Regulation of Phenylalanine and Tyrosine Biosynthesis in *Pseudomonas aureofaciens* ATCC 15926

By ERICH BLUMENSTOCK,† OLGA SALCHER
AND FRANZ LINGENS*

*Institut für Mikrobiologie,
Universität Hohenheim, Garbenstrasse 30, D-7000 Stuttgart 70,
Federal Republic of Germany*

(Received 24 May 1979; revised 25 September 1979)

Association patterns and regulatory properties of chorismate mutase, prephenate dehydratase and prephenate dehydrogenase from *Pseudomonas aureofaciens* ATCC 15926 were studied. Prephenate dehydrogenase (molecular weight 95000) was separated by Sephadex G-100 chromatography from both the chorismate mutase–prephenate dehydratase I complex (molecular weight 75000) and from a second, low molecular weight prephenate dehydratase (prephenate dehydratase II; molecular weight 30000). The chorismate mutase–prephenate dehydratase complex persisted after DEAE-Sephadex A-50 chromatography. With the exception of prephenate dehydratase II, enzyme activities were influenced by end-products. Chorismate mutase was competitively inhibited by L-phenylalanine ($K_i = 3.5 \mu\text{M}$). Prephenate dehydratase I was inhibited by L-phenylalanine ($K_i = 8 \mu\text{M}$) and activated by L-tyrosine ($K_a = 5 \mu\text{M}$). Prephenate dehydrogenase was feedback-inhibited by L-tyrosine. Substrate saturation curves of chorismate mutase and of prephenate dehydratase II were hyperbolic with K_m values of 0.31 mM for chorismate and 0.015 mM for prephenate, respectively. The substrate saturation curve of the complexed prephenate dehydratase I was sigmoid; a K_m value of 0.18 mM was calculated for prephenate. Chorismate mutase, prephenate dehydratase and prephenate dehydrogenase were not repressed by aromatic amino acids.

INTRODUCTION

The terminal reactions in phenylalanine and tyrosine biosynthesis are accomplished by the conversion of chorismate through prephenate to phenylpyruvate or 4-hydroxyphenylpyruvate, which are then transaminated to the corresponding amino acids (Fig. 1). In micro-organisms many patterns for the regulation of these reaction sequences have evolved (Lingens, 1968). They may be used for an additional characterization of bacterial families, e.g. the *Enterobacteriaceae* (Cotton & Gibson, 1970).

In *Pseudomonadaceae*, results from three wild-type strains of *Pseudomonas aeruginosa* (Ahmed & Campbell, 1973; Calhoun *et al.*, 1973; Stenmark-Cox & Jensen, 1975; Waltho, 1973) and from *Pseudomonas* sp. ATCC 11299a (Cerutti & Guroff, 1965) are available. Aggregation and regulation patterns of enzymes involved in phenylalanine and tyrosine biosynthesis differ amongst the *P. aeruginosa* strains ATCC 9027, PAO1 and PAT. Such a diversity is not known amongst strains of *Escherichia coli*. Our studies have focused on the questions of which types of regulation patterns are found in *P. aureofaciens* and whether they are related to the patterns observed in *P. aeruginosa* strains.

† Present address: Max-Planck-Institut für Immunbiologie, Stübeweg 51, D-7800 Freiburg, Federal Republic of Germany.

Fig. 1. Final steps in phenylalanine and tyrosine biosynthesis. Trivial names of enzymes: chorismate mutase (1), prephenate dehydratase (2), prephenate dehydrogenase (3), phenylalanine aminotransferase (4), tyrosine aminotransferase (5).

METHODS

Organism and growth conditions. *Pseudomonas aureofaciens* ATCC 15926 was grown at 30 °C with vigorous aeration in a minimal medium containing (g l⁻¹): glycerol, 30; K₂HPO₄, 3; KH₂PO₄, 0.5; (NH₄)₂SO₄, 1; NaCl, 5; MgSO₄·7H₂O, 0.5; pH 7.0. Cells for the preparation of enzymes were harvested in the late-exponential phase of growth by centrifugation. The yield was approximately 2.5 g wet weight cells per litre medium. The cells were stored at -18 °C.

Enzyme assays. Buffer A (50 mM-Tris/HCl, pH 7.7, containing 0.1 mM-EDTA) was used in the measurement of chorismate mutase (EC 5.4.99.5) activity by the method of Cotton & Gibson (1965), prephenate dehydratase (EC 4.2.1.51) activity by the method of Cotton & Gibson (1970) and prephenate dehydrogenase (EC 1.3.1.12) activity by the technique of Cotton & Gibson (1967).

Prephenate dehydratase associated with chorismate mutase was designated prephenate dehydratase I, whereas the non-associated enzyme was termed prephenate dehydratase II.

Protein determination. Protein was determined by the biuret method (Layne, 1957) using crystalline bovine serum albumin as standard.

Extraction and purification procedures. All procedures were carried out at 4 °C using buffer A and buffer B (0.15 M-Tris/HCl, pH 7.7, containing 0.1 mM-dithioerythritol and 0.1 mM-EDTA). When necessary, buffers and protein solutions were supplemented with the protease inhibitor phenylmethanesulphonyl fluoride at a final concentration of 1 mM (Fahrney & Gold, 1963).

Preparation of crude extracts. Frozen cells were ground with one and a half times their weight of aluminium oxide powder (Alcoa 305). The viscous paste was diluted with 4 vol. buffer A. The suspension was centrifuged at 37000 g for 20 min and the supernatant (15 to 20 mg protein ml⁻¹) was used in further studies.

Ammonium sulphate fractionation. Powdered (NH₄)₂SO₄ (enzyme grade) was slowly added to the crude extract (15 mg protein ml⁻¹). After stirring for 20 min, the precipitate was collected by centrifugation (20000g, 20 min) and dissolved in buffer A. The resulting protein solution (35 mg ml⁻¹) was dialysed for 2 h against 1000 vol. buffer A and stored at -18 °C, if not used immediately.

Sephadex G-100 chromatography. Dialysed protein solution (1 ml, containing 25 to 35 mg protein) obtained by (NH₄)₂SO₄ fractionation (0 to 80% saturation) was layered directly on to the gel surface of a Sephadex G-100 column (2.5 × 80 cm) previously equilibrated with buffer A. A flow rate of 17 ml h⁻¹ of buffer A was maintained and 2.5 ml fractions were collected. Active fractions that had to be rechromatographed were pooled and concentrated by addition of (NH₄)₂SO₄ up to 90% saturation before being applied to a Sephadex G-100 or a DEAE-Sephadex column.

DEAE-Sephadex chromatography. An (NH₄)₂SO₄ fraction (0 to 80% saturation) was prepared as described above, but using buffer B. The resulting protein solution (35 mg ml⁻¹) was dialysed against 500 vol. buffer B with two equal changes during 8 h. Dialysed protein solution (1 ml, containing 20 mg

Fig. 2. Sephadex G-100 chromatography of a 0 to 80% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction of crude extract from *P. aureofaciens*. Activities of chorismate mutase ($\circ$), prephenate dehydratase ($\bullet$) and prephenate dehydrogenase ($\square$) were measured in all fractions (see Methods). All procedures were carried out in presence of 1 mM-phenylmethanesulphonyl fluoride.

protein) was applied to a DEAE-Sephadex A-50 column (1.6×25 cm) previously equilibrated with buffer B. After the column had been washed with buffer B, enzymes were eluted by a linear gradient of 0 to 0.4 M-NaCl in buffer B. Fractions of 2.5 ml were collected at a flow rate of 15 ml h^{-1} .

Estimation of molecular weights. The molecular weights of chorismate mutase, prephenate dehydratase and prephenate dehydrogenase were estimated by gel filtration (Andrews, 1964). Approximately 20 mg protein from a 0 to 80% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction of crude extract in 1 ml buffer A were applied to a Sephadex G-100 column (2.5×80 cm). The column was calibrated using the following marker proteins of known molecular weight: bovine serum albumin (dimer form, 134000; monomer form, 67000), bovine chymotrypsinogen A (25000), and cytochrome *c* from horse heart (12400). Proteins were eluted with buffer A at a flow rate of 17 ml h^{-1} and 2.5 ml fractions were collected. The void volume was determined with blue dextran.

Chemicals and biochemicals. All reagents used were of the highest purity grade available. Chorismate (Gibson, 1970) and prephenate (Dayan & Sprinson, 1970) were prepared by standard methods.

RESULTS AND DISCUSSION

Ammonium sulphate fractionation

The distribution of chorismate mutase and prephenate dehydratase activities was determined in protein fractions obtained by precipitation with increasing concentrations of $(\text{NH}_4)_2\text{SO}_4$ (0 to 30, 30 to 40, 40 to 50, 50 to 70 and 70 to 90% saturation). Chorismate mutase activity was present in the first three fractions, whereas prephenate dehydratase was distributed throughout the fractions. In the course of feedback inhibition experiments it was found that prephenate dehydratase (prephenate dehydratase I) in $(\text{NH}_4)_2\text{SO}_4$ fractions up to 50% saturation was inhibited by 0.1 mM-L-phenylalanine and activated by 0.1 mM-L-tyrosine, whereas prephenate dehydratase activities from $(\text{NH}_4)_2\text{SO}_4$ fractions of higher saturations (50 to 90%) were not influenced by either amino acid (prephenate dehydratase II). The possible existence of two differently regulated prephenate dehydratase activities was therefore studied in detail by gel filtration.

Sephadex G-100 chromatography

The elution profile (Fig. 2) of an $(\text{NH}_4)_2\text{SO}_4$ fraction (0 to 80% saturation) on Sephadex G-100 showed a single peak of prephenate dehydrogenase activity followed by a peak containing chorismate mutase as well as prephenate dehydratase activity. An additional peak exhibiting prephenate dehydratase activity was eluted separately. The recovery of

Fig. 3. DEAE-Sephadex chromatography of a 0 to 80% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction of crude extract from *P. aureofaciens*. Activities of chorismate mutase (○) and prephenate dehydratase (●) were assayed as described in Methods. All procedures were carried out in presence of 1 mM-phenylmethanesulphonyl fluoride.

chorismate mutase–prephenate dehydratase I and prephenate dehydratase II activity was usually 80%, while that of prephenate dehydrogenase was 60%. The regulatory properties of these enzyme activities in the eluates were identical to those of the enzyme activities from $(\text{NH}_4)_2\text{SO}_4$ fractions. Prephenate dehydrogenase was inhibited by 0.1 mM-L-tyrosine.

Similar results were obtained when crude extracts were directly applied to the Sephadex G-100 column. This indicates that splitting of prephenate dehydratase activity was not caused by $(\text{NH}_4)_2\text{SO}_4$ fractionation. The preparation of enzyme extracts and the Sephadex G-100 chromatography were also done in the presence of 1 mM-phenylmethanesulphonyl fluoride. The sequence of elution as well as the regulatory properties of the enzymes were not altered, but the elution profile showed a more regular shape due to inhibition of proteolytic activities.

DEAE-Sephadex chromatography

The elution patterns of chorismate mutase and prephenate dehydratase activities were also studied by ion-exchange chromatography (Fig. 3). The phenylalanine-insensitive prephenate dehydratase II was eluted from the DEAE-Sephadex column in two successive peaks (recovery of enzyme activity 75%) followed by the phenylalanine-sensitive prephenate dehydratase I, which co-eluted with chorismate mutase (recovery of enzyme activity 62%).

Rechromatographed fractions of prephenate dehydratase II (recovery of enzyme activity 90%) from Sephadex G-100 or A-50 columns eluted as single peaks whose position corresponded to the first chromatographic profiles. Fractions of the chorismate mutase–prephenate dehydratase I aggregate (recovery of enzyme activity 85%) again showed two small successive peaks of prephenate dehydratase II activity upon rechromatography. The same results were obtained in the presence of phenylmethanesulphonyl fluoride. A working hypothesis might therefore be that prephenate dehydratase II is a dissociation product of prephenate dehydratase I. In *Pseudomonas* sp. ATCC 11299a (Cerutti & Guroff, 1965) and in *P. aeruginosa* strain PAT (Waltho, 1973) only one enzyme activity of chorismate mutase or prephenate dehydratase was detected. For *P. aeruginosa* strain PAO1 an enzyme complex of chorismate mutase and prephenate dehydratase was reported, as well as a second chorismate mutase and prephenate dehydratase, which were not aggregated (Calhoun *et al.*, 1973; Patel *et al.*, 1977). Two enzyme complexes each containing chorismate mutase and prephenate dehydratase activity were found in *P. aeruginosa* ATCC 9027 (Ahmed & Campbell, 1973). Data from column chromatography of prephenate dehydrogenase are available for *P. aeruginosa* strain PAO1 and *P. aeruginosa* ATCC 9027. No aggregation with other enzymes of the pathway (Ahmed & Campbell, 1973; Stenmark-Cox & Jensen, 1975) was observed. For *Pseudomonas* sp. ATCC 11299a a biosynthetic role of

Table 1. *Effect of aromatic amino acids on chorismate mutase, prephenate dehydratase I and II and prephenate dehydrogenase activity from P. aureofaciens*

For assaying chorismate mutase and prephenate dehydratase I a 0 to 40% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction of crude extract was used and for prephenate dehydratase II a 60 to 90% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction of crude extract was used. The prephenate dehydrogenase assay contained protein from crude extract. Enzyme activities were assayed as described in Methods and are expressed relative to the activities without added amino acids. The specific activities in the control assays were $[\text{nmol min}^{-1} (\text{mg protein})^{-1}]$: chorismate mutase, 78; prephenate dehydratase I, 71; prephenate dehydratase II, 23; prephenate dehydrogenase, 12.

L-Amino acid(s) added (0.1 mM)	Relative enzyme activity			
	Chorismate mutase	Prephenate dehydratase I	Prephenate dehydratase II	Prephenate dehydrogenase
None	1.00	1.00	1.00	1.00
Phenylalanine	0.66	0.38	0.97	1.00
Tryptophan	0.93	1.02	1.00	1.01
Tyrosine	0.93	1.5	1.00	0.51
Phenylalanine + tyrosine	0.68	0.41	1.02	0.5
Phenylalanine + tryptophan	0.68	0.38	1.00	1.00
Tryptophan + tyrosine	0.99	1.45	1.00	0.5
Phenylalanine + tryptophan + tyrosine	0.68	0.4	0.98	0.54

phenylalanine hydroxylase was discussed. However, this inducible catabolic enzyme was not detected in *P. aureofaciens* ATCC 15926 (O. Salcher & F. Lingens, unpublished results).

Molecular weight estimation

Molecular weights were estimated by gel filtration with Sephadex G-100. Prephenate dehydrogenase was eluted at a position corresponding to a molecular weight of 95000. The chorismate mutase-prephenate dehydratase I aggregate and prephenate dehydratase II had molecular weights of 75000 and 30000, respectively.

For *P. aeruginosa* strain PAO1, the molecular weights of the corresponding enzymes determined by the same method were: prephenate dehydrogenase, 150000; the chorismate mutase-prephenate dehydratase I aggregate, 134000; the second prephenate dehydratase, 76000 (Calhoun *et al.*, 1973); the second chorismate mutase, 27000 (Patel *et al.*, 1977).

Regulation of enzyme activity by aromatic amino acids

The influence of aromatic amino acids on enzyme activities was studied using protein from $(\text{NH}_4)_2\text{SO}_4$ fractions (Tables 1 and 2). Similar results to those described below were obtained with fractions purified by gel filtration and ion-exchange chromatography. Chorismate mutase activity was feedback-inhibited by 0.1 mM-L-phenylalanine, but the enzyme activity was not influenced by 0.1 mM-L-tyrosine. L-Tryptophan exerted no regulatory control on the enzymes of phenylalanine or tyrosine synthesis. D-Tyrosine was almost as efficient (30% activation) as L-tyrosine in activating prephenate dehydratase I. Other aromatic D-amino acids were inactive. Inhibition of prephenate dehydratase I by 10 μM -L-phenylalanine was reversed by 50 μM -L-tyrosine. Prephenate dehydratase II was not inhibited by aromatic amino acids, even when low concentrations of prephenate (0.012 to 0.04 mM) were used as substrate. Kinetic studies revealed further distinguishing features of the two prephenate dehydratase activities. The K_m value for prephenate of prephenate dehydratase II was about tenfold lower than that of prephenate dehydratase I (Table 2).

Plots of prephenate dehydratase I activity against various prephenate concentrations were sigmoid. Sigmoidicity was intensified by the inhibitor L-phenylalanine. The activator

Table 2. Comparison of kinetic properties of the chorismate mutase-prephenate dehydratase I aggregate and the prephenate dehydratase II from *P. aureofaciens*

Assays for chorismate mutase and prephenate dehydratase I contained protein from a 0 to 30% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction of crude extract. Assays for prephenate dehydratase II were performed with a 60 to 90% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction of crude extract. Enzyme activities were assayed as described in Methods. The K_i value of L-phenylalanine in the chorismate mutase reaction was determined at 0.9 mM-chorismate. The K_i value of L-phenylalanine in the prephenate dehydratase I reaction as well as the K_a value of L-tyrosine were each determined at 0.34 mM-prephenate. Values of K_m , K_i and K_a were generally determined from Lineweaver-Burk plots except those for prephenate dehydratase I which were calculated from curves by the method of Koshland *et al.* (1966).

Enzyme	K_m value (mM)	Substrate saturation curve	Inhibitor	K_i value (μM)	Activator	K_a value (μM)
Chorismate mutase	0.31	Hyperbolic	L-Phenylalanine	3.5	None	0
Prephenate dehydratase I	0.18	Sigmoid	L-Phenylalanine	8	L-Tyrosine	5
Prephenate dehydratase II	0.015	Hyperbolic	None	0	None	0

Fig. 4. Effects of L-phenylalanine and L-tyrosine on the substrate saturation curve of prephenate dehydratase I from *P. aureofaciens*. The assay for prephenate dehydratase I was done with protein (55 μg) from a 0 to 40% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction of crude extract as described in Methods. Assays contained 4 μM (○) or 40 μM (●) L-tyrosine, 8 μM (□) or 14 μM (■) L-phenylalanine, or no added amino acids (Δ).

L-tyrosine converted the substrate saturation curve to a simple Michaelis-Menten hyperbola (Fig. 4).

In *P. aeruginosa* PAO1 the bifunctional enzyme chorismate mutase-prephenate dehydratase was inhibited by L-phenylalanine (Patel *et al.*, 1977). However, inhibition of chorismate mutase was lost during purification by either gel filtration or ion-exchange chromatography (Calhoun *et al.*, 1973). Chorismate mutase from strain PAT was also inhibited by L-tyrosine (Waltho, 1973). In crude extracts of strain ATCC 9027 both chorismate mutase-prephenate dehydratase aggregates were insensitive to aromatic amino acids. After purification by ion-exchange chromatography, the so-called second prephenate dehydratase activity had become sensitive to inhibition by L-phenylalanine, and the inhibition was reversed by L-tyrosine (Ahmed & Campbell, 1973). The latter effect was also reported for the other *P. aeruginosa* strains studied and for *Pseudomonas* sp. ATCC 11299a (Cerutti & Guroff, 1965). The non-complexed chorismate mutase and the prephenate dehydratase from *P. aeruginosa* strain PAO1 were not influenced by either amino acid (Calhoun *et al.*, 1973; Patel *et al.*, 1977). This was also observed for chorismate mutase from *Pseudomonas* sp. ATCC 11299a (Cerutti & Guroff, 1965). With the exception of *Pseudomonas* sp. ATCC 11299a (Cerutti & Guroff, 1965), prephenate dehydrogenases of the above-mentioned *P. aeruginosa* strains were inhibited by L-tyrosine. So far the two

forms of prephenate dehydratase found in strains of *P. aeruginosa* have not been compared in kinetic studies. In our study the enzymes of phenylalanine and tyrosine biosynthesis were not repressed by aromatic amino acids (3 mM), which were also used as sole sources of carbon by *P. aureofaciens*. Extracts prepared from bacteria grown under conditions of potential repression were also tested for effects of L-phenylalanine and L-tyrosine on enzyme activities. No differences in the pattern of inhibition or activation were apparent, suggesting the absence of isoenzymes altered in regulatory properties. These results are consistent with those described for other pseudomonads.

We thank the Fonds der Chemischen Industrie for support and Mrs M. Vann for reading the manuscript.

REFERENCES

- AHMED, S. I. & CAMPBELL, J. J. R. (1973). A bi-functional enzyme in *Pseudomonas aeruginosa*: a new pattern in the organization of enzymes concerned with phenylalanine and tyrosine biosynthesis. *Journal of Bacteriology* **115**, 205–212.
- ANDREWS, P. (1964). Estimation of the molecular weights of proteins by Sephadex gel-filtration. *Biochemical Journal* **91**, 222–233.
- CALHOUN, D. H., PIERSON, D. L. & JENSEN, R. A. (1973). Channel-shuttle mechanism for the regulation of phenylalanine and tyrosine synthesis at a metabolic branch point in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **113**, 241–251.
- CERUTTI, P. & GUROFF, G. (1965). Enzymatic formation of phenylpyruvic acid in *Pseudomonas* sp. (ATCC 11299a) and its regulation. *Journal of Biological Chemistry* **240**, 3034–3038.
- COTTON, R. G. H. & GIBSON, F. (1965). The biosynthesis of phenylalanine and tyrosine: enzymes converting chorismic acid into prephenic acid and their relationships to prephenate dehydratase and prephenate dehydrogenase. *Biochimica et biophysica acta* **100**, 76–88.
- COTTON, R. C. H. & GIBSON, F. (1967). The biosynthesis of tyrosine in *Aerobacter aerogenes*: partial purification of the T protein. *Biochimica et biophysica acta* **147**, 222–237.
- COTTON, R. G. H. & GIBSON, F. (1970). Tyrosine and phenylalanine biosynthesis: the T and P proteins (*Aerobacter aerogenes*), chorismate mutase (*Pisum sativum*). *Methods in Enzymology* **17A**, 564–574.
- DAYAN, J. & SPRINSON, D. B. (1970). Preparation of prephenic acid. *Methods in Enzymology* **17A**, 559–561.
- FAHRNEY, D. E. & GOLD, A. M. (1963). Sulfonyl fluorides as inhibitors of esterases. I. Rates of reaction with acetylcholinesterase, α -chymotrypsin and trypsin. *Journal of the American Chemical Society* **85**, 997–1000.
- GIBSON, F. (1970). Preparation of chorismic acid. *Methods in Enzymology* **17A**, 362–364.
- KOSHLAND, D. E., JR, NÉMETHY, G. & FILMER, D. (1966). Comparison of experimental binding data and theoretical models in proteins containing subunits. *Biochemistry* **5**, 365–385.
- LAYNE, E. (1957). Spectrometric and turbidimetric methods for measuring proteins. *Methods in Enzymology* **3**, 447–454.
- LINGENS, F. (1968). The biosynthesis of aromatic amino acids and its regulation. *Angewandte Chemie (International Edition in English)* **7**, 350–360.
- PATEL, N., PIERSON, D. L. & JENSEN, R. A. (1977). Dual enzymatic routes to L-tyrosine and L-phenylalanine via pretyrosine in *Pseudomonas aeruginosa*. *Journal of Biological Chemistry* **252**, 5839–5846.
- STENMARK-COX, S. & JENSEN, R. A. (1975). Prephenate dehydrogenase from *Pseudomonas aeruginosa* is a regulated component of the channel-shuttle mechanism controlling tyrosine-phenylalanine synthesis. *Archives of Biochemistry and Biophysics* **167**, 540–546.
- WALTHO, J. A. (1973). Regulation of phenylalanine biosynthesis in *Pseudomonas aeruginosa*. *Biochimica et biophysica acta* **320**, 232–241.