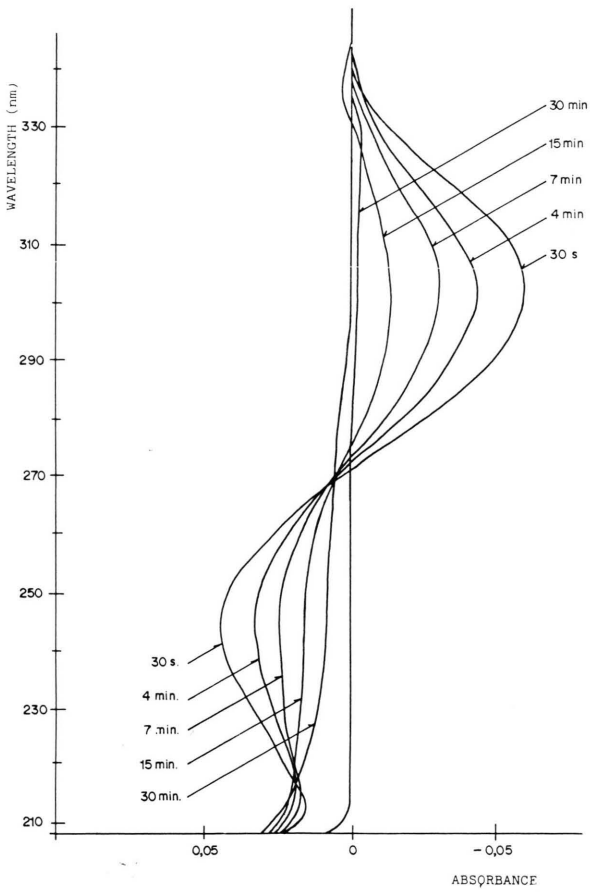

Fig. 4. Difference spectrum between the enzymic and spontaneous isomerisation reaction.

A freshly prepared solution of 0.05 nM 5-carboxymethyl-2-hydroxymuconate in 0.1 M Na phosphate buffer pH 8.0 was placed in two cuvettes and the difference spectrum determined. Then 0.25 µg of highly purified 5-carboxymethyl-2-hydroxymuconate isomerase was added to the experimental cuvette and the difference spectrum measured after various time intervals up to 30 min. For further details see text.

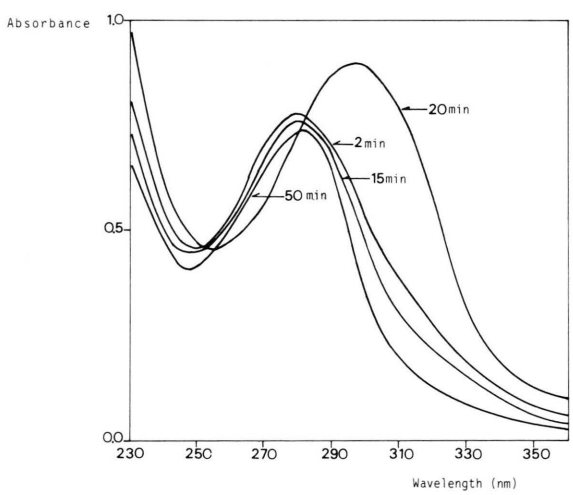

Fig. 5. The ultraviolet absorption spectra associated to 2-hydroxyhepta-2,4-diene-1,7-dioate formation.

To a freshly prepared solution of 0.05 nM 5-carboxymethyl-2-hydroxymuconate in 0.1 M Na phosphate buffer pH 8.0 was added 0.25 µg of 5-carboxymethyl-2-hydroxymuconate isomerase, 5 µmol $MgSO_4$ and 0.21 µg of 5-carboxymethyl-2-oxo-hex-3-ene-1,6-dioate decarboxylase and the spectrum measured after various times of incubation at 30 °C.

Discussion

5-Carboxymethyl-2-hydroxymuconate isomerase and 5-carboxymethyl-2-oxo-hex-3-ene-1,6-dioate decarboxylase were readily isolated from *P. putida* by conventional chromatography methods. Analysis of the enzymes by Sephadex G 100 chromatography and sodium dodecylsulphate-polyacrylamide gel electrophoresis suggested that the enzymes exist as single polypeptide chains with approximated molecular weights of 37,000. The apparent molecular weights of these enzymes were similar to those obtained by gel filtration from *E. coli* enzyme extracts, those being 36,000 for the isomerase and 41,000 for the decarboxylase (Garrido-Pertierra and Cooper, data unpublished). When the subunit molecular weights for the *E. coli* enzymes were determined by electrophoresis the value for the decarboxylase was similar to the apparent molecular weight value for the native enzyme. However, the isomerase showed an apparent subunit molecular weight of 64,000, suggesting that the protein was behaving anomolously on the gel. Such behaviour does not seem to occur in the case of the isomerase of *P. putida*.

The purification procedure employed yielded proteins that were over 98% purity. This degree of purity was used to emphasize the physiologically important role of the isomerase enzyme in order to favour the isomerisation reaction, because otherwise the spontaneous isomerisation would be a limiting step of the pathway.

The profile pH activity curve for *P. putida* isomerase was similar to that obtained for *E. coli* [2] although the maximum pH was slightly less alkaline. The high affinity of the isomerase and decarboxylase for their substrates is of the same order as that described for other enzymes in the 4-hydroxy-phenylacetate pathway [2, 11, 12]; this characteristic may reflect the need to keep the normal cellular concentrations of these compounds low. The spectral characteristics of the isomerase and decarboxylase substrates reported here are different from those described by Barbour and Bayly [3, 4], in *P. putida* mutants extracts. The availability of highly purified enzymes meant that they could be used to investigate the spectral characteristics of the substrates and products of the reactions without themselves adding significantly to the total absorbance of the solutions. This is in contrast to the experiments of Barbour and Bayly, where the presence of crude bacterial extract and NAD^+ in the reaction mixture meant that the total absorbance of the solutions was high and may have led to difficulties in making precise spectral measurements.

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