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Pseudomonas aeruginosa Possesses Two Novel Regulatory Isozymes of 3-Deoxy-D-*arabino*-heptulosonate 7-Phosphate Synthase*

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In *Pseudomonas aeruginosa* the initial enzyme of aromatic amino acid biosynthesis, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase, has been known to be subject to feedback inhibition by a metabolite in each of the three major pathway branchlets. Thus, an apparently balanced multieffector control is mediated by L-tyrosine, by L-tryptophan, and by phenylpyruvate. We have now resolved DAHP synthase into two distinctive regulatory isozymes, herein denoted DAHP synthase-tyr (M_r = 137,000) and DAHP synthase-trp (M_r = 175,000). DAHP synthase-tyr comprises >90% of the total activity. L-Tyrosine was found to be a potent effector, inhibiting competitively with respect to both phosphoenolpyruvate (K_i = 23 μM) and erythrose 4-phosphate (K_i = 23 μM). Phenylpyruvate was a less effective competitive inhibitor: phosphoenolpyruvate (K_i = 2.55 mM) and erythrose 4-phosphate (K_i = 1.35 mM). DAHP synthase-trp was found to be inhibited noncompetitively by L-tryptophan with respect to phosphoenolpyruvate (K_i = 40 μM) and competitively with respect to erythrose 4-phosphate (K_i = 5 μM). Chorismate was a relatively weak competitive inhibitor: phosphoenolpyruvate (K_i = 1.35 mM) and erythrose 4-phosphate (K_i = 2.25 mM). Thus, each isozyme is strongly inhibited by an amino acid end product and weakly inhibited by an intermediary metabolite.

The allosteric enzyme, DAHP[1] synthase, catalyzes the initial common reaction that is committed to the biosynthesis of aromatic compounds, the major ones being L-phenylalanine, L-tyrosine, and L-tryptophan. The control patterns for DAHP synthase which exist in nature to regulate the multibranched pathway of aromatic biosynthesis have been recognized for some time for the diversity of alternative mechanisms (1). More recently, these variations have become better understood in a context of appreciation that the catalytic steps of phenylalanine and tyrosine biosynthesis are also diverse in nature (2). Allosteric control of DAHP synthase by single end product effectors is common and has recently been interpreted (3) as reflective of those organisms in which exogenous amino acids are unimportant. The need for in-depth studies is illustrated by the fact that DAHP synthase of *Pseudomonas aeruginosa* was originally classified as such a unimetabolite-controlled enzyme (4). In cases where exogenous amino acids play a significant role in the nutrition and physiology of the organism (3), multieffector patterns of regulation are known. These include isozymic feedback inhibition, sequential feedback inhibition, cumulative feedback inhibition, and concerted feedback inhibition (1, 2, 4).

Since 1973 (5) the DAHP synthase activity of *P. aeruginosa* has been known to be feedback inhibited by L-tryptophan and by phenylpyruvate in addition to the earlier known (4) feedback inhibition by L-tyrosine. This pattern comprised a novel, but apparently balanced allosteric pattern, in which a single metabolite within each terminal branchlet was an allosteric effector of DAHP synthase. It is interesting that with this report of two isozymic DAHP synthases in *P. aeruginosa*, the feedback pattern can be regarded as not only isozymic but as cumulative and sequential as well. Each regulatory isozyme is controlled by two effectors which inhibit cumulatively in combination, and each isozyme is regulated by an intermediary metabolite.

MATERIALS AND METHODS

Microbiological Aspects—Strain 1 of *P. aeruginosa*, originally obtained from B. W. Holloway (6), has been subcultured in our laboratories for about 18 years. Cultures were grown at 37 °C as previously described (7) in a minimal salts/glucose medium (8) and harvested during the late exponential phase of growth by centrifugation. For adequate amounts of starting enzyme prior to partial purification, 16-liter batches of cells were cultured in 20-liter carboys, continuously sparged with humidified air. Stock cultures were maintained as stab cultures (9). A tryptophan auxotroph (*trpA1*) deficient in anthranilate synthase (10), and a 4-fluorotryptophan-resistant mutant (4FT1) that excretes tryptophan due to the constitutive formation of anthranilate synthase, 5-phosphorylribose-1-pyrophosphate phosphoribosyl transferase, and indolglycerol-phosphate synthase (10) were obtained from D. H. Calhoun (Mount Sinai School of Medicine, New York, NY).

Preparation of Crude Extracts—Whole cell pellets were suspended in 50 mM potassium phosphate buffer (pH 7.0) containing 1.0 mM dithiothreitol and disrupted by sonication, using a Lab-Line Ultra-tip sonicator. Centrifugation at 150,000 × *g* was carried out to remove cell debris, and the extract was passed through a Sephadex G-25 column (1.5 × 20 cm) equilibrated in starting buffer to remove small molecules that might interfere with enzyme assays.

Analytical Procedures—DAHP synthase assays were carried out according to the method of Calhoun *et al.* (11). In crude extracts, as well as partially purified samples of each isozyme, conditions of proportionality with respect to both time and protein concentration were observed. When compounds under test as inhibitors were used, appropriate blanks were included to insure against interference of the chemical assay by effector molecules (11). Optimal DAHP synthase activity commonly requires the addition of divalent metal ions (12). Stock solutions of cobalt sulfate, magnesium sulfate, and manganese sulfate prepared in 50 mM potassium phosphate buffer (pH 7.0) were added to reaction mixtures to a final concentration of 1.0 mM to test for optimal activity in each enzyme sample (crude extract and partially purified samples of DAHP synthase-tyr and DAHP synthase-trp). The appropriate metal ion was then included in subsequent assays. A typical reaction mixture (200 μl) contained 50 mM potassium

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[1] The abbreviation used is: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate.

phosphate buffer (pH 7.0), 1.0 mM erythrose 4-phosphate, 1.0 mM phosphoenolpyruvate, 1.0 mM metal ion, and enzyme. All reactions were carried out at 37 °C and the absorbance measured at 549 nm. The concentration of DAHP was estimated using a molar extinction coefficient of 45,000 (5).

Anthranilate synthase was assayed by the method of Calhoun *et al.* (10). A 400-μl reaction mixture contained 50 mM potassium phosphate (pH 7.0), 10 mM $MgSO_4$, 20 mM glutamine, 2 mM chorismate, and enzyme. When aminase activity was assayed, 20 mM NH_4Cl replaced glutamine in the reaction mixture. The rate of anthranilate formation (at 37 °C) was followed continuously by measuring fluorescence (excitation wavelength of 313 nm; emission wavelength of 393 nm) using an Aminco-Bowman spectrophotofluorometer. A standard curve relating known concentrations of authentic anthranilate to fluorescence was used.

Protein concentration was determined by the method of Bradford (13).

DEAE-cellulose Chromatography—A crude extract containing 440 mg of protein was applied at 4 °C to a DE52 column (2.5 × 20 cm) equilibrated in 50 mM potassium phosphate buffer (pH 7.0) and 1.0 mM dithiothreitol. The protein was washed on the column with 300 ml of buffer. The wash effluent containing unbound protein was collected in 6.0-ml fractions. The column was eluted with a linear salt gradient of KCl, one reservoir containing 450 ml of potassium phosphate buffer and the other 450 ml of potassium phosphate buffer plus 0.5 M KCl. Fractions of 6.0 ml were collected.

Gel Filtration—The DAHP synthase isozyme bands recovered from DEAE-cellulose were pooled and concentrated by Amicon PM-10 filtration. These concentrates were applied separately to an Ultrogel AcA34 gel filtration column (2.5 × 60 cm) equilibrated in 50 mM potassium phosphate buffer (pH 7.0) containing 1.0 mM dithiothreitol. The column was calibrated for molecular weight determination with the following molecular weight standards: chymotrypsin (225,000), catalase (210,000), aldolase (158,000), albumin (67,000), and ovalbumin (45,000). The void volume was determined with blue dextran. Fractions were collected at 4 °C.

Biochemicals—Protein standards for molecular weight determination were obtained from Pharmacia Fine Chemicals. Amino acids, erythrose 4-phosphate, phenylpyruvate, phosphoenolpyruvate, dithiothreitol, and Sephadex G-25 were obtained from Sigma. DEAE-cellulose (DE52) was obtained from Whatman. Ultrogel AcA34 was obtained from LKB. Chorismate was isolated from the accumulation medium of a triple auxotroph of *Klebsiella pneumoniae* 62-1 and purified as the free acid (14). All chemicals were of the best grade commercially available.

RESULTS

Resolution of Two DAHP Synthase Isozymes—When a crude extract of wild type *P. aeruginosa* was fractionated by ion exchange chromatography on DEAE-cellulose, the majority of the activity (94.2%) for DAHP synthase passed into the wash eluate without retardation (left of the *dotted line* in Fig. 1). Unexpectedly, about 6% of the activity eluted in the gradient. Samples from each peak of activity were tested for sensitivity to feedback inhibition by L-tyrosine, L-tryptophan, and phenylpyruvate, previously characterized as allosteric effectors of DAHP synthase when assayed in unfractionated extracts (10). The major, leftward band of activity was sensitive to inhibition by L-tyrosine and by phenylpyruvate, but not by L-tryptophan. On the other hand, the minor band of DAHP synthase was sensitive to inhibition by L-tryptophan, but not to inhibition by either L-tyrosine or phenylpyruvate. Thus, it was qualitatively apparent that regulatory isozymes had been separated.

Fractions comprising each of the isozyme bands were pooled, concentrated by ultrafiltration, and applied to a gel filtration column as described under "Materials and Methods." Each sample eluted as a symmetrical peak in a different molecular weight position. The major isozyme, denoted as DAHP synthase-tyr, had a calculated molecular weight of 137,000. The minor isozyme, denoted as DAHP synthase-trp, had a calculated molecular weight of 175,000.

The data given in Table I show that inhibition data from

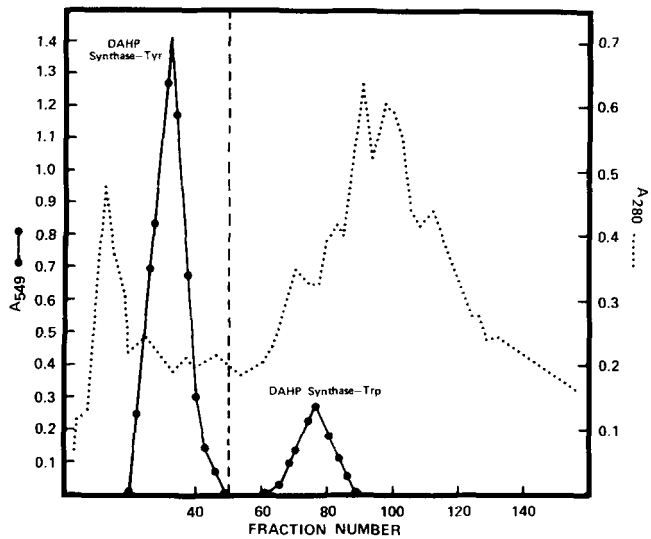

FIG. 1. **Separation of DAHP synthase-tyr and DAHP synthase-trp following DEAE-cellulose chromatography.** A 440-mg amount of crude extract protein was applied to a DE52 column as described under "Materials and Methods." DAHP synthase-tyr (838 nmol/min) was resolved as the major fraction of the activity applied while DAHP synthase-trp (51 nmol/min) represented only about 6% of total DAHP synthase activity. The *vertical dotted line* marks the boundary between the end of the wash eluate and the beginning of the salt gradient eluate.

crude, unfractionated extract fit reasonably well with expectations for a mixture of isozymes having the individual allosteric sensitivities shown. In addition, a new allosteric specificity, that of DAHP synthase-trp for chorismate, was identified. Note that inhibition by chorismate is hardly apparent in crude extracts. In each of the three preparations (Table I), DAHP synthase activity was stimulated in the presence of a metal ion. Surprisingly, while each of the partially purified isozymes was activated best by cobalt, crude extracts were activated best by manganese.

Characterization of DAHP Synthase-tyr—The enzymological characterization of this isozyme was done with preparations partially purified as in Fig. 1. A pH optimum of 6.8 was determined, and all characterizations were done at this pH. A 1.0 mM concentration of cobalt stimulated activity 4-fold at saturating substrate concentrations. Under the same conditions manganese stimulated activity 3-fold. The apparent substrate affinities ($K_{m\ app}$) of DAHP synthase-tyr for either of the two substrates was estimated to be 1.0 mM (see Fig. 2) from double reciprocal plots. Inhibition by L-tyrosine was found to be competitive (Fig. 2*a*) with respect to phosphoenolpyruvate (K_i = 23 μM) and also competitive (Fig. 2*d*) with respect to erythrose 4-phosphate (K_i = 23 μM). Phenylpyruvate inhibited competitively (Fig. 2*b*) with respect to phosphoenlopyruvate (K_i = 2.55 mM) and competitively (Fig. 2*e*) with respect to erythrose 4-phosphate (K_i = 1.35 mM). The inhibition curves obtained at saturating substrate concentrations show (Fig. 2*c*) that L-tyrosine is a more potent inhibitor of DAHP synthase-tyr than is phenylpyruvate by at least an order of magnitude.

Characterization of DAHP Synthase-trp—DEAE-cellulose fractionated isozyme recovered as shown in Fig. 1 was used to study properties of DAHP synthase-trp. All characterizations were done at the pH optimum, which was found to be 7.2. Cobalt stimulation was relatively modest (1.8-fold) at substrate saturation, and other metal ions including manganese had little effect upon activity. The apparent substrate affinities of DAHP synthase-trp for phosphoenolpyruvate (1.11 mM) and erythrose 4-phosphate (0.56 mM) were found as the

TABLE I

Comparison of DAHP synthase activity in crude extract and partially purified isozyme preparations

Enzyme preparation[a]	Specific activity[b]	Metal ion activation[c]	Inhibition by effectors[d] (%)							
			PHE	PPY	TYR	TRP	ARO	CHA	TYR + PPY	CHA + TRP
Crude extract	8.77	Mn^{2+}	0	24	95	10	97	3		
DAHP synthase-tyr	55.90	$Co^{2+} > Mn^{2+}$	0	25	95	3	97	0	99	
DAHP synthase-trp	4.86	Co^{2+}	0	0	0	70	64	40		81

[a] Crude extract was prepared as described under "Materials and Methods." Preparations of DAHP synthase-tyr and DAHP synthase-trp were obtained after DE52 chromatography (see Fig. 1).

[b] Specific activities for each enzyme preparation are expressed as nanomoles of DAHP/min/mg of protein.

[c] Enzyme activity was measured in the presence and absence of various metal ions to determine optimal activity. All subsequent assays were performed with the metal ion producing maximum activity.

[d] Assays were performed with a final concentration of erythrose 4-phosphate and phosphoenolpyruvate at 1.0 mM. The final concentration of each effector was 0.5 mM. PHE, L-phenylalanine; PPY, phenylpyruvate; TYR, L-tyrosine; TRP, L-tryptophan; ARO, L-phenylalanine + L-tyrosine + L-tryptophan; CHA, chorismate.

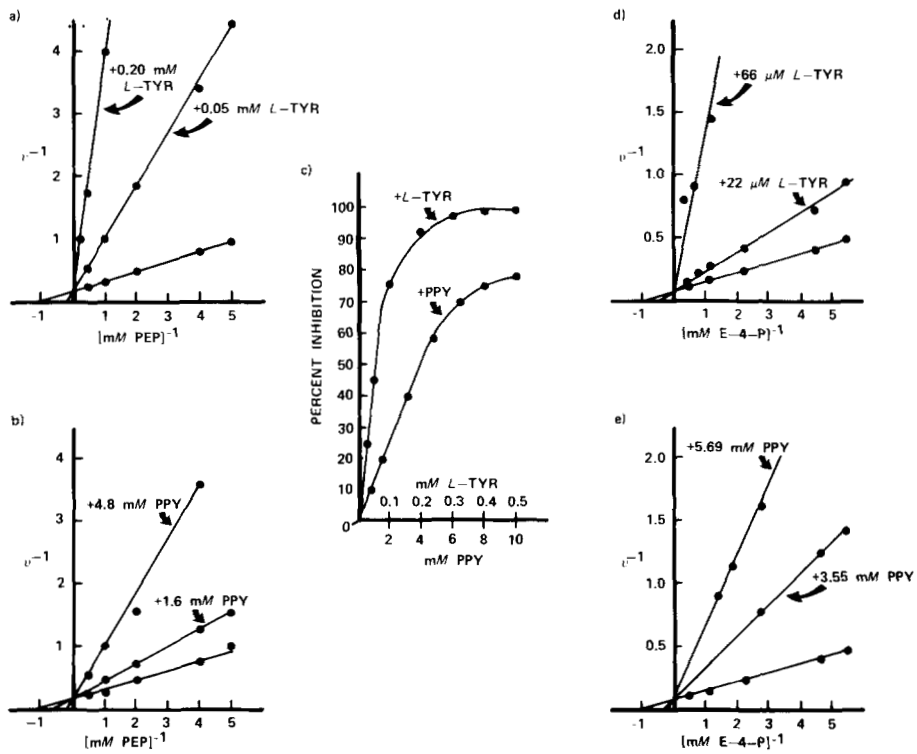

FIG. 2. **Characterization of DAHP synthase-tyr.** This major isozyme species was recovered from DAHP synthase-trp by DEAE-cellulose chromatography (Fig. 1) and concentrated to a protein concentration of 3.0 mg/ml (specific activity: 55.2 nmol/min/mg). All assays in this kinetic analysis were carried out with an enzyme concentration of 30 μg/ml, which was well within the linear portion of an activity *versus* protein curve. Each reaction mixture contained 1.0 mM Co^{2+} and was buffered to pH 6.8 with 200 mM potassium phosphate to insure maximal activity (see Table II). Double reciprocal plots of substrate saturation curves obtained with phosphoenolpyruvate (*PEP*) as the variable substrate and erythrose 4-phosphate (*E-4-P*) fixed at 2.0 mM were constructed (*a* and *b*). Substrate saturation curves were also done in the presence of the L-tyrosine (*L-TYR*) concentrations indicated in (a), or of the phenylpyruvate (*PPY*) concentrations indicated in (*b*). (*c*) shows inhibition curves obtained with L-*TYR* or with *PPY* when both *E-4-P* and *PEP* were present at 1 mM concentration. Substrate saturation curves were also obtained with *E-4-P* as the variable substrate and *PEP* concentration fixed at 2.0 mM. The data obtained in the presence or absence of L-*TYR* or of *PPY* were plotted in double reciprocal form as shown in (*d*) and (*e*), respectively.

appropriate extrapolation values taken from double reciprocal plots (Fig. 3). Inhibition by L-tryptophan was found to be noncompetitive (Fig. 3*a*) with respect to phosphoenolpyruvate (K_i = 40 μM) and competitive (Fig. 3*d*) with respect to erythrose 4-phosphate (K_i = 5 μM). Chorismate inhibited competitively (Fig. 3*b*) with respect to phosphoenolpyruvate (K_i = 1.35 mM) and competitively (Fig. 3*e*) with respect to erythrose 4-phosphate (K_i = 2.25 mM). The inhibition curve obtained at saturating substrate concentrations showed (Fig. 3*c*) that L-tryptophan is a more potent inhibitor of DAHP synthase-trp than is chorismate by an order of magnitude. Thus, 0.46 mM chorismate was required for 50% inhibition compared to only 0.04 mM for L-tryptophan.

The foregoing results are compiled as a summary table to facilitate a comparison of the two regulatory isozymes (Table II).

Synonymy of DAHP Synthase-trp and Anthranilate Synthase?—Since the aminase subunit of anthranilate synthase binds both chorismate (substrate) and L-tryptophan (feedback inhibitor), we considered the possibility that DAHP synthase-trp might consist of a catalytic subunit aggregated with a regulatory subunit, the latter also being the catalytic subunit of anthranilate synthase. (A multifunctional anthranilate synthase/DAHP synthase-trp seemed ruled out *a priori* because of molecular weight data: anthranilate synthase aminase, M_r = 64,000 (15) compared to DAHP synthase-trp, M_r = 175,000.)

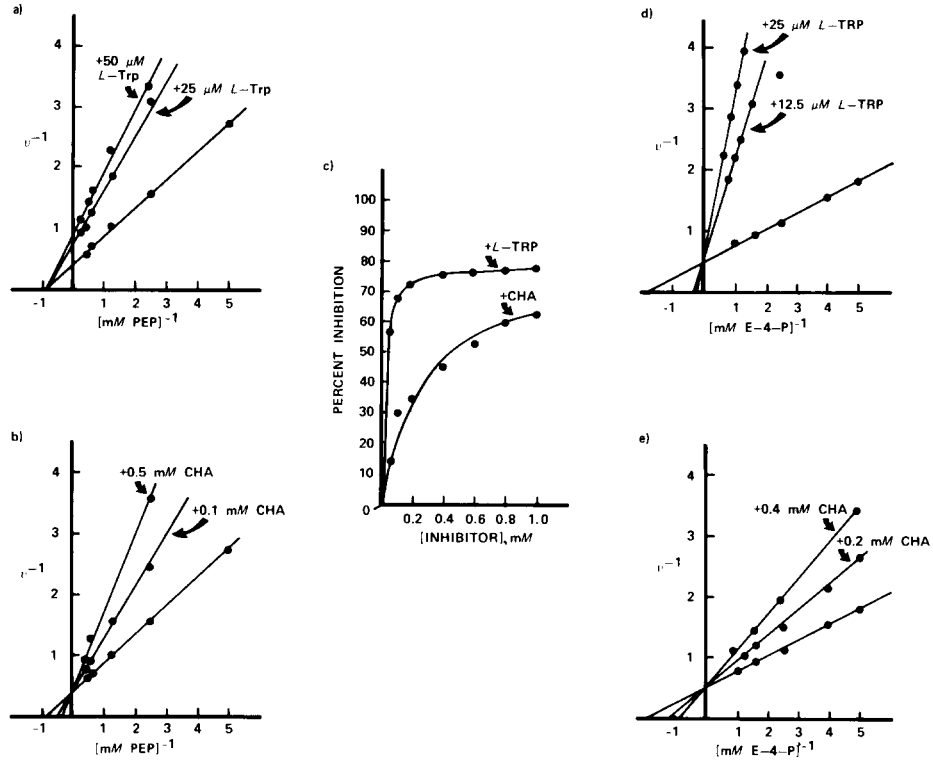

FIG. 3. **Characterization of DAHP synthase-trp.** This minor isozyme species was obtained from DEAE-cellulose chromatography (Fig. 2), and concentrated to a protein concentration of 3.3 mg/ml (specific activity: 4.86 nmol/min/mg). All assays were performed with an enzyme concentration of 190 μg/ml, which was well within the linear portion of an activity *versus* protein curve. Each reaction mixture contained 1.0 mM Co^{2+} and was buffered to pH 7.2 with 200 mM potassium phosphate to insure maximal activity (see Table II). Double reciprocal plots of substrate and erythrose 4-phosphate (*E-4-P*), fixed at 2.0 mM, were constructed (*a* and *b*). Substrate saturation curves were also done in the presence of the L-tryptophan (*L-TRP*) concentrations indicated in (*a*), or of the chorismate (*CHA*) concentrations indicated in (*b*). (*c*) shows inhibition curves obtained with *L-TRP* or with *CHA* when both *E-4-P* and *PEP* were present at 1 mM concentration. Substrate saturation curves were also obtained at 1 mM concentration. Substrate saturation curves were also obtained with *E-4-P* as the variable substrate and *PEP* concentration fixed at 2.0 mM. The data obtained in the presence or absence of *L-TRP* and *CHA* were plotted in double reciprocal form as shown in (*d*) and (*e*), respectively.

TABLE II

Enzymological characterization of DAHP synthase isozymes

Isozyme species	pH optimum[a]	Co^{2+} activation[b]	Inhibition	Phosphoenolpyruvate[c]			Type of inhibition[d]	Erythrose 4-phosphate[c]			Type of inhibition[d]
				K_m	K_i	V_m		K_m	K_i	V_m	
		mM		mM				mM			
DAHP synthase-tyr	6.8	0.40		1.0		1.67		1.0		14.3	
(M_r = 137,000)			L-Tyrosine		23 μM		C		23 μM		C
			Phenylpyruvate		2.55 mM		C		1.35 mM		C
DAHP synthase-trp	7.2	0.25		1.11		2.08		0.56		6.67	
(M_r = 175,000)			L-Tryptophan		40 μM		N		5 μM		C
			Chorismate		1.35 mM		C		2.25 mM		C

[a] Samples of each isozyme were obtained from DEAE-cellulose and assayed for DAHP synthase activity from pH 6.0 to 8.0 using 200 mM potassium phosphate buffer.

[b] DAHP synthase activity was measured with increasing cobalt concentrations (0.025–3.0 mM). The concentrations indicated represent the points at which the activation curves plateaued.

[c] K_m and V_m were determined by plotting the reciprocal of velocity (v^{-1}) against the reciprocal of the substrate concentration (mM^{-1}). This procedure was repeated at various inhibitor concentrations (I), and apparent K_m and V_m values were determined. A plot of I as a function of $K_{m\ app}/V_{m\ app}$ will give $-K_i$ at the intercept of the ordinate.

[d] The type of inhibition (C = competitive; N = noncompetitive) was deduced from Figs. 2 and 3.

This idea was tested by examination of possible effects upon DAHP synthase-trp as the result of mutant deficiency in *trpA*, the structural gene for anthranilate synthase aminase (10), or as a result of derepression (100-fold) of the *trpABD* operon in a constitutive regulatory mutant (4FT1) that is resistant to 4-fluorotryptophan (10). In either case, whether anthranilate synthase activity was lost by mutation or whether anthranilate synthase was highly elevated, we saw no suggestive influence upon the properties of DAHP synthase-trp (Table III). Isolated DAHP synthase-trp did not exhibit any anthranilate synthase (aminase) or anthranilate synthase (amidotransferase) activity.

DISCUSSION

Metal Ion Stimulation of DAHP Synthase—The comparative enzymology and regulation of aromatic amino acid biosynthesis among members of the *Pseudomonadaceae* allow recognition of five distinct taxonomic groupings (12, 16, 17). With the exception of the Group V or xanthomonad species, there was a general pattern of divalent metal ion stimulation

TABLE III

Specific activities of wild type, trpA1, and mutant 4FT1

All three preparations were prepared by partial purification from DEAE-cellulose as in Fig. 1. Elution positions of DAHP synthase-tyr and DAHP synthase-trp were comparable for each preparation.

Strain[a]	Specific activity[b]		
	Crude extract	DAHP synthase-tyr	DAHP synthase-trp
Wild type	8.8	55.9	4.9
trpA1	6.3	43.7	5.0
4FT1	6.9	46.2	3.8

[a] The mutant strains of *P. aeruginosa* were described in Ref. 5.
[b] Expressed as nanomoles of DAHP/min/mg of protein.

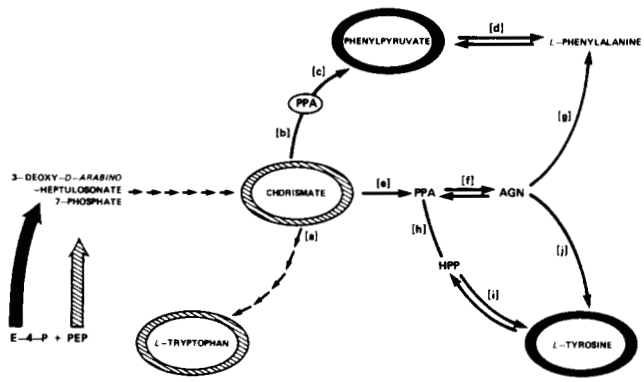

FIG. 4. **Multimetabolite control of early step reaction of aromatic amino acid biosynthesis.** DAHP synthase activity is represented by two isozymes. The major species, DAHP synthase-tyr, shown as a *solid arrow* on the left, is regulated by both L-tyrosine and phenylpyruvate as illustrated symbolically. The minor isozyme, DAHP synthase-trp (depicted by a *striped arrow*), is regulated by L-tryptophan and by chorismate. Dual enzyme sequences to L-phenylalanine utilize the activities of [*b*]-[*c*], chorismate mutase-prephenate (*PPA*) dehydratase (a multifunctional protein); [*d*], phenylpyruvate aminotransferase; [*e*], chorismate mutase; [*f*], prephenate aminotransferase; and [*g*], arogenate (*AGN*) dehydratase. Dual enzyme sequences to L-tyrosine utilize the activities of the above cited enzymes [*e*] and [*f*]; [*j*], arogenate dehydrogenase; [*h*], prephenate dehydrogenase; and [*i*], 4-hydroxyphenylpyruvate (*HPP*) aminotransferase. L-Tryptophan also feedback inhibits anthranilate synthase [*a*]; L-phenylalanine feedback inhibits [*c*]; and L-tyrosine feedback inhibits enzymes [*h*] and [*j*].

of DAHP synthase activity (12). In each of these species, either cobalt or manganese produced optimal enzyme activity. In no case were these metal ions equivalent in ability to activate DAHP synthase activity. In crude extracts of *P. aeruginosa*, total DAHP synthase was activated about 20-fold by manganese, whereas isolated DAHP synthase-tyr was only activated 3-fold and DAHP synthase-trp was not activated at all by manganese. Interestingly, partially purified preparations of each isozyme of DAHP synthase were best activated by cobalt (Table I). This apparent discrepancy may be explained by the presence of 3-dehydroquinate synthase in crude extract preparations. This activity is responsible for the cobalt-dependent conversion of DAHP to 3-dehydroquinate, the second intermediate of the common portion of the aromatic pathway (18). Therefore, when assaying crude extract preparations for DAHP synthase activity, the addition of cobalt ions may enhance 3-dehydroquinate synthase activity, thereby removing DAHP and partially masking the true cobalt-mediated stimulation of DAHP synthase. The addition of manganese ions would not be expected to substitute for cobalt in the 3-dehydroquinate synthase reaction, thus allowing the full potential of manganese-stimulated DAHP synthase activity to be measured. The partially purified isozyme preparations are assumed to be uncontaminated by 3-dehydroquinate synthase activity, thus allowing the uncomplicated measurement of cobalt activation of DAHP synthase.

DAHP Synthase-tyr—This major isozyme, like most biosynthetic pathway enzymes of *P. aeruginosa* (19), is not repressible by end products.[2] This distinguishes it from any of the three *Escherichia coli* isozymic DAHP synthases (20, 21), in which both transcriptional control and allosteric control mechanisms are exploited. Allosteric sites for L-tyrosine and phenylpyruvate must be separate since desensitization to phenylpyruvate but not to L-tyrosine has been observed *in vitro* (5), and because a regulatory mutant has been isolated which is resistant to L-tyrosine-mediated feedback inhibition while retaining phenylpyruvate sensitivity.[2] The unstable, phenylpyruvate-desensitized DAHP synthase previously isolated (5) only has a molecular weight of 52,000 compared to the much larger (137,000) and stable DAHP synthase reported here. Hence, DAHP synthase-tyr may eventually prove to consist of two or more types of polypeptide chain subunits.

DAHP Synthase-trp—This minor isozyme does not derepress in response to L-tryptophan limitation of tryptophan auxotrophs and does not repress in the presence of excess levels of aromatic end products. Consideration of the evolution of allosteric regulation for DAHP synthase-trp brings to mind anthranilate synthase, since this protein binds both chorismate (substrate) and tryptophan (feedback inhibitor). Gene duplication of *trpA* (encodes the aminase protein) followed by fusion of the duplicate *trpA* to a gene encoding a catalytic subunit of DAHP synthase is a feasible scenario of the evolutionary recruitment (22) of the regulatory portion of the contemporary DAHP synthase-trp. The possibility of a multifunctional DAHP synthase-trp/anthranilate synthase (aminase) is eliminated by their failure to co-purify during purification. If DAHP synthase-trp is a protein-protein aggregate in which the regulatory subunit is in fact anthranilate synthase (aminase), then (i) anthranilate synthase must be inactive in the aggregated state (*i.e.* DAHP synthase-trp lacked anthranilate synthase activity), (ii) mutant *trpA1* which lacks anthranilate synthase activity must still make a protein capable of functioning as a regulatory subunit in the DAHP synthase aggregate (*i.e.* DAHP synthase-trp was not altered in mutant *trpA1*), and (iii) anthranilate synthase protein must not be limiting to assembly of the hypothetical aggregate (*i.e.* DAHP synthase-trp was not altered in constitutive mutant 4FT1).

We recently determined[3] that *Xanthomonas campestris* possesses a single species of DAHP synthase. This enzyme is reminiscent of the DAHP synthase-trp isozyme of *P. aeruginosa*, except that in *X. campestris* chorismate is a much more effective feedback inhibitor than is L-tryptophan. In this case, it would appear that the basic pattern of allosteric control is sequential feedback inhibition, chorismate levels presumably indirectly reflecting all three end product levels (following appropriate end product regulation of allosteric enzymes linking chorismate with the terminal amino acid branchlets).

Regulatory Roles of DAHP Synthase Isozymes—The differential regulation of the two DAHP synthase isozymes seems to reconcile with a feasible mechanism for partitioning the control of total DAHP synthase activity between metabolites of the divergent pathway branchlets. The basic outline of the aromatic biosynthetic pathway is shown in Fig. 4, which emphasizes the pattern of end product control for DAHP synthase. It is appropriate that the major isozyme is regulated by metabolites of the tyrosine and phenylalanine branchlets, since primary pathway flow is toward these amino acids. Likewise, it seems appropriate that the minor isozyme is

[2] R. Whitaker and R. Jensen, unpublished data.

[3] R. Whitaker, G. Byng, and R. Jensen, unpublished data.

controlled by L-tryptophan, an amino acid formed in quantitatively minor amounts.

Clearly, the most sensitive control signals are L-tyrosine for DAHP synthase-tyr and L-tryptophan for DAHP synthase-trp. It should be possible to gain a fuller appreciation of the *in vivo* significance of phenylpyruvate inhibition of DAHP synthase-tyr and chorismate inhibition of DAHP synthase-trp through examination of various classes of deregulated mutants. Since the intermediary metabolites (especially chorismate) may indirectly reflect end product levels, aspects of sequential feedback inhibition may operate in this most complicated pathway. Each isozyme can also be shown to be inhibited cumulatively by a combination of two inhibitors. Thus, this system has features to employ simultaneously what can be regarded as isozymic, cumulative, and sequential modes of feedback inhibition.

REFERENCES

1. Jensen, R. A., and Rebello, J. L. (1970) *Dev. Ind. Microbiol.* **11,** 105-121
2. Byng, G. S., Kane, J. F., and Jensen, R. A. (1982) *Crit. Rev. Microbiol.* **9,** 227-252
3. Jensen, R. A., and Hall, G. C. (1982) *Trends Biochem. Sci.* **7,** 177-180
4. Jensen, R. A., Nasser, D. S., and Nester, E. W. (1967) *J. Bacteriol.* **94,** 1582-1593
5. Jensen, R. A., Calhoun, D. H., and Stenmark, S. L. (1973) *Biochim. Biophys. Acta* **293,** 256-268
6. Holloway, B. W. (1955) *J. Gen. Microbiol.* **13,** 572-581
7. Patel, N., Pierson, D. L., and Jensen, R. A. (1977) *J. Biol. Chem.* **252,** 5839-5846
8. Calhoun, D. H., and Jensen, R. A. (1972) *J. Bacteriol.* **109,** 365-372
9. Patel, N., Stenmark-Cox, S. L., and Jensen, R. A. (1978) *J. Biol. Chem.* **253,** 2972-2978
10. Calhoun, D. H., Pierson, D. L., and Jensen, R. A. (1973) *Mol. Gen. Genet.* **121,** 117-132
11. Calhoun, D. H., Pierson, D. L., and Jensen, R. A. (1973) *J. Bacteriol.* **113,** 241-251
12. Whitaker, R. J., Byng, G. S., Gherna, R. L., and Jensen, R. A. (1981) *J. Bacteriol.* **145,** 752-759
13. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248-254
14. Gibson, F. (1964) *Biochem. J.* **90,** 256-261
15. Queener, S. W., Queener, S. F., Meeks, J. R., and Gunsalus, I. C. (1973) *J. Biol. Chem.* **248,** 151-161
16. Byng, G. S., Whitaker, R. J., Gherna, R. L., and Jensen, R. A. (1980) *J. Bacteriol.* **144,** 247-257
17. Whitaker, R. J., Byng, G. S., Gherna, R. L., and Jensen, R. A. (1981) *J. Bacteriol.* **147,** 526-534
18. Haslam, E. (1974) In *The Shikimate Pathway*, pp. 3-48, John Wiley & Sons, New York
19. Jensen, R. A., and Pierson, D. L. (1975) *Nature (Lond.)* **254,** 667-671
20. Jensen, R. A., and Nasser, D. S. (1968) *J. Bacteriol.* **95,** 188-196
21. Jensen, R. A., and Byng, G. S. (1981) in *Isozymes* (Ratazzi, M. C., Scandalios, J. G., and Whitt, G. S., eds) Vol. 5, pp. 143-174, Alan R. Liss, Inc., New York
22. Jensen, R. A. (1976) *Annu. Rev. Microbiol.* **30,** 409-425