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Clues from *Xanthomonas campestris* about the Evolution of Aromatic Biosynthesis and its Regulation

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Summary. The recent placement of major Gram-negative prokaryotes (Superfamily B) on a phylogenetic tree (including, e.g., lineages leading to *Escherichia coli, Pseudomonas aeruginosa,* and *Acinetobacter calcoaceticus*) has allowed initial insights into the evolution of the biochemical pathway for aromatic amino acid biosynthesis and its regulation to be obtained. Within this prokaryote grouping, *Xanthomonas campestris* ATCC 12612 (a representative of the Group V pseudomonads) has played a key role in facilitating deductions about the major evolutionary events that shaped the character of aromatic biosynthesis within this grouping. *X. campestris* is like *P. aeruginosa* (and unlike *E. coli*) in its possession of dual flow routes to both L-phenylalanine and L-tyrosine from prephenate. Like all other members of Superfamily B, *X. campestris* possesses a bifunctional P-protein bearing the activities of both chorismate mutase and prephenate dehydratase. We have found an unregulated arogenate dehydratase similar to that of *P. aeruginosa* in *X. campestris*. We separated the two tyrosine-branch dehydrogenase activities (prephenate dehydrogenase and arogenate dehydrogenase); this marks the first time this has been accomplished in an organism in which these two activities coexist. Superfamily B organisms possess 3-deoxy-D-*arabino*-heptulosonate 7-P (DAHP) synthase as three isozymes (e.g., in *E. coli*), as two isozymes (e.g., in *P. aeruginosa*), or as one enzyme (in *X. campestris*). The two-isozyme system has been deduced to correspond to the ancestral state of Superfamily B. Thus, *E. coli* has gained an isozyme, whereas *X. campestris* has lost one. We conclude that the single, chorismate-sensitive DAHP synthase enzyme of *X. campestris* is evolutionarily related to the tryptophan-sensitive DAHP synthase present throughout the rest of Superfamily B. In *X. campestris,* arogenate dehydrogenase, prephenate dehydrogenase, the P-protein, chorismate mutase-F, anthranilate synthase, and DAHP synthase are all allosteric proteins; we compared their regulatory properties with those of enzymes of other Superfamily B members with respect to the evolution of regulatory properties. The network of sequentially operating circuits of allosteric control that exists for feedback regulation of overall carbon flow through the aromatic pathway in *X. campestris* is thus far unique in nature.

Key words: Metabolic evolution — Aromatic biosynthesis — Regulatory enzymes — *Xanthomonas campestris*

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Introduction

Obvious constraints exist in nature with respect to biochemical alternatives for the evolutionary assembly of major metabolic pathways. Against this background of conservatism, allosteric regulation and certain gene–enzyme relationships (e.g., existence of multifunctional enzymes that channel key pathway intermediates) may exhibit considerable diversity. In prokaryotes, the pathway for biosyn-

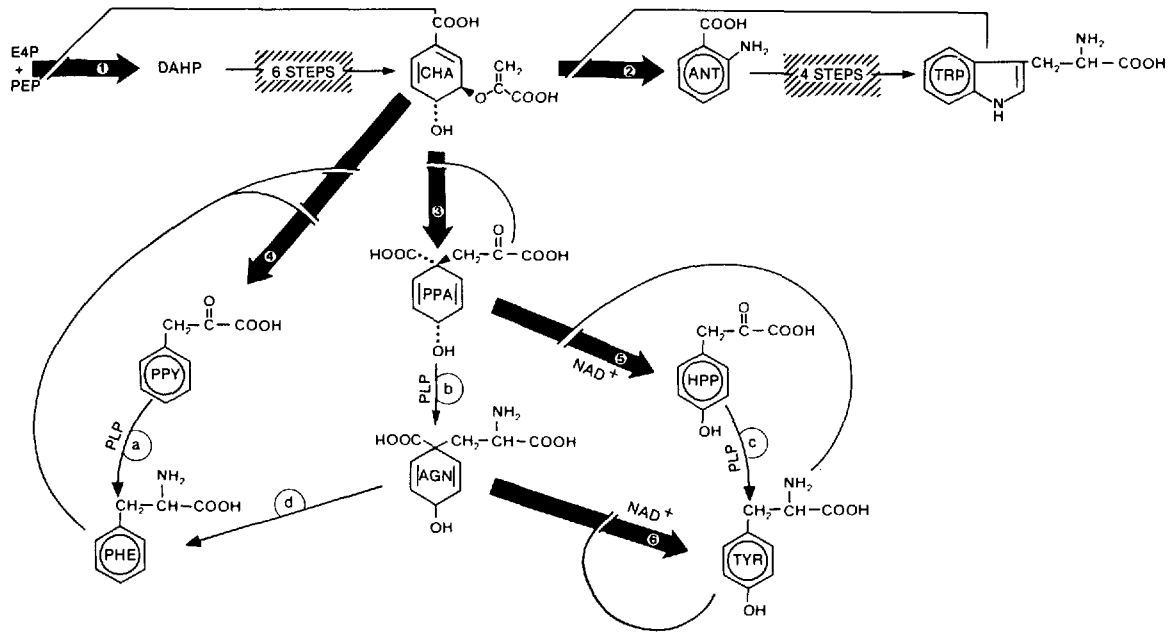

Fig. 1. Biochemical pathway for biosynthesis of aromatic amino acids in *Xanthomonas campestris*. Enzymes subject to feedback inhibition are represented by heavy solid arrows, numbered as follows: 3-deoxy-D-*arabino*-heptulosonate 7-P (DAHP) synthase (1) catalyzes the condensation of erythrose-4-P (E4P) and phosphoenolpyruvate (PEP) to yield DAHP, and is feedback inhibited by chorismate (CHA). Anthranilate (ANT) synthase (2) is feedback inhibited by L-tryptophan (TRP). Chorismate mutase-F (3) is product inhibited by prephenate (PPA). The multifunctional P-protein (4) carries catalytic sites for chorismate mutase (chorismate mutase-P) and for prephenate dehydratase, and each of these activities is feedback inhibited by L-phenylalanine (PHE). Prephenate dehydrogenase (5) is feedback inhibited by L-tyrosine (TYR), as is arogenate (AGN) dehydrogenase (6). Each dehydrogenase requires nicotinamide adenine dinucleotide (NAD^+) as cofactor. Enzyme a is phenylpyruvate (PPY) aminotransferase; enzyme b is prephenate aminotransferase; and enzyme c is 4-hydroxyphenylpyruvate (HPP) aminotransferase. Each aminotransferase functions well with L-glutamate as the amino-donor reactant and requires pyridoxal-5'-P (PLP) as cofactor. Enzyme d is arogenate dehydratase. Prephenate molecules formed by chorismate mutase-P are fated to become transformed only to L-phenylalanine via phenylpyruvate. In contrast, prephenate molecules formed by chorismate mutase-F may be transformed to L-phenylalanine via arogenate or to L-tyrosine via either arogenate or 4-hydroxyphenylpyruvate

thesis of aromatic amino acids has been the richest source of data exemplifying such diversity (Jensen and Rebello 1970; Byng et al. 1982). More recently, it has become clear that even the enzymic construction of the aromatic pathway exhibits evolutionary diversity (Byng et al. 1982). A third area of diversity is the specificity of aromatic-pathway dehydrogenases for pyridine nucleotide cofactor, with some requiring nicotinamide adenine dinucleotide (NAD^+), some requiring NAD phosphate ($NADP^+$), and some accepting either (Byng et al. 1982).

Although such information about pathway diversity allowed some degree of organization of feasible evolutionary groups, no sense of evolutionary direction was possible without a phylogenetic tree. If the pathways studied belonged to organisms having established positions on a credible phylogenetic tree, then the evolution of any suitable biochemical pathway possessed by these organisms could be deduced. The advent of oligonucleotide cataloging (Fox et al. 1980), a technique that allows the computer-assisted estimation of evolutionary similarity between any given pair of organisms, has stimulated probing of evolutionary directions in aromatic-pathway construction and regulation.

The most extensively studied prokaryote grouping for which a phylogeny based on oligonucleotide cataloging exists and for which the aromatic pathway and its regulation have been characterized is an assemblage of Gram-negative bacteria denoted as Superfamily B (Jensen 1985). Major lineages within Superfamily B are the enteric bacteria, Group I pseudomonads, Group V pseudomonads, and *Acinetobacter* species. The Group V pseudomonads are mostly named as species of *Xanthomonas*; some phytopathogenic species currently named as *Pseudomonas* are clearly Group V pseudomonads on the criterion of rRNA homology (Palleroni et al. 1973). Information about Group V pseudomonads has already heavily influenced the emerging picture of evolutionary steps in the history of aromatic biosynthesis and regulation in Superfamily B prokaryotes (Byng et al. 1983a,c; Jensen 1985). This paper provides detailed enzyme characterizations of *Xanthomonas campestris*.

Figure 1 illustrates the multibranched pathway

responsible for aromatic biosynthesis in *Xanthomonas* species. Minor branches to vitamin-like compounds such as *p*-aminobenzoate are not shown. Figure 1 shows the dual pathways that coexist in both Group I pseudomonads and Group V pseudomonads. In other organisms, either L-arogenate or phenylpyruvate may be an exclusive intermediate of L-phenylalanine biosynthesis. Similarly, either L-arogenate or 4-hydroxyphenylpyruvate may be an exclusive intermediate of L-tyrosine biosynthesis. Three levels of allosteric regulation commonly exist to control the formation of aromatic amino acids in prokaryotes. At the early-pathway level, 3-deoxy-D-*arabino*-heptulosonate 7-P (DAHP) synthase usually is a target of allosteric control. At the midpathway level, chorismate mutase is a second possible target. Finally, late-pathway control is commonly exercised at initial enzyme steps within each of the three terminal amino acid branches.

Materials and Methods

Microbiological Procedures

X. campestris ATCC 12612 was obtained from the American Type Culture Collection (Rockville, Maryland) and maintained on double-strength Difco nutrient agar at 28°C. When culturing cells for extract preparation, we used a 250-ml culture in late-exponential phase to inoculate 10 l of double-strength nutrient broth containing 2% glucose. The culture was grown at 28°C under constant aeration, harvested during the late-exponential phase of growth by centrifugation, washed with three volumes of 50 mM potassium phosphate buffer (pH 7.0), and stored at −80°C until needed.

Extract Preparation

Whole cells were suspended in 50 mM potassium phosphate buffer containing 1.0 mM dithiothreitol (pH 7.0), sonicated using a Lab-line ultra-tip sonicator, and centrifuged for 1 h at 150,000 × g to remove cell debris. Small molecules were then removed from the extract by passage through a 1.5 × 20 cm Sephadex G-25 column that had been equilibrated in starting buffer. The extract at this stage is denoted as crude extract. For the isolation and characterization of the bifunctional P-protein, extracts were prepared as above except that the starting buffer was 50 mM potassium phosphate (pH 7.0) containing 1.0 mM L-tyrosine.

DEAE-Cellulose Chromatography

Crude extract (120 mg protein) was applied to a 1.5 × 20 cm DE-52 column equilibrated in 50 mM potassium phosphate buffer (pH 7.0) containing 1.0 mM dithiothreitol. The column was washed with 100 ml of buffer, and bound protein was then eluted with a 300-ml linear salt gradient (0 to 0.5 M KCl) prepared in the same starting buffer. Fractions (2.0 ml) were collected and assayed for the appropriate enzyme activities. The bifunctional P-protein was found to require L-tyrosine for stability. Therefore, for the partial purification and characterization of these activities, a DE-52 column was run in which 50 mM potassium phosphate buffer (pH 7.0) containing 1.0 mM L-tyrosine was substituted for the buffer described above.

Hydroxylapatite Chromatography

A 100-mg portion of crude extract was loaded onto the bed of a 1.5 × 15 cm column of Bio-Gel HTP previously equilibrated in 10 mM potassium buffer (pH 7.0) containing 1.0 mM dithiothreitol. The column was washed with 100 ml of starting buffer and then eluted by application of a salt gradient of between 10 mM and 400 mM potassium phosphate (pH 7.0, containing 1.0 mM dithiothreitol). Fractions (2.0 ml) were collected.

Analytical Procedures

All enzyme data were obtained under assay conditions of proportionality with respect to the variables of reaction time and protein concentration. All reaction mixtures were incubated at 37°C.

DAHP Synthase. DAHP synthase activity was assayed as described by Jensen et al. (1973). A pH optimum of 7.0 was identified by measuring enzyme activity in potassium phosphate buffer within a range of pH 6.0–8.0 (data not shown). All subsequent DAHP synthase assays were carried out at pH 7.0. For the assay of crude extracts or chromatographic eluates, the reaction mixture (200 μl) contained 1.0 mM phosphoenolpyruvate, 1.0 mM erythrose-4-phosphate, 50 mM potassium phosphate buffer (pH 7.0), and enzyme. The substrate and inhibitor concentrations used for kinetic analyses are indicated in the appropriate figure legends. In all assays the appropriate blanks were included to insure against interference with the chemical assay by effector molecules (Jensen et al. 1967). The concentration of DAHP was calculated by relating absorbance at 549 nm to a molar extinction coefficient of 45,000.

Chorismate Mutase. Chorismate mutase activity was assayed as described by Patel et al. (1977). Reaction mixtures (200 μl) contained 0.5 mM chorismate, 50 mM potassium phosphate (pH 7.0), and enzyme. After 20 min at 37°C, enzyme activity was halted by acidification and the absorbance of phenylpyruvate was measured in base at 320 nm using a molar extinction coefficient of 17,500 for calculations.

Prephenate Dehydratase. Prephenate dehydratase was assayed as described by Patel et al. (1977). Typical reaction mixtures (200 μl) contained 1.0 mM prephenate, 50 mM potassium phosphate (pH 7.0), and enzyme. After incubation at 37°C for 20 min, 800 μl of 2.5 N NaOH was added and phenylpyruvate was measured spectrophotometrically at 320 nm.

Arogenate Dehydratase. Reaction mixtures (150 μl) contained 50 mM potassium phosphate (pH 7.5), 1.0 mM arogenate, and enzyme. Formation of L-phenylalanine was determined by the methods of Shapiro et al. (1981).

Prephenate and Arogenate Dehydrogenase Assays. Prephenate dehydrogenase and arogenate dehydrogenase were assayed by the method of Byng et al. (1980). Reaction mixtures (200 μl) contained 0.5 mM NAD^+, 1.0 mM prephenate or 0.4 mM arogenate, 50 mM potassium phosphate buffer (pH 7.0), and enzyme. The continuous formation of NADH was followed using an Aminco-Bowman spectrophotofluorometer (excitation $\lambda = 340$ nm, emission $\lambda = 460$ nm). The change in fluorescence was compared with the fluorescence of known concentrations of authentic NADH.

Anthranilate Synthase. Anthranilate synthase was assayed according to Calhoun et al. (1973b). Reaction mixtures (400 μl) contained 1.0 mM $MgSO_4$, 20 mM glutamine, 1.0 mM chorismate, 50 mM potassium phosphate buffer (pH 7.0), and enzyme. The rate of anthranilate formation was followed continuously by

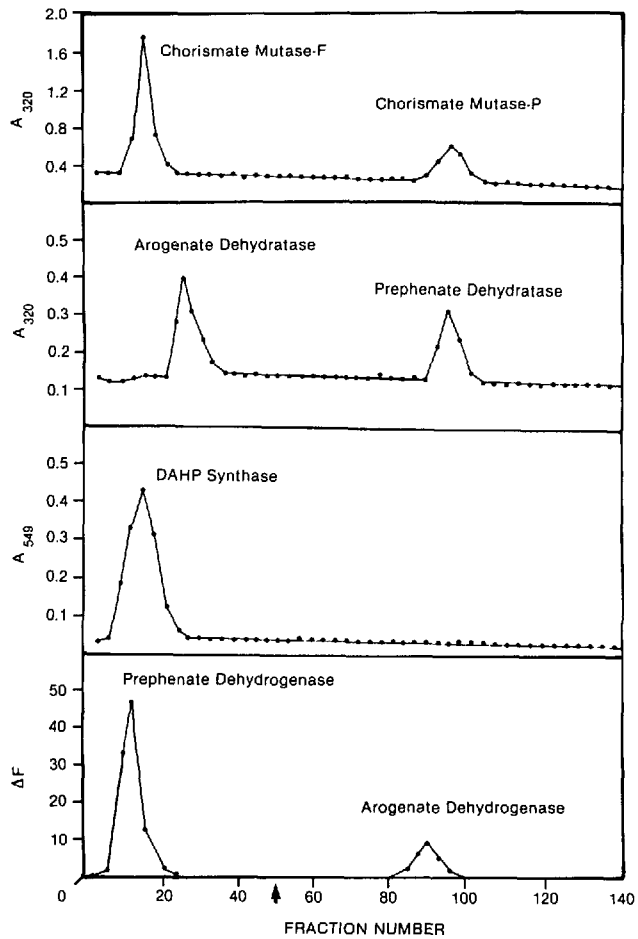

Fig. 2. DEAE-cellulose (DE-52) fractionation of key aromatic-pathway enzymes from crude extract. The arrow at fraction 50 indicates the beginning of the application of the linear gradient as described in Methods. Arogenate dehydratase and arogenate dehydrogenase profiles were initially recognized for convenience by their activities using prephenate as substrate. Table 1 lists the specific activities measured subsequently using L-arogenate as substrate. The multifunctional P-protein consists of chorismate mutase-P and prephenate dehydratase activities. The monofunctional chorismate mutase species is termed chorismate mutase-F

measuring its fluorescence with an Aminco-Bowman spectrophotofluorometer (excitation λ = 313 nm, emission λ = 393 nm). A standard curve was prepared using authentic anthranilate.

Protein. Protein concentration was estimated by the method of Bradford (1976) as described in *Bio-Rad Technical Bulletin* 1051.

Biochemicals

Amino acids, erythrose-4-phosphate, phosphoenolpyruvate, dithiothreitol, cellulose phosphate, NAD^+, NADH, and Sephadex G-25 were obtained from Sigma Chemical Company. DE-52 was obtained from Whatman, and hydroxylapatite was from BioRad. Prephenate was prepared as the barium salt from culture supernatants of a tyrosine auxotroph of *Salmonella typhimurium* (Dayan and Sprinson 1970) and converted to the potassium salt with excess K_2SO_4 prior to use. Arogenate was prepared from culture supernatants of a triple auxotroph of *Neurospora crassa* ATCC 36373 (Jensen et al. 1977). The isolation and purification were modified according to Zamir et al. (1980). Chorismate was isolated from the accumulation medium of the triple auxotroph *Klebsiella pneumoniae* 62-1 (ATCC 25306) and purified as the free acid (Gibson 1964). All chemicals were of the best grade commercially available.

Results

Chromatographic Separations

A crude extract of *X. campestris* was fractionated by chromatography over DEAE-cellulose. The elution positions of key enzymes are shown in Fig. 2. Two peaks of activity were recovered for chorismate mutase, prephenate dehydratase, and prephenate dehydrogenase. The peak of prephenate dehydratase that washed through the column without retardation proved capable of reaction with L-arogenate as substrate and was designated arogenate dehydratase by analogy with the enzyme of Group I pseudomonads (Whitaker et al. 1981b; Byng et al. 1983b). One of the two NAD^+-dependent prephenate dehydrogenase activities was also active with L-arogenate as substrate and was accordingly named arogenate dehydrogenase. The specific activities of the peak fractions are listed in Table 1.

Because chorismate mutase-F, DAHP synthase, and prephenate dehydrogenase activities coeluted in the wash eluate fractions (Fig. 2), the possible existence of several multifunctional proteins was considered. Figure 3 shows the results of hydroxylapatite chromatography. The smallest peak of chorismate mutase was exactly coincident with that of prephenate dehydratase, as after DEAE-cellulose fractionation (Fig. 2). Hence, this enzyme appears to be synonymous with the multifunctional P-protein found in enteric bacteria (Schmit and Zalkin 1969; Davidson et al. 1972) and in Group I pseudomonads (Calhoun et al. 1973a; Whitaker et al. 1981b). In one experiment the P-protein recovered from DEAE-cellulose was concentrated by Amicon PM-10 filtration and applied to a hydroxylapatite column, whereupon chorismate mutase-P and prephenate dehydratase activities eluted with exact coincidence.

The results of Fig. 2 also allowed the initial possibilities that a multi-functional T-protein (i.e., one possessing catalytic centers for both chorismate mutase and prephenate dehydrogenase) might exist, as in enteric bacteria (Cotton and Gibson 1967), or that a multifunctional protein carrying catalytic centers for DAHP synthase and chorismate mutase might exist, as in *Bacillus* species (Huang et al. 1974). These possibilities were ruled out because chorismate mutase-F (Fig. 3) separated from both DAHP synthase and prephenate dehydrogenase. This monofunctional chorismate mutase was designated

Table 1. Enzyme activities eluted from DEAE-cellulose

Protein	Reactions catalyzed	Specific activity[a]	Percentage of total activity[b]
Chorismate mutase-F	1. Chorismate mutase	1.68	69%
P-protein	1. Chorismate mutase-P	0.80	31%
	2. Prephenate dehydratase	0.64	43%
Arogenate dehydratase	2. Prephenate dehydratase	1.53	57%
	3. Arogenate dehydratase	0.25	100%
Prephenate dehydrogenase	4. Prephenate dehydrogenase	2.09	84%
Arogenate dehydrogenase	4. Prephenate dehydrogenase	0.76	16%
	5. Arogenate dehydrogenase	1.71	100%
DAHP synthase	6. DAHP synthase	4.96	100%
Anthranilate synthase[c]	7. Anthranilate synthase	1.63	100%

[a] Specific activities (peak fractions) are expressed as nmol/min/mg protein
[b] In three cases a reaction listed in column 2 is catalyzed by two of the proteins listed in column 1. The total activities recovered (and assayed at saturating substrate) equal 100% for each of the seven enzyme activities (column 2)
[c] Anthranilate synthase (amidotransferase) was assayed in crude extracts

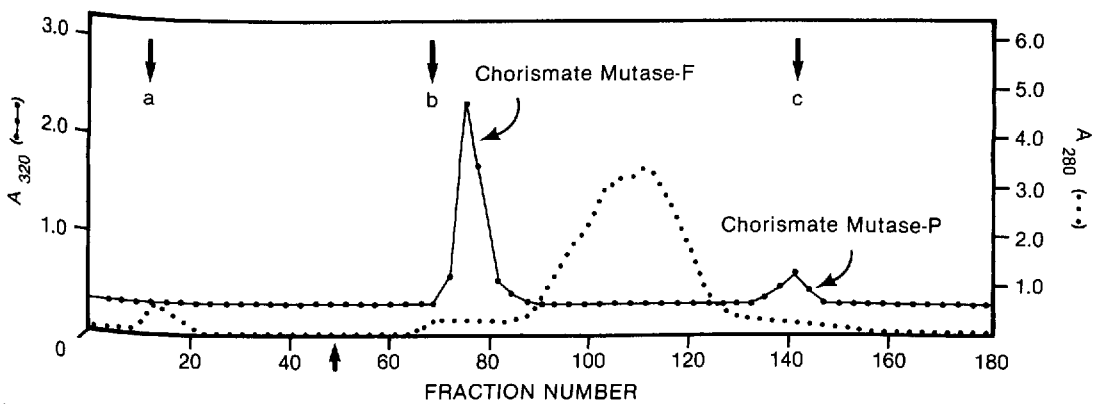

Fig. 3. Hydroxylapatite chromatography of key aromatic-pathway enzymes. Crude extract was applied to a column as described in Methods. Arrow at fraction 50 indicates the starting point for the potassium phosphate gradient. The peak positions of DAHP synthase (a), prephenate dehydrogenase (b), and prephenate dehydratase (c) are indicated by arrows showing their relationships to the elution positions of chorismate mutase-F and chorismate mutase-P

chorismate mutase-F by virtue of its similarity to the enzyme present in Subgroup Ib pseudomonads (Byng et al. 1983c).

Phenylalanine Branch

Chorismate mutase-P and prephenate dehydratase coeluted from both DEAE-cellulose and hydroxylapatite chromatography columns, consistent with properties of the P-protein present elsewhere within Superfamily B. Partially purified P-protein was fairly labile. Since prephenate dehydratase was slightly stimulated by L-tyrosine, we tested stabilization of enzyme activity in the presence of L-tyrosine. Both activities of the bifunctional P-protein were indeed stabilized when 1.0 mM L-tyrosine was added prior to extract preparation and then maintained during further fractionation protocols. Both activities were higher in the absence of dithiothreitol. In one experiment a sample of P-protein that had been partially purified (DEAE-cellulose) in the presence of 1.0 mM L-tyrosine was divided into two portions. One portion was dialyzed overnight to remove L-tyrosine. The dialyzed sample lost 44% of the original prephenate dehydratase activity and 21% of chorismate mutase-P activity. These activity losses were not restored by addition of L-tyrosine. However, the residual prephenate dehydratase activity retained its normal sensitivity to L-tyrosine-mediated activation (about 25%).

Inhibition studies of the P-protein were carried out in the presence of 0.4 mM L-tyrosine, a concentration adequate for maximal protective effects. Double reciprocal plots of substrate saturation data showed prephenate dehydratase to have a K_m(app) of 0.19 mM for prephenate, and chorismate mutase-P had a K_m(app) of 0.36 mM for chorismate. At substrate saturation, prephenate dehydratase was invulnerable to feedback inhibition. On the other

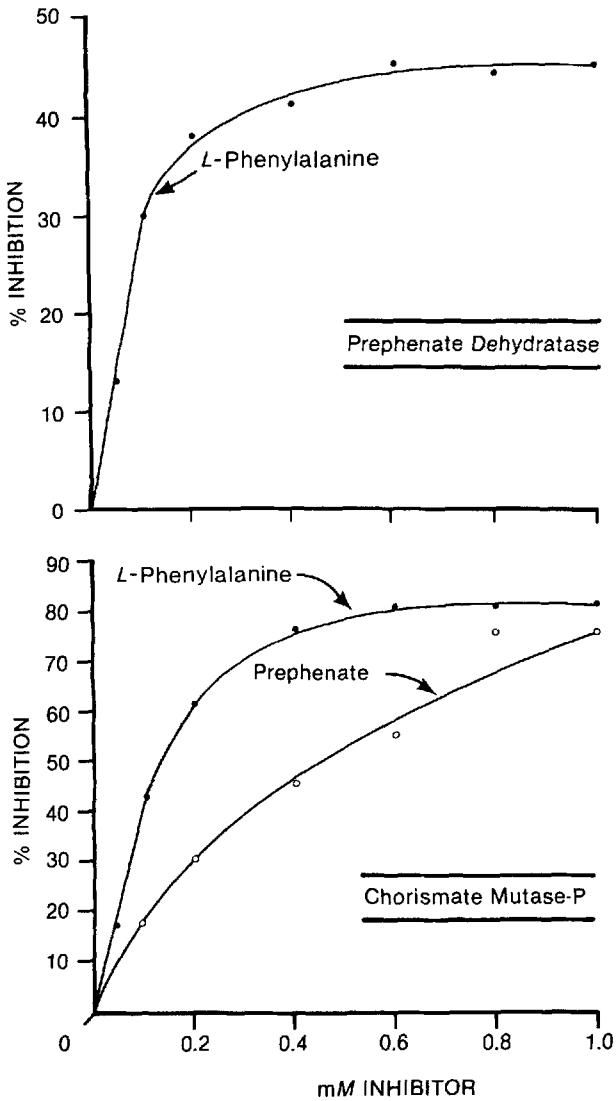

Fig. 4. Allosteric regulation of bifunctional P-protein activities by L-phenylalanine and/or prephenate. The P-protein was recovered from a DE-52 column (see Fig. 1). Substrate concentrations used for chorismate mutase-P (bottom) and prephenate dehydratase (top) were 0.35 mM and 0.20 mM, respectively

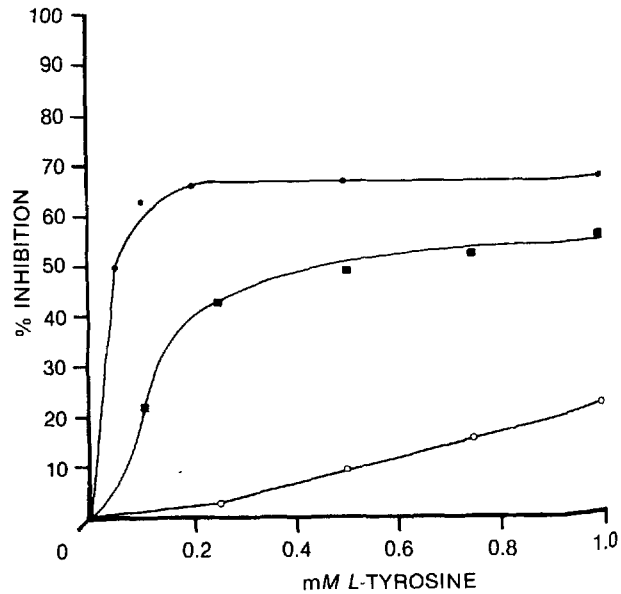

Fig. 5. Sensitivity of cyclohexadienyl dehydrogenases to feedback inhibition by L-tyrosine. Peak fractions of prephenate dehydrogenase and arogenate dehydrogenase that were separated by DE-52 chromatography were used for inhibition studies. The substrate concentrations of prephenate (PPA) and arogenate (AGN) were 1.0 mM and 0.10 mM, respectively. Inhibition of prephenate dehydrogenase is shown by filled circles. Inhibition of arogenate dehydrogenase was followed during catalysis with either of its substrates: L-arogenate (filled diamonds) or prephenate (open circles)

hand, chorismate mutase-P was inhibited 22% by 0.5 mM L-phenylalanine and 61% by 0.5 mM prephenate. Figure 4 shows inhibition curves obtained when substrate concentrations were fixed at about K_m levels. Under these conditions, significant sensitivity of prephenate dehydratase to feedback inhibition by L-phenylalanine became apparent. Chorismate mutase-P also exhibited dramatically greater sensitivity to feedback inhibition by L-phenylalanine at lower substrate levels. Sensitivity to inhibition by prephenate, on the other hand, was not affected significantly by substrate levels.

Arogenate dehydratase is active with prephenate or L-arogenate. The enzyme is insensitive to feedback inhibition by L-phenylalanine when assayed with prephenate. Technical limitations of the arogenate dehydratase assay (Shapiro et al. 1981) hinder the direct assessment of arogenate dehydratase activity in the presence of L-phenylalanine.

Tyrosine Branch

Two NAD^+-dependent dehydrogenases were cleanly separated following DEAE-cellulose chromatography. One enzyme (prephenate dehydrogenase) was specific for prephenate, whereas the second (arogenate dehydrogenase) was reactive with both prephenate and L-arogenate. The latter was more active on L-arogenate (Table 1). Neither dehydrogenase was able to utilize $NADP^+$ at all in satisfaction of the cofactor requirement. Substrate saturation data for prephenate dehydrogenase were plotted in double reciprocal form, yielding a K_m(app) of 0.09 mM for prephenate and a K_m(app) of 0.20 mM for NAD. Arogenate dehydrogenase was contaminated with an oxidase, which prevented valid kinetic determinations.

Prephenate dehydrogenase was more sensitive than arogenate dehydrogenase to feedback inhibition by L-tyrosine by an order of magnitude (Fig. 5). A 50% level of inhibition for prephenate dehydrogenase was obtained at a concentration of 0.05

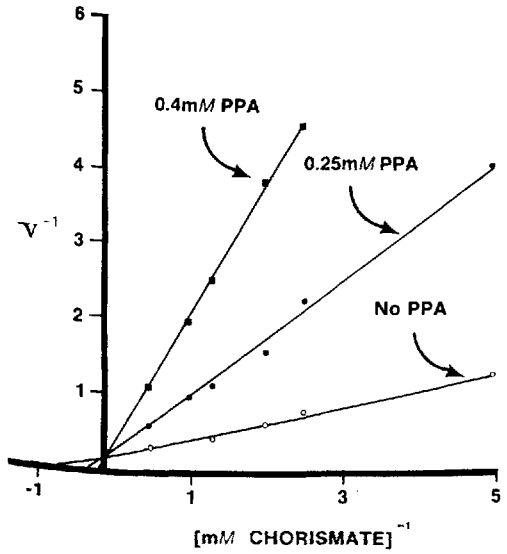

Fig. 6. Double reciprocal plot of chorismate mutase-F initial velocities as a function of substrate concentration in the presence of several fixed concentrations of prephenate (PPA). An ordinate value of 1.0 equals a velocity of 1.0 μmol produced/min/mg protein

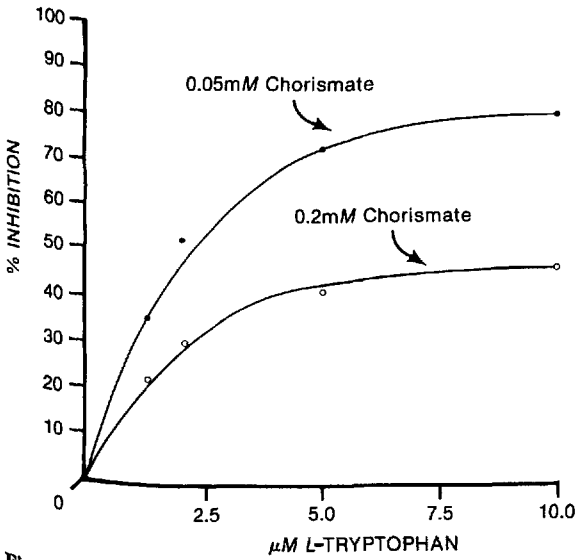

Fig. 7. Sensitivity of anthranilate synthase (amidotransferase) to feedback inhibition by L-tryptophan. Initial rates of enzyme activity were determined in the presence of 20 mM glutamine and either 0.05 mM or 0.20 mM chorismate as indicated. The source of enzyme was unfractionated crude extract

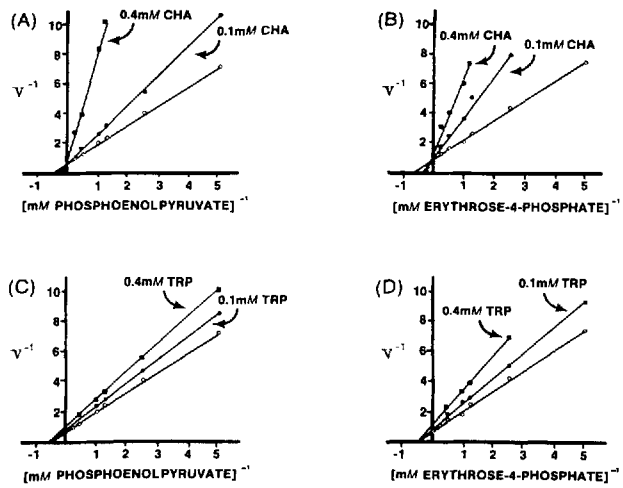

Fig. 8A–D. Double reciprocal plots of DAHP synthase velocities as a function of **A,C** phosphoenolpyruvate or **B,D** erythrose-4-phosphate as the variable substrate. The fixed substrate was present at 2.0 mM, while the variable substrate concentration ranged between 0.20 and 3.0 mM. Two concentrations of L-tryptophan (TRP) or of chorismate (CHA) were used for analysis of inhibition kinetics. DAHP synthase was recovered from DEAE-cellulose as shown in Fig. 2

mM L-tyrosine, whereas 0.50 mM L-tyrosine was required to inhibit arogenate dehydrogenase by 50%. When arogenate dehydrogenase was assayed for sensitivity to inhibition using prephenate as substrate, an even more reduced (by about tenfold) sensitivity was observed.

Since chorismate mutase-P channels most or all molecules of prephenate toward L-phenylalanine by virtue of its association with prephenate dehydratase, it is reasonable to assume that chorismate mutase-F is the primary source of prephenate molecules that support L-tyrosine biosynthesis. It also would generate prephenate molecules for whatever fraction of L-phenylalanine is formed via L-arogenate. Partially purified chorismate mutase-F was stable, and the double reciprocal plot shown in Fig. 6 yielded a K_m(app) of 1.67 mM for chorismate. Like chorismate mutase-P (Fig. 4), chorismate mutase-F was inhibited by prephenate (Fig. 6), with a K_{is} value of 0.12 mM calculated. L-arogenate, L-phenylalanine, L-tyrosine, and L-tyrptophan were tested as possible allosteric effectors. No inhibitory or activating effects were found, regardless of whether the compounds were present individually or in combination.

Tryptophan Branch

Anthranilate synthase was chosen for examination because of its usual key role as a regulatory enzyme. This activity proved to be completely labile following column chromatography procedures employed in this study. However, anthranilate synthase was quite stable in crude extracts (Table 1). When assayed with glutamine as amino-donor substrate (amidotransferase), the specific activity was about 3.5-fold greater than when ammonia served as the amino-donor substrate (aminase). Figure 7 shows that anthranilate synthase was sensitive to feedback inhibition in the presence of micromolar levels of L-tryptophan. The increased effectiveness of L-tryptophan as a feedback inhibitor when chorismate

Table 2. Chorismate and L-tryptophan as inhibitors of DAHP synthase

Inhibitor	Substrate	K_i[a]	Type of inhibition[b]
Chorismate	Phosphoenolpyruvate	0.40 mM	Competitive
	Erythrose-4-phosphate	0.10 mM	Competitive
L-Tryptophan	Phosphoenolpyruvate	0.44 mM	Noncompetitive
	Erythrose-4-phosphate	0.58 mM	Noncompetitive

[a] K_i values were determined as K_{is} by plotting the slope $K_m(app)/V_m(app)$(slope) against the concentration of inhibitor. In the case of noncompetitive inhibition, K_{is} values shown agreed well with K_{ii} values obtained by plotting $V_m(app)$ reciprocals against inhibitor concentrations

[b] Determined from double reciprocal plots (Fig. 8)

levels were lowered indicates a competitive mode of inhibition.

Early-Pathway Regulation

The activity of DAHP synthase in crude extracts was found to be feedback inhibited by either chorismate or L-tryptophan, with 0.5 mM concentrations of these effectors producing 96% and 70% inhibition, respectively. Other potential effectors of DAHP synthase activity, such as L-tyrosine, L-phenylalanine, phenylpyruvate, and prephenate, were ineffective. DAHP synthase activity recovered from DEAE-cellulose was stable and retained the allosteric sensitivities noted in crude extracts. Activity in the latter preparations was not activated by divalent cations, confirming previous results obtained with crude extracts (Whitaker et al. 1981a); this feature distinguishes *Xanthomonas* DAHP synthase from that of all other pseudomonad groups (Whitaker et al. 1981a). Substrate saturation curves were generated and the data plotted in double reciprocal form (Fig. 8). The $K_m(app)$ values for erythrose-4-phosphate and phosphoenolpyruvate were 1.67 mM and 2.0 mM, respectively. Similar results were obtained from a partially purified fraction of DAHP synthase recovered from a cellulose phosphate column (specific activity = 5.46 nmol/min/mg protein). In this experiment DAHP synthase separated completely from both of the chorismate mutase activities. Inhibition by chorismate was competitive against either substrate (Fig. 8A and B), whereas L-tryptophan inhibited with non-competitive kinetics when either erythrose-4-phosphate or phosphoenolpyruvate was the variable substrate (Fig. 8C and D). An inspection of the inhibitor constants (K_i) listed in Table 2 shows chorismate to be the more potent inhibitor on a molar basis.

Discussion

Biochemical Arrangement of Aromatic Biosynthesis in Xanthomonas

Dual flow routes to L-phenylalanine and to L-tyrosine exist in *X. campestris*. The phenylpyruvate route to L-phenylalanine employs the bifunctional P-protein, which catalyzes the reactions of both chorismate mutase and prephenate dehydratase. The P-protein is relatively ancient and has been found throughout Superfamily B (Jensen 1985). Figure 9 presents a comparison of the enzyme arrangements found in some of the major lineages of Superfamily B. *X. campestris* lacks the fused T-protein present in enterics, and prephenate dehydrogenase and chorismate mutase-F are separable proteins. As in Subgroup Ib pseudomonads, *X. campestris* is able to form L-phenylalanine and L-tyrosine from L-arogenate by utilizing arogenate dehydratase and arogenate dehydrogenase, respectively. Arogenate dehydrogenase and arogenate dehydratase exhibit substrate ambiguity, both being capable of reaction with prephenate as an alternative substrate. Arogenate dehydrogenase is more active with arogenate, whereas arogenate dehydratase is more active with prephenate. Although a variety of reasons have been advanced in support of the idea that arogenate dehydrogenase and prephenate dehydrogenase are separate gene products in *Pseudomonas aeruginosa* (Patel et al. 1978), *X. campestris* provides the first rigorous evidence for separate dehydrogenase enzymes in Superfamily B. Within Superfamily B, no variations in the steps of tryptophan biosynthesis are known.

The Group V pseudomonad lineage that contains *X. campestris* is the only Superfamily B section that lacks isozyme forms of DAHP synthase. The *X. campestris* DAHP synthase is called DAHP synthase-trp because it is sensitive to inhibition by L-tryptophan as well as by chorismate. It is noteworthy that DAHP synthase-trp of *P. aeruginosa* exhibits a minor sensitivity to inhibition by chorismate (Whitaker et al. 1982). In contrast, *Escherichia coli* DAHP synthase-trp totally lacks sensitivity to inhibition by chorismate (Whitaker, unpublished data).

Sequential Feedback Inhibition

The pattern of allosteric control whereby an early branchpoint enzyme is feedback inhibited by an intermediate that accumulates because of inhibition

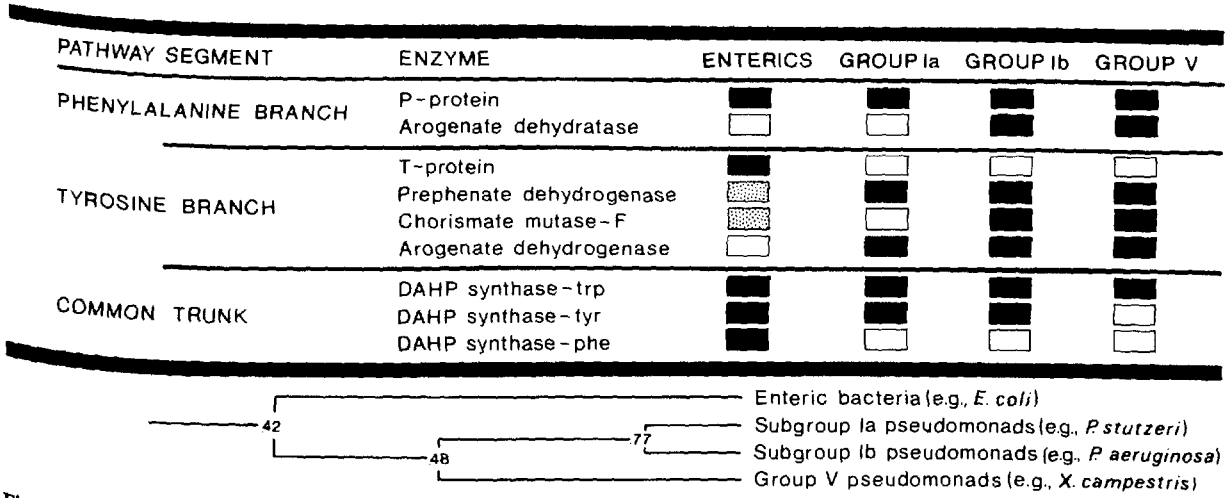

Fig. 9. Profile of aromatic biosynthesis in the *X. campestris* lineage in relationship to other major branches of Superfamily B. Shaded and open boxes indicate the presence or absence, respectively, of the indicated enzyme. The stipled boxes shown under "ENTERICS" indicate that prephenate dehydrogenase and chorismate mutase-F activities are present only as components of the multifunctional T-protein, the latter presumably having arisen by fusion of genes originally specifying chorismate mutase-F and prephenate dehydrogenase. A simplified dendrogram of major Superfamily B branches is shown at the bottom of the figure. Numbers given at the branch points are S_{AB} values established by oligonucleotide cataloging (Fox et al. 1980; Stackebrandt and Woese 1981). Information obtained from Group V pseudomonads has played an instrumental role in determining that chorismate mutase-F and arogenate dehydratase were lost relatively recently in the pseudomonad Subgroup Ia lineage (Byng et al. 1983c) and that DAHP synthase-trp and DAHP synthase-tyr were present in an early Superfamily B ancestor (Byng et al. 1983a)

imposed within a second feedback loop is called sequential feedback inhibition. This pattern, first shown for aromatic biosynthesis in *Bacillus subtilis* (Jensen and Nester 1965; Nester and Jensen 1966), depends on end-product regulation of each of the three terminal branches. This promotes the secondary backup of midpathway metabolites, which in turn feedback inhibit the initial pathway enzyme. In *B. subtilis* a single gene specifies both DAHP synthase and chorismate mutase (Huang et al. 1974). The catalytic site for chorismate mutase is identical with the allosteric site for DAHP synthase (which is very sensitive to inhibition by prephenate and less sensitive to inhibition by chorismate). This synonymy of sites was deduced from results obtained through the use of selective trypsin digestion (Huang et al. 1974). This multifunctional protein thus provides an effective mechanism by which an intermediary metabolite, normally expected to have a relatively small range of concentration variation, can function as an allosteric effector. In *B. subtilis*, prephenate levels indirectly reflect tyrosine and phenylalanine levels, and intracellular chorismate is thought to be roughly proportional to the level of tryptophan. The sequential pattern of DAHP synthase control by prephenate and chorismate is a conservative feature of *Bacillus* species (Jensen et al. 1967; Jensen 1970; Jensen and Rebello 1970) and has also been found in *Staphylococcus* (Jensen et al. 1967). In *X. campestris* it is possible that anthranilate synthase and DAHP synthase exist as a multifunctional protein. If so, then the allosteric sites for chorismate and L-tryptophan would be sites conferred by the anthranilate synthase moiety. The pursuit of this possibility awaits the successful stabilization of anthranilate synthase activity during partial purification.

The mechanism of sequential feedback inhibition in *X. campestris* demands that physiological conditions of end-product excess trigger elevated levels of intracellular chorismate, the most effective feedback inhibitor of DAHP synthase. The allosteric control specificities cited in this paper do indeed appear to be oriented to accomplish chorismate backup under conditions of end-product excess (see Fig. 1). Inhibition of chorismate mutase-P by L-phenylalanine or of anthranilate synthase by L-tryptophan would accomplish this directly. Although inhibition of prephenate dehydrogenase by L-tyrosine and of prephenate dehydratase by L-phenylalanine lead directly to prephenate accumulation, both chorismate mutase-P and chorismate mutase-F are in turn sensitive to inhibition by prephenate.

DAHP Synthase-trp in Relationship to Evolution of Overall Pathway Control

An interesting evolutionary question is whether the ancestral enzyme utilized tryptophan or chorismate as the primary signal of control. We suggest for two reasons that the chorismate sensitivity of *X. campestris* DAHP synthase-trp is a relatively recent allostery that emerged from an ancestor having DAHP synthase isozymes similar to those of contemporary

P. aeruginosa. First, given the present isozyme arrangements in Superfamily B lineages that have been ordered on the phylogenetic tree, it is most probable that *X. campestris* diverged from a lineage possessing DAHP synthase-tyr in addition to DAHP synthase-trp (Jensen 1985). Second, a case has been made that early-pathway regulation (i.e., at the level of DAHP synthase) evolved prior to late-pathway regulation of the phenylalanine and tyrosine branches because (i) early-pathway regulation is intuitively the most efficient single evolutionary change that could occur, and (ii) in cases where overall allosteric regulation of the aromatic pathway is weakly developed, early-pathway regulation exists whereas late-pathway regulation does not (Jensen and Hall 1983).

One can envision that the initial chorismate sensitivity of the allosteric site may have been the fortuitous result of origin of the tryptophan-binding site from the anthranilate synthase gene (by gene fusion). At this time direct end-product control of DAHP synthase-trp by tryptophan conferred a selective advantage, as did the analogous direct end-product control exerted by L-tyrosine on DAHP synthase-tyr. Following establishment of early-pathway control, the several late-pathway controls then evolved. Once the late-pathway loops of feedback inhibition became established, regulation of a single DAHP synthase by chorismate became feasible for the first time. If DAHP synthase-trp retained the original chorismate-binding site (perhaps similar to that still retained by *P. aeruginosa*), then sensitivity to chorismate inhibition may have become accentuated in conjunction with the loss of DAHP synthase-tyr.

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