Arogenate (Pretyrosine) Pathway of Tyrosine and Phenylalanine Biosynthesis in *Pseudomonas aureofaciens* ATCC 15926

By BRIGITTE KELLER, EBERHARD KELLER, OLGA SALCHER
AND FRANZ LINGENS*

*Institute of Microbiology, University of Hohenheim, Garbenstrasse 30, D-7000 Stuttgart 70,
Federal Republic of Germany*

(Received 5 October 1981)

Assays of enzyme activities suggest that arogenate, the product of prephenate transamination, is an intermediate in the biosynthesis of both phenylalanine and tyrosine in *Pseudomonas aureofaciens* ATCC 15926. In addition to prephenate dehydratase and prephenate dehydrogenase, arogenate dehydratase and arogenate dehydrogenase activities were demonstrated. This pattern of aromatic amino acid biosynthesis in pseudomonads had previously been demonstrated only in *P. aeruginosa*. Arogenate dehydrogenase from *P. aureofaciens* differs from that in *P. aeruginosa* in its utilization of either NAD⁺ or NADP⁺ as cofactor and its inhibition by L-tyrosine. During ammonium sulphate fractionation, arogenate dehydratase co-precipitated with prephenate dehydratase I activity and not with prephenate dehydratase II. The pattern of regulation of the arogenate route to tyrosine in *P. aureofaciens* ATCC 15926 differed from that previously reported for strain ATCC 13986.

INTRODUCTION

In a recent paper, we reported that the biosynthesis of phenylalanine and tyrosine in *Pseudomonas aureofaciens* ATCC 15926 (Blumenstock *et al.*, 1980) proceeds by a route identical with that previously established in such micro-organisms as *Escherichia coli* and *Bacillus subtilis* (Gibson & Pittard, 1968). This route goes via phenylpyruvate and 4-hydroxyphenylpyruvate with a subsequent transamination to phenylalanine and tyrosine, respectively (Fig. 1).

The presence of an alternative pathway for the biosynthesis of tyrosine was originally described in cyanobacteria (Stenmark *et al.*, 1974). In this pathway, prephenate is first transaminated to pretyrosine (now named arogenate), and the last step of tyrosine synthesis is a dehydrogenation. This route was later found in *P. aeruginosa* (Patel *et al.*, 1977) and more recently in coryneform bacteria (Fazel & Jensen, 1979). In *P. aeruginosa* there is an enzymic sequence to phenylalanine via pretyrosine in addition to the phenylpyruvate pathway (Patel *et al.*, 1977), hence the adoption of the name arogenate for the common precursor.

The present paper deals with the dual pathways to tyrosine and to phenylalanine in *P. aureofaciens* and the regulation of the biosynthesis of these aromatic amino acids.

METHODS

Organisms and growth conditions. *Pseudomonas aureofaciens* ATCC 15926 was grown in a minimal medium and harvested in the late-exponential phase of growth (Blumenstock *et al.*, 1980).

Enzyme assays. Crude extracts of bacteria were prepared as described by Blumenstock *et al.* (1980) except that cells were suspended in 100 mM-potassium phosphate buffer, pH 8.0 (buffer A) for the dehydratase enzyme, or buffer A supplemented with 0.1 mM-dithioerythritol (buffer B) for the dehydrogenase enzyme, and disrupted in a French press at 110 MPa. Cell debris was removed by centrifuging at 150 000 g for 1 h.

Fig. 1. Final steps in phenylalanine and tyrosine biosynthesis in *Pseudomonas aureofaciens*. Trivial names of enzymes: prephenate dehydratase (A), phenylalanine aminotransferase (B), arogenate aminotransferase (C), arogenate dehydratase (D), prephenate dehydrogenase (E), tyrosine aminotransferase (F), arogenate dehydrogenase (G).

Prephenate dehydratase [prephenate hydro-lyase (decarboxylating); EC 4.2.1.51] was measured by the method of Cotton & Gibson (1970) and prephenate dehydrogenase [prephenate:NAD⁺ oxidoreductase (decarboxylating); EC 1.3.1.12] by the technique of Cotton & Gibson (1967). Arogenate dehydratase was determined by measuring the enzymic product, phenylalanine, spectrofluorimetrically according to Shapiro *et al.* (1981).

For the assay of arogenate dehydrogenase, the continuous formation of NADH or NADPH was followed spectrophotometrically at 340 nm. The reaction mixture contained protein (80–130 µg), 4 mM-NAD⁺ or NADP⁺, 500 mM-(NH₄)₂SO₄, 0.4 mM-prephenate or 0.1 mM-arogenate and buffer B to a final volume of 0.5 ml. A blank containing the same compounds but omitting prephenate or arogenate was used as the control. The reaction was started by the addition of prephenate or arogenate. The reaction mixture was incubated at 30 °C.

Protein was determined by the Lowry method using bovine serum albumin as standard.

Chemicals. All reagents were of the highest purity available. Arogenate was prepared from the culture supernatants of a triple mutant of *Neurospora crassa* ATCC 36373 (Jensen *et al.*, 1977). Its purification and isolation was done essentially by the method of Zamir *et al.* (1980). Arogenate concentrations were determined after acid conversion to phenylalanine as described by Ambrose (1969). Prephenate was prepared as described by Dayan & Sprinson (1970).

RESULTS AND DISCUSSION

Prephenate and arogenate dehydrogenase

The regulation of prephenate dehydrogenase has already been described (Blumenstock *et al.*, 1980). As pseudomonads exhibit diverse patterns of enzyme regulation in tyrosine biosynthesis (Byng *et al.*, 1980), we extended our previous studies to include the enzymes of the arogenate pathway to tyrosine in *P. aureofaciens* ATCC 15926.

The regulation of arogenate dehydrogenase was found to be identical with that of prephenate dehydrogenase, i.e. the enzyme was neither repressed by tyrosine nor induced or

activated by phenylalanine. The sole regulatory effector was tyrosine which at 1 mM caused 60% inhibition.

NAD⁺ and NADP⁺ were equally effective as cofactor, with a specific activity of 9 nmol min⁻¹ (mg protein)⁻¹ in a crude extract from cells grown in minimal medium.

The variation of cofactor specificity and regulatory properties of the prephenate/arogenate dehydrogenase allow the family of *Pseudomonadaceae* to be divided into five groups (Byng *et al.*, 1980) which corresponded strikingly with the five ribosomal RNA (rRNA) homology groups established by rRNA-DNA hybridization (Palleroni *et al.*, 1973). The dehydrogenase pattern observed with *P. aureofaciens* ATCC 15926 permits its assignment to group II of the pseudomonads (Byng *et al.*, 1980). The characteristics of this group are as follows: an arogenate dehydrogenase using either NAD⁺ or NADP⁺ as hydrogen acceptor and sensitive to feedback inhibition by L-tyrosine. Byng *et al.* (1980) assigned *P. aureofaciens* ATCC 13986 to group I of the pseudomonad classification on the basis of an arogenate dehydrogenase inactive with NADP⁺. Moreover, group I species have an NAD⁺-linked arogenate dehydrogenase highly sensitive to inhibition by L-tyrosine and an NAD⁺-linked prephenate dehydrogenase relatively insensitive to tyrosine inhibition.

Prephenate and arogenate dehydratase

In *P. aureofaciens*, Blumenstock *et al.* (1980) distinguished two prephenate dehydratases (prephenate dehydratases I and II) separable by (NH₄)₂SO₄ fractionation, DEAE-Sephadex or Sephadex G-100 chromatography, and exhibiting differences in their molecular weights and regulatory behaviour. Prephenate dehydratase I was found in (NH₄)₂SO₄ fractions up to 50% saturation, whereas prephenate dehydratase II activity was detected in (NH₄)₂SO₄ fractions of 50–90% saturation. Two different prephenate dehydratases were already known in *P. aeruginosa* PAO1 (Calhoun *et al.*, 1973; Patel *et al.*, 1977), but these enzymes were distinguishable by their molecular weights from those detectable in *P. aureofaciens*. Prephenate dehydratase I associated with chorismate mutase is activated by tyrosine and inhibited by phenylalanine, whereas prephenate dehydratase II is not influenced by either end-product (Blumenstock *et al.*, 1980; Patel *et al.*, 1977). Prephenate dehydratase I from *P. aeruginosa* did not show any activity with arogenate as substrate, whereas prephenate dehydratase II was able to react with both prephenate and arogenate (Patel *et al.*, 1977).

In a crude extract of *P. aureofaciens* ATCC 15926 the specific activity of arogenate dehydratase was determined to be 8 nmol h⁻¹ (mg protein)⁻¹. Under the same conditions the prephenate dehydratase activity was 2000 nmol h⁻¹ (mg protein)⁻¹. After (NH₄)₂SO₄ fractionation arogenate dehydratase activity was found in the precipitate formed between 0 and 50% saturation; the fraction precipitated between 50 and 90% saturation exhibited only prephenate dehydratase activity. These results demonstrated the co-fractionation of arogenate dehydratase with prephenate dehydratase I and its non-identity with prephenate dehydratase II.

Phenylalanine and tyrosine synthesis in *P. aureofaciens* ATCC 15926

The results suggest that *P. aureofaciens* synthesizes tyrosine via parallel routes, the 4-hydroxyphenylpyruvate and arogenate pathways, as well as phenylalanine via the phenylpyruvate or the arogenate branchlet (Fig. 1). In the view of the 250-fold excess of prephenate dehydratase I activity, the phenylpyruvate branch seems to be the main route of phenylalanine synthesis.

REFERENCES

AMBROSE, J. A. (1969). A shortened method for the fluorometric determination of phenylalanine. <i>Clinical Chemistry</i> 15 , 15–23.	BLUMENSTOCK, E., SALCHER, O. & LINGENS, F. (1980). Regulation of phenylalanine and tyrosine biosynthesis in <i>Pseudomonas aureofaciens</i>

- ATCC 15926. *Journal of General Microbiology* **117**, 81–87.
- BYNG, G. S., WHITAKER, R. J., GHERNA, R. L. & JENSEN, R. A. (1980). Variable enzymological patterning in tyrosine biosynthesis as a means of determining natural relatedness among the *Pseudomonadaceae*. *Journal of Bacteriology* **144**, 247–257.
- 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.
- COTTON, R. G. 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.
- FAZEL, A. M. & JENSEN, R. A. (1979). Obligatory biosynthesis of L-tyrosine via the pretyrosine branchlet in coryneform bacteria. *Journal of Bacteriology* **138**, 805–815.
- GIBSON, F. & PITTARD, J. (1968). Pathways of biosynthesis of aromatic amino acids and vitamins and their control in microorganisms. *Bacteriological Reviews* **32**, 465–492.
- JENSEN, R. A., ZAMIR, L., ST PIERRE, M., PATEL, N. & PIERSON, D. L. (1977). The isolation and preparation of pretyrosine accumulated as a dead-end metabolite of *Neurospora crassa*. *Journal of Bacteriology* **132**, 896–903.
- PALLERONI, N. J., KUNISAWA, R., CONTOPOULOU, R. & DOUDOROFF, M. (1973). Nucleic acid homologies in the genus *Pseudomonas*. *International Journal of Systematic Bacteriology* **23**, 333–339.
- 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.
- SHAPIRO, C. L., JENSEN, R. A., WILSON, K. A. & BOWEN, J. R. (1981). An assay for activity of aroenate dehydratase based upon the selective oxidation of aroenate. *Analytical Biochemistry* **110**, 27–30.
- STENMARK, S. L., PIERSON, D. L., GLOVER, G. J. & JENSEN, R. A. (1974). Blue-green bacteria synthesize L-tyrosine by the pretyrosine pathway. *Nature, London* **247**, 290–292.
- ZAMIR, L. O., ARISON, B., DOUGLAS, A., ALBERS-SCHÖNBERG, G., BOWEN, J. R. & JENSEN, R. A. (1980). Structure of aroenate (pretyrosine), an amino acid intermediate of aromatic biosynthesis. *Journal of the American Chemical Society* **102**, 4499–4504.