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The Ancient Origin of a Second Microbial Pathway for L-Tyrosine Biosynthesis in Prokaryotes

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Summary. The 4-hydroxyphenylpyruvate pathway of L-tyrosine biosynthesis is well known. The presence of a second pathway appears to be a group characteristic of the blue-green algae (bacteria) which accordingly synthesize L-tyrosine by transamination of prephenate followed by a dehydrogenation reaction. An amino acid metabolite, pretyrosine, is the novel biochemical intermediate of the new pathway (denoted the pretyrosine pathway). All species of blue-green bacteria tested possessed only the pretyrosine pathway, while a variety of non-photosynthetic taxa exhibited only the 4-hydroxyphenylpyruvate pathway. A cell type that may reflect some properties of an ancient evolutionary intermediate is the pseudomonad group, a possibility suggested by the ability of *Pseudomonas aeruginosa* extracts to carry out both sets of pathway reactions.

Key words: Pretyrosine - Metabolic Pathways - Blue-Green Algae - Transamination - Prokaryotes - L-Tyrosine.

Introduction

Substantial data that sample the entire spectrum of biological life forms have accumulated over the past few decades, documenting major biochemical pathways that are central to the metabolic process. The outcome is the general observation that major biochemical pathways usually are qualitatively identical in the sequence of reaction steps. Hence, the sequence of reactions that define fundamental metabolic pathways appears to have been strongly conserved during evolution. This may be in marked contrast to the variability of the enzyme proteins that function within these highly conserved pathways, e.g., differing patterns of multi-enzyme aggregates and/or differing regulatory patterns[1].

[1] In the case of some enzymes, alternative types of regulatory patterns appear to be conserved at about the generic level. The molecular approach of determining the comparative regulation of branch-point enzymes such as 3-deoxy-D-arabino heptulosonate-7-phosphate (DAHP) synthetase has useful application for the resolution of certain taxonomic and phylogenetic controversies (Jensen & Rebello, 1970).

In instances where different metabolic sequences are known to provide alternative pathways to an important metabolite, the distribution of those pathways in nature appears to reflect an ancient evolutionary event that is faithfully retained in derivative cell types that have survived to modern times. Hence the presence of either the diaminopimelate or the α-aminoadipate pathway for L-lysine biosynthesis appears to mark broad phylogenetic relationships, undoubtedly reflecting an ancient evolutionary divergence (Rodwell, 1969). We recently found that prokaryotes possess either of two pathways for L-tyrosine biosynthesis (Stenmark et al., 1974). The 4-hydroxyphenylpyruvate and pretyrosine pathways are illustrated in Fig.1. The uniform occurrence of the pretyrosine pathway in species of such a diverse group as the blue-green "algae" (more appropriately referred to as bacteria: order *Chroococcales*, Stanier et al., 1971) suggests an ancient origin for the pretyrosine pathway.

Materials and Methods

Microorganisms. All strains of blue-green bacteria were grown by L.O. Ingram as described by Stenmark et al. (1974). The origins of the strains of blue-green bacteria listed in Table 2 were referenced by Stenmark et al. (1974). *Serratia marcescens* strain Nima was obtained from Dr. R. Williams (Williams & Hearn, 1967). The K-12 strain of *Escherichia coli* was obtained from T.Matney as UTH 432, synonymous with W2979 (J. Lederberg) having the genotype: F^- *ara gal malA xyl mtl* λ^+/λ^R. Cells of *Clostridium butyricum* were grown by R. Twarog, shipped to Houston in dry ice, and prepared as crude extract as previously described (Jensen & Twarog, 1972). Strain 168 of *Bacillus subtilis* (Armstrong et al., 1970) and strain 1 of *Pseudomonas aeruginosa* (Calhoun & Jensen, 1972) were used.

Biochemicals. Prephenate, converted to the potassium salt immediately before use,was prepared and stored as the barium salt by the method of Gibson (1964). Purity, uncorrected for solvation, was greater than 95%. Pretyrosine was prepared as recently described (Stenmark et al., 1974). Phenylpyruvate, 4-hydroxyphenylpyruvate, and all amino acids were obtained from Sigma. Other chemicals were of the best reagent grade available from commercial sources. Sephadex was obtained from Pharmacia.

Extract Preparation. Cell pellets obtained by centrifugation of cultures harvested in the exponential phase of growth were stored at -20° prior to extract preparation. The whole cells were resuspended in 4 ml of 0.04 M

potassium phosphate buffer, pH 6.8, at 4°. Cells were disrupted with four 30-sec pulses of ultrasound energy at ice temperature using a Bronwill sonifier. (Cells of *A. quadruplicatum* were lysed with lysozyme as described under Fig.2.) Each extract volume was increased to 6 ml with buffer, and the preparation was centrifuged for 2 hrs at 35,000 RPM in a type 65 fixed-angle rotor of a Beckman preparative ultracentrifuge. About 4 ml of clear extract were decanted. The extract was percolated through a small Sephadex G-25 column in order to remove small molecules. The resulting preparation is denoted as crude extract. Such crude extracts yielded protein concentrations ranging from 2-20 mg per ml.

Enzymology. Initial rates of enzyme reactions were determined under conditions of proportionality with respect to reaction time and protein concentration. Occasionally, as noted in the text, partial purification was necessary. Prephenate dehydrogenase activity was assayed by measurement of NADH formation (Champney & Jensen, 1970). Pretyrosine dehydrogenase was also measured by continuous formation of the NADH product as described by Stenmark et al. (1974). The assay of prephenate transaminase by measuring absorbance of phenylpyruvate at 320 nm was also described (Stenmark et al., 1974). Unless otherwise indicated, the reaction mixture contained 0.05 ml of 40 mM L-phenylalanine, 0.04 ml of 50 mM α-ketoglutarate, 0.01 ml of 1 mM PLP, and 0.05 ml of enzyme preparation. The reaction was terminated after 15 min at 37° by addition of 0.8 ml of 1 N NaOH. The method of Silbert et al. (1963) was used for assay of aromatic transaminase activity. Protein concentration was estimated by the procedure of Lowry et al. (1951).

Thin-Layer Chromatography. Pretyrosine, other amino acids, and keto acids were identified qualitatively by the following procedure. A 10 µl-volume of sample was applied to the surface of a cellulose thin-layer plate (Eastman Kodak Co.). Subsequent chromatography was carried out using a 1-propanol: water (70:30) solvent. After movement of the solvent front 8 cm from the origin, the plate was dried, sprayed with ninhydrin, and developed at 100°. The R_f value for pretyrosine in this solvent is 0.26.

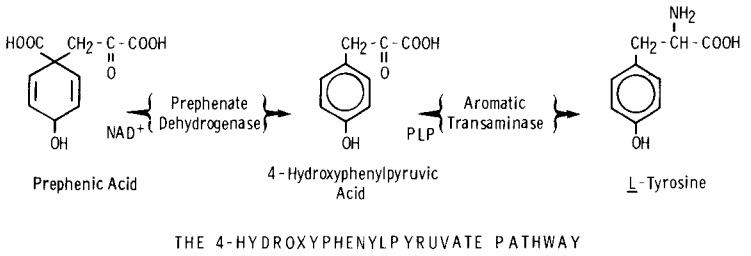
Results

Data given in Table 1 obtained with partially purified enzyme preparations support the conclusions that *B. subtilis* possesses only the 4-hydroxyphenylpyruvate pathway, while the blue-green bacterium *Agmenellum quadruplicatum* has only the pretyrosine pathway. *B. subtilis* has an active prephenate

Table 1. Enzyme activities of L-tyrosine biosynthesis in microorganisms

Enzymological assays are described under "Materials and Methods". Enzyme activities were partially purified following gel-filtration on a Sephadex G-200 column. The specific activities found in crude *B. subtilis* extract for prephenate dehydrogenase and aromatic transaminase were 17.9 and 23.8, respectively. The specific activity for pretyrosine dehydrogenase in the crude *A. quadruplicatum* extract was 6.7. *B. subtilis* extracts and column buffers contained 30% (v/v) glycerol, a condition which completely stabilizes the otherwise labile prephenate dehydrogenase (Champney & Jensen, 1970).

Microorganism	Specific activity (nmoles/min/mg)			
	Prephenate dehydro-genase	Aromatic trans-aminase	Prephenate trans-aminase	Pretyrosine dehydro-genase
Bacillus subtilis	49.7	78.3	0	0
Agmenellum quadruplicatum	0	0.9	10.2	87.0



THE 4-HYDROXYPHENYLPYRUVATE PATHWAY

THE PRETYROSINE PATHWAY

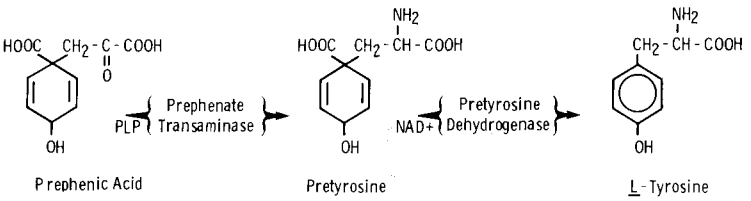


Fig.1. Biochemical pathways for L-tyrosine biosynthesis. NAD^+ = nicotinamide adenine dinucleotide; PLP = pyridoxal-5'-phosphate

dehydrogenase (previously characterized by Champney & Jensen, 1970) but lacks detectable pretyrosine dehydrogenase activity. Likewise, while an aromatic transaminase capable of reaction with 4-hydroxyphenylpyruvate was found, a prephenate-reactive transaminase activity was not detected. The inverse results were obtained with extracts of *A. quadruplicatum* which possesses only the enzyme activities of the pretyrosine pathway (shown in Fig.1).

In *A. quadruplicatum*, L-leucine and L-isoleucine were comparable to L-phenylalanine (qualitatively by thin-layer chromatography) as the most active amino donors for transamination when prephenate was used as the keto substrate. With L-leucine as the amino donor reactant, phenylpyruvate (but not 4-hydroxyphenylpyruvate) was an excellent keto acid substrate. It appears that a single transaminase may catalyze the first unique step of L-tyrosine biosynthesis as well as the last step of L-phenylalanine biosynthesis (Stenmark et al., 1974). Fig. 2 shows the elution profile of pretyrosine pathway enzymes of *A. quadruplicatum* obtained from a gel-filtration (Sephadex G-200) column. The following molecular weight estimates were obtained: prephenate transaminase ($\bar{M}$ 130,000) and pretyrosine dehydrogenase ($\bar{M}$ 65,000). The pretyrosine dehydrogenase activity eluted as shown in Fig. 2, possessed characteristics that were similar to those measured in crude extracts. In contrast prephenate transaminase could not be properly characterized in crude extracts owing to the presence of interfering activities. Because of the

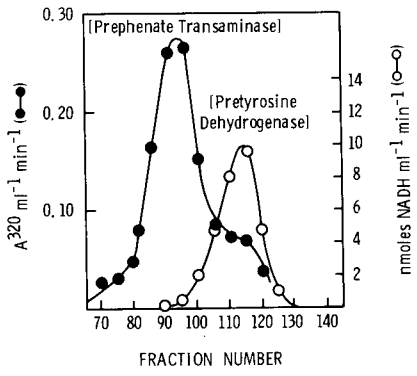


Fig.2. Separation of prephenate transaminase and pretyrosine dehydrogenase activities of *A. quadruplicatum* by gel-filtration. Cells of *A. quadruplicatum* were suspended in potassium phosphate buffer (40 mM, pH 6.8) containing 40 µM PLP. Lysozyme (Worthington, 2X crystallised) was added to a final concentration of 5 mg per ml. After lysis for 30 min at 37°, the crude extract was frozen at dry ice temperature, thawed at 37°, and centrifuged at 40,000 rpm in a type 65 fixed-angle rotor of a Beckman preparative ultracentrifuge. A 3.2-ml volume of extract protein (164 mg) was applied to a 2.5 x 100 cm Sephadex G-200 column equilibrated with 40 mM phosphate buffer (pH 6.8) containing 40 µM PLP.Eluate fractions of 2.5-ml volume were collected. The column was calibrated by the procedure of Andrews (1965) using chymotrypsinogen ($\bar{M}$ 25,000), ovalbumin ($\bar{M}$ 45,000), bovine serum albumin ($\bar{M}$ 67,000) and γ-globulin ($\bar{M}$ 160,000) as molecular weight standards. All standards were obtained from Mann Biochemicals. Fractions containing the leading portion of prephenate transaminase were pooled,concentrated by means of an Amicon diaflow cell(PM 10 filter,and used subsequently to characterize the enzyme. Fractions containing the pretyrosine dehydrogenase activity were treated similarly. Prephenate transaminase was assayed as previously described (Stenmark et al., 1974). The assay of column eluates differed from our standard procedure (Stenmark et al., 1974) in that 0.04 ml (rather than 0.10 ml) of 5 mM pretyrosine was added to the reaction mixture. Pretyrosine dehydrogenase was purified about five-fold following gel-filtration to yield a specific activity of 40.3 nmoles NADH per min per mg protein at 37°. Owing to the difficulties for accurate assay of transaminase activity in crude extracts, the purification achieved for prephenate transaminase is uncertain

greater difficulty generally encountered with transaminase assays, comparative studies designed to qualitatively identify the pathway existing for L-tyrosine biosynthesis were done in crude extracts by characterizing dehydrogenase activity.

Comparative enzymological results of dehydrogenase assays in crude extracts are shown in Table 2 which contains a summary of data obtained from both prokaryotes and eukaryotes. The six species of blue-green algae shown exhibit only the dehydrogenase activity of the pretyrosine pathway. Neither dehydrogenase activity was found in extracts of a number of other species of blue-green algae tested: *Plectonema terebrans* strain Jam (filamentous, marine), *Fisherella ambigua* strain HB (filamentous, freshwater), *Oscillatoria williamsii* strain Mev (filamentous, marine), and *Schizothrix calcicola* strain Man (filamentous, marine). Since none of these strains require L-tyrosine for growth, the negative data are almost certainly attributable to trivial factors of extract and assay conditions used. A comprephensive search for more optimal enzymological conditions in these strains was not attempted.

In contrast, only the enzymes of the 4-hydroxyphenylpyruvate pathway were found in *B. subtilis*, species representing two genera of *Enterobacteriaceae*, and *C. butyricum*. These enzymes have also been shown to exist in other species of eubacteria (Table 2), but the probable absence in these organisms of the pretyrosine pathway remains to be confirmed. *P.aeruginosa* possesses the enzyme activities of both pathways in vitro. (We have obtained similar results with *P. putida*.) Partial purification yielded a single dehydrogenase and a single transaminase in *P. aeruginosa*, each exhibiting substrate ambiguity that accounts for the catalytic potential to synthesize L-tyrosine by the two different sequences shown in Fig. 1. The 4-hydroxyphenylpyruvate route probably is at least the preferred sequence in vivo since the K_m values of the dehydrogenase calculated for prephenate and pretyrosine in *P. aeruginosa* were 0.06 mM and 0.50 mM, respectively. The 4-hydroxyphenylpyruvate pathway has also been found in fungi (*N. crassa* and *S. cerevisiae*) and in bean cotyledon (*P. vulgaris*) preparations (Table 2). The possible partitioning of these pathways in mitochondrial or chloroplast organelles of eukaryotic cells is unknown and merits further examination in view of the speculation that eukaryotic organelles evolved from ancient prokaryotes.

Table 2. Taxonomic distribution of L-tyrosine pathway dehydrogenases

Assays for prephenate dehydrogenase and pretyrosine dehydrogenase activities were done as described under "Materials and Methods", and these data are expressed as specific activities. Because of the variable assay conditions used, activities reported by others in the literature are simply indicated qualitatively as '+' and referenced at the right. Lack of data about possible pretyrosine dehydrogenase activity is indicated by '?'. The organisms listed at the left are organized from top to bottom as the following groups: blue-green bacteria, non-photosynthetic prokaryotes, fungi, and plants.

Organism	Specific activity (nmoles NADH/min/mg)	
	Pretyrosine dehydrogenase	Prephenate dehydrogenase
Agmenellum quadruplicatum	6.5	0
Anacystis nidulans	2.3	0
Coccochloris elabens	4.6	0
Nostoc muscorum	0.8	0
Lyngbya lagerheimii	3.0	0
Oscillatoria sp.	1.0	0
Bacillus subtilis	0	30
Escherichia coli	0	397
Serratia marcescens[a]	0	63
Salmonella typhimurium	?	+ (Zalkin, 1967)
Aerobacter aerogenes	?	+ (Cotton & Gibson, 1967)
Brevibacterium flavum	?	+ (Sugimoto et al., 1973)
Clostridium butyricum	0	+[b]
Pseudomonas aeruginosa	7.7	7.7
Neurospora crassa	?	+ (Catcheside, 1969)
Saccharomyces cerevisiae	?	+ (Lingens, 1968)
Phaseola vulgaris	?	+ (Gamborg & Keeley, 1966)

[a]Extract was partially purified by gel-filtration (Sephadex G-200) owing to interferring activities that were present in crude extracts.

[b]Activity is qualitatively denoted as '+' because activity constantly decreased as a function of time, and an initial rate could not be determined in order to calculate a valid specific activity.

Discussion

The separation of blue-green algae and other prokaryotes from a common ancestor is estimated to have occurred 3-4 billion years ago (Barghoorn, 1971), making this a most ancient event. Since the pretyrosine pathway appears to be a "group-characteristic" of the blue-green algae while a variety of non-photosynthetic prokaryotes so far studied utilize the 4-hydroxyphenylpyruvate pathway for L-tyrosine biosynthesis, intriguing evolutionary possibilities exist. The presence of either the pretyrosine or the 4-hydroxyphenylpyruvate pathway may provide evolutionary clues about the era when blue-green algae and other prokaryotes diverged.

The terrestrial origin of life and the subsequent sequence of evolutionary events followed a time of abiotic synthesis (denoted chemical evolution by Ponnamperuma). Presumably the early forms of life were heterotrophic anaerobes (perhaps like species of modern *Clostridium*). This was followed eventually by the appearance of autotrophic species that were no longer dependent upon organic nutrients. A photoheterotrophic bacterium is hypothesized (Olson, 1970) to have been the common ancestor of modern photosynthetic bacteria and blue-green algae at a time when a largely anoxygenic environment contained H_2, N_2, CH_4, NH_3, CO_2 and water (Cloud, 1965). Under conditions of decreasing nutrient concentration in the ancient organic soup, ability to utilize organic compounds existing in a more oxidized state would confer a selective advantage upon photoheterotrophic organisms. In turn, photoautotrophs (similar to blue-green algae) evolved, and the generation of oxygen by photoautotrophs provided conditions suitable for the evolution of aerobic heterotrophs and autotrophs.

The evolution of the prokaryotes has been reviewed comprehensively from the vantage points of energy metabolism (Kelly, 1971) and carbon dioxide assimilation (McFadden, 1973), and there seems to be a consensus that the general sequence of evolutionary events cited above is a probable one (see Hall, 1971). Since clostridial species are the best candidates for resemblence to the ancestral cell type that was common both to modern blue-green algae and other prokaryotes, the 4-hydroxyphenylpyruvate pathway may be the most ancient. The 4-hydroxyphenylpyruvate pathway has been documented most extensively in heterotrophic organisms such as species of *Enterobacteriaceae* and *Bacillus*. It will be of considerable interest to see whether insight into prokaryote evolution can be gained by extending our comparative data to include photoheterotrophic and autotrophic organisms. In this connec-

tion the ambiguity of the *P. aeruginosa* enzymes is of special interest since the pseudomonad group includes autotrophic organisms. For example, Stanier et al. (1966) have renamed species such as *Hydrogenomonas facilis* (now *P. facilis*). Thus, taxonomic analysis revealed that the former *H. facilis* resembled *P. acidovorans* more than it resembled other autotrophic pseudomonads. Recent data based upon the determination of ribosomal RNA homology groups substantiate previous conclusions (Palleroni et al., 1973). Perhaps the substrate ambiguities of *P. aeruginosa* dehydrogenase and transaminase reflect the properties of the ancestral enzymes. Evolution toward greater specificity could then have resulted in either the pretyrosine-specific sequence or the 4-hydroxyphenylpyruvate-specific sequence.

In spite of the incontestable conservatism that has attended the evolution of biochemical pathways, it is possible that more variation in pathway sequences may exist than previously supposed. For example, a novel pathway of isoleucine biosynthesis was recently found in the spirochete organism, *Leptospira interrogans* (Charon et al., 1974).

Most rigorous enzymology and biochemistry in prokaryotic microorganisms has been done in a relatively small group of heterotrophs such as enteric bacteria, pseudomonads and *B. subtilis*. Perhaps it is no accident that the new pathways found for L-tyrosine biosynthesis and L-isoleucine biosynthesis occur in microbial groups (blue-green algae and *Leptospira*, respectively) in which very little enzymology has been done.

An additional example of pathway variation that may possibly be of interest is the joint existence of the phosphorylated and non-phosphorylated routes of serine biosynthesis in *Rhodopseudomonas capsulata*(Schmidt & Sojka, 1973) and in *Pseudomonas* AM1 (Heptinstall & Quayle, 1970). However, the fact that no organism has been found to have only the non-phosphorylated pathway in the absence of the phosphorylated pathway is consistent with the evidence (Feld & Sallach, 1973) that the non-phosphorylated pathway functions in the gluconeogenic direction (serine ↔ hydroxypyruvate ↔ D-glycerate ↔ 2-P-glycerate) rather than in the biosynthetic direction.

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