JOURNAL OF BACTERIOLOGY, Dec. 1980, p. 1034-1042
0021-9193/80/12-1034/09$02.00/0

Vol. 144, No. 3

Enzymological Basis for Growth Inhibition by L-Phenylalanine in the Cyanobacterium *Synechocystis* sp. 29108

GERALDINE C. HALL[1] AND ROY A. JENSEN[2]*

Department of Biological Sciences[1] and Center for Somatic-Cell Genetics and Biochemistry,[2] State University of New York at Binghamton, Binghamton, New York 13901

The pattern of allosteric control in the biosynthetic pathway for aromatic amino acids provides a basis to explain vulnerability to growth inhibition by L-phenylalanine (0.2 mM or greater) in the unicellular cyanobacterium *Synechocystis* sp. 29108. We attribute growth inhibition to the hypersensitivity of 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase to feedback inhibition by L-phenylalanine. Hyperregulation of this initial enzyme of aromatic biosynthesis depletes the supply of precursors needed for biosynthesis of L-tyrosine and L-tryptophan. Consistent with this mechanism is the total reversal of phenylalanine inhibition by a combination of tyrosine and tryptophan. Inhibited cultures also contained decreased levels of phycocyanin pigments, a characteristic previously correlated with amino acid starvation in cyanobacteria. L-Phenylalanine is a potent noncompetitive inhibitor (with both substrates) of 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase, whereas L-tyrosine is a very weak inhibitor. Prephenate dehydratase also displays allosteric sensitivity to phenylalanine (inhibition) and to tyrosine (activation). Both 2-fluoro and 4-fluoro derivatives of phenylalanine were potent analog antimetabolites, and these were used in addition to L-phenylalanine as selective agents for resistant mutants. Mutants were isolated which excreted both phenylalanine and tyrosine, the consequence of an altered 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase no longer sensitive to feedback inhibition. Simultaneous insensitivity to L-tyrosine suggests that L-tyrosine acts as a weak analog mimic of L-phenylalanine at a common binding site. Prephenate dehydratase in the regulatory mutants was unaltered. Surprisingly, in view of the lack of regulation in the tyrosine branchlet of the pathway, such mutants excrete more phenylalanine than tyrosine, indicating that L-tyrosine activation dominates L-phenylalanine inhibition of prephenate dehydratase in vivo. In mutant Phe r19 the loss in allosteric sensitivity of 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase was accompanied by a threefold increase in specific activity. This could suggest that existence of a modest degree of repression control (autogenous) over 3-deoxy-D-arabinoheptulosonate synthase, although other explanations are possible. Specific activities of chorismate mutase, prephenate dehydratase, shikimate/nicotinamide adenine dinucleotide phosphate dehydrogenase, and arogenate/nicotinamide adenine dinucleotide phosphate dehydrogenase in mutant Phe r19 were identical with those of the wild type.

A diverse array of microorganisms is vulnerable to growth inhibitory effects of exogenous metabolites such as amino acids, even though endogenous synthesis is obligatory for growth (5). Although such nutritional phenomena may be most familiar with autotrophic microbes (30), heterotrophs are probably more susceptible than is generally appreciated (5). The sensitivity of *Escherichia coli* K-12 to bacteriostasis in the presence of L-valine (25) is the best-known case in point. Phototrophs, lithotrophs, and methylotrophs have been termed specialist strains because of their failure in common to grow on organic media, growth being inhibited by certain organic compounds, especially amino acids (30).

Phenomena of metabolite inhibition often involve branched biosynthetic pathways such as the aromatic and branched-chain amino acid pathways. Disproportionately great allosteric control of an early shared enzyme by one of multiple end products provides an obvious molecular basis for inhibition. Among the diversity of allosteric control patterns that have been discerned in nature for 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase (16, 19), unimetabolite (denoted retro-Phe, retro-Tyr, or retro-Trp [19]) patterns are surprisingly common. Thus, several strains of *Thiobacillus* possessing a retro-Phe pattern of DAHP synthase allostery were indeed found to be growth

inhibited by L-phenylalanine, an inhibition fully reversible by a combination of L-tyrosine and L-tryptophan (23). An interesting mutant selected for phenylalanine resistance proved to have an altered DAHP synthase insensitive to L-phenylalanine inhibition, provided both L-tyrosine and L-tryptophan were also present (24).

Species of cyanobacteria elaborate DAHP synthase enzymes subject to unimetabolite control, either retro-Phe or retro-Tyr in pattern. Thus far, species having the retro-Tyr pattern are not growth inhibited by exogenous tyrosine (34). On the other hand, retro-Phe-patterned species are likely to exhibit sensitivity of growth to phenylalanine. Among these species, *Agmenellum quadruplicatum* is particularly sensitive to bacteriostasis in the presence of phenylalanine (14). Although DAHP synthase of *A. quadruplicatum* is very sensitive to phenylalanine inhibition, the basis of inhibition seems to involve at least in part the accumulation of a toxic pathway intermediate (20).

A less-complicated experimental system is offered by *Synechocystis* sp. 29108, a species which is also growth inhibited by phenylalanine. Enzymological comparisons of the wild type with phenylalanine-resistant mutants presented in this communcation show that phenylalanine toxicity can be attributed to hyperregulation of DAHP synthase and subsequent starvation for L-tyrosine and L-tryptophan. It is of interest that loss of allosteric regulation of DAHP synthase in mutants causes excretion of both tyrosine and phenylalanine, even though the terminal branchlet of phenylalanine biosynthesis retains an intact feedback loop of allosteric control.

MATERIALS AND METHODS

Growth of cultures. The unicellular cyanobacterium, *Synechocystis* sp. 29108, was obtained from the American Type Culture Collection (Rockville, Md.) and was routinely maintained at room temperature on Cg 10 medium (32) solidified with 1.4% (wt/vol) Difco agar which was sterilized separately. Phenylalanine-resistant mutants were maintained on Cg 10 medium supplemented with 1 mM L-phenylalanine.

Growth responses to the addition of aromatic amino acids were determined by cultivation in 50 ml of Dm medium (33) in 25- by 200-mm tubes incubated in a 34°C water bath under fluorescent light bulbs and aerated with a 2% (vol/vol) CO_2-air mixture. At selected time intervals, samples were aseptically removed, and the turbidity was measured at 600 nm with a Bausch and Lomb Spectronic 20.

Selection of mutants. Spontaneous mutants resistant to L-phenylalanine were obtained by spreading a lawn of 10^6 to 10^8 cells of *Synechocystis* sp. 29108 on Cg 10 medium supplemented with 1 mM L-phenylalanine. Resistant colonies arose after 5 to 7 days of incubation at 34°C and were then purified by single-colony isolation. Isolated single colonies were checked for phenotypic stability and excretion of aromatic amino acids.

Identification of excreted amino acids. Phenylalanine-resistant mutants initially were examined for amino acid excretion by their ability to cross-feed auxotrophic strains on solid media. *Synechococcus* 602 *pheA1* was used as an auxotrophic screen for detection of excreted phenylalanine, and a tyrosine auxotroph of *Bacillus subtilis* was used for the detection of excreted tyrosine as previously described (11). Quantitative assays for excreted aromatic amino acids were carried out as described before (11).

Extract preparation. Actively growing cultures were centrifuged, and the cell pellets were washed three times with 50 mM potassium phosphate buffer, pH 7.5. Cell pellets were stored at −40°C before extract preparation. Pellets were suspended at 0 to 4°C in 50 mM potassium phosphate buffer, pH 7.5 (or pH 6.8 when used for DAHP synthase assays), containing 1 mM dithiothreitol (DTT). Cells were disrupted with six 30-s bursts of ultrasound energy with a Lab-Line Ultratip sonicator at 70 mV. The resulting preparation was centrifuged at 150,000 × *g* for 1 h in a Beckman preparative ultracentrifuge. The supernatant was either passed through a Sephadex G-25 column equilibrated with extract buffer, or dialyzed 20 to 22 h against three 1-liter changes of buffer.

Analytical procedures. Protein concentrations were estimated by the method of Bradford (2). DAHP synthase was assayed by the procedure of Srinivasan and Sprinson (31) as modified by Jensen and Nester (17).

Arogenate dehydrogenase (previously called pretyrosine dehydrogenase [36]) and shikimate dehydrogenase were assayed at 37°C by following the formation of NADPH in an Aminco-Bowman spectrophotofluorometer (excitation at 340 nm, emission at 460 nm). Reaction mixtures contained 50 mM potassium phosphate buffer (pH 7.5), 1 mM NADP, 1 mM shikimate or 0.5 mM arogenate, 50 to 100 μg of extract protein, and 0.25 mM dithiothreitol. L-Malate dehydrogenase was determined by following the formation of NADH fluorometrically as above.

Chorismate mutase and prephenate dehydratase were determined by measuring the formation of phenylpyruvate as previously described (26). Reaction mixtures at 37°C contained 50 mM potassium phosphate buffer (pH 7.5), 1 mM chorismate or prephenate, 100 to 200 μg of extract protein, and 0.5 mM dithiothreitol.

Biochemicals. Chorismate was isolated as the barium salt from culture supernatants of *Enterobacter aerogenes* 62-1 as described by Gibson (10). Barium prephenate was isolated from culture supernatants of *Salmonella typhimurium* (6). Before use, barium chorismate and barium prephenate were converted to the corresponding potassium salts by addition of excess potassium sulfate. Arogenate was isolated from culture supernatants of a multiple aromatic mutant of *Neurospora crassa* (21) and purified by a modified procedure (36). All other biochemicals were obtained from Sigma Chemical Co.

RESULTS

Growth inhibition by L-phenylalanine. The presence of L-phenylalanine in solid growth

medium at concentrations exceeding 0.2 mM produced a visible growth inhibition of *Synechocystis* sp. 29108, until at 1 mM concentration only resistant colonies appeared. L-Tyrosine alone reversed the inhibition by L-phenylalanine. The growth response of *Synechocystis* sp. 29108 to the presence of aromatic amino acids was examined in more detail in liquid growth medium. The addition of 1 mM L-phenylalanine increased the initial doubling time from 6 to 16 h and also reduced the total growth yield reached at stationary phase by a factor of 10 (Fig. 1). The addition of 0.1 mM L-tyrosine partially reversed the L-phenylalanine inhibition, and at 1.0 mM L-tyrosine the wild-type growth rate was almost restored. L-Tryptophan at 1 mM concentration partially reversed inhibition, but not as efficiently as L-tyrosine.

The L-phenylalanine-inhibited cells were deficient in phycocyanin pigments, as evidenced by pronounced yellow-green coloration and by a decrease in the major absorbance peak at 620 nm. In cultures grown in the presence of 1 mM L-phenylalanine plus 0.1 mM L-tyrosine, the absorbance at 620 nm was 25% lower than that of a wild-type culture with identical absorbancies in the 400 to 520 nm range. The contribution of phycobiliprotein absorbance to turbidity in experiments such as illustrated in Fig. 1 was less than 10%. Decreased phycobiliprotein content is most often associated with conditions of nitrogen starvation (1).

Regulatory enzymes in the aromatic pathway. The above results suggested that L-phenylalanine, by inhibiting or repressing a common-pathway enzyme, led to starvation for L-tyrosine and L-tryptophan. To identify the possible enzymatic target of L-phenylalanine, those enzymes known to be sites of regulation in other organisms were assayed in the presence and absence of possible effector molecules (Table 1). No prephenate dehydrogenase activity was detected with either NAD or NADP. Both shikimate dehydrogenase and arogenate dehydrogenase were specific for the cofactor NADP. The only enzymes displaying significant sensitivities to effector molecules were DAHP synthase and prephenate dehydratase. The inhibition of prephenate dehydratase by exogenous L-phenylalanine would not be expected to cause growth inhibition since, if anything, that would maximize the availability of prephenate for L-tyrosine synthesis, i.e., a sparing effect. The inhibition of DAHP synthase by exogenous L-phenylalanine, however, would promote starvation for all remaining aromatic end products since DAHP synthase catalyzes the first step in the common aromatic pathway.

Allosteric regulation of DAHP synthase.

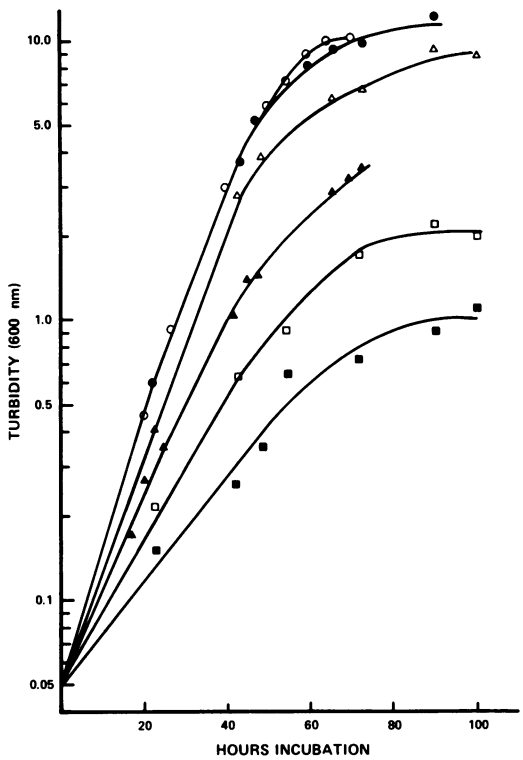

FIG. 1. *L-Phenylalanine growth inhibition of wild-type Synechocystis sp. 29108 and reversal of inhibition by L-tryptophan and L-tyrosine. Growth tubes contained no additions (●), 1 mM L-phenylalanine and 0.5 mM each L-tryptophan and L-tyrosine (○), 1 mM each L-phenylalanine and L-tyrosine (△), 1 mM each L-phenylalanine and L-tryptophan (▲), 1 mM L-phenylalanine and 0.1 mM tyrosine (□), 1 mM L-phenylalanine (■).*

The data in Table 1 show that the allosteric regulation of DAHP synthase in *Synechocystis* sp. 29108 is of the unimetabolite (retro-Phe) type (19). L-Phenylalanine was a potent inhibitor of DAHP synthase, producing 90% inhibition at a concentration of 0.05 mM. Kinetic analysis (Fig. 2A) showed that L-phenylalanine inhibition is noncompetitive with respect to the substrate phosphoenolpyruvate and probably noncompetitive with respect to the second substrate, erythrose 4-phosphate, as well. This conclusion is consistent with the finding that doubling the amount of substrate present (in a limiting range) did not decrease the percent inhibition by L-phenylalanine in a range of inhibition expected to vary responsively if inhibition were competitive. Kinetic analyses could not be extended to lower ranges of substrate concentration owing to limitations of the low activities obtained.

Analysis of phenylalanine-resistant mutants. Several spontaneous, phenylalanine-re-

TABLE 1. *Effects of aromatic pathway metabolites on the catalytic activities of aromatic pathway enzymes*

Enzyme	Addition[a]	% Relative activity[b]
DAHP synthase	None	100
	L-Phenylalanine	10
	L-Tyrosine	90
	L-Tryptophan	101
	Prephenate	95
	Phenylpyruvate and chorismate	117
	L-Arogenate	137
Shikimate dehydrogenase	None	100
	L-Phenylalanine, L-tyrosine and L-tryptophan	86
Chorismate mutase	None	100
	L-Phenylalanine	105
	L-Tyrosine	111
	L-Phenylalanine and L-tyrosine	105
	L-Arogenate	100
Arogenate dehydrogenase	None	100
	L-Phenylalanine	80
	L-Tyrosine	68
Prephenate dehydratase	None	100
	L-Phenylalanine	9
	L-Tyrosine	618
	L-Phenylalanine and L-tyrosine	165

[a] With DAHP synthase, phenylalanine, tyrosine, and tryptophan were added to final concentrations of 0.1 mM; other additions were at 0.5 mM concentrations. Shikimate dehydrogenase: each addition was at 0.1 mM concentration. Chorismate mutase: each addition was present at 0.5 mM. Arogenate dehydrogenase: each addition was at 1.0 mM. Prephenate dehydratase: each addition at 0.125 mM concentration.

[b] Relative activities of 100 for each enzyme shown (top to bottom) correspond to specific activities of 1.34, 2.5, 6.8, 9.4, and 2.1 nmol/min per mg of crude extract protein, respectively.

sistant mutants were selected from Cg 10 agar plates containing 1 mM L-phenylalanine. None of the resistant mutants supported a growth halo of wild-type cells. However, when these same mutants were spot inoculated onto a lawn of a phenylalanine-requiring mutant (*Synechococcus* 602 *pheA1*) on unsupplemented Cg 10 plates, all of the resultant colonies were surrounded by a halo of cross-fed auxotrophic cells. This phenomenon, i.e., the absence of cross-fed wild-type growth surrounding metabolite-excreting colonies on antimetabolite-containing media, is generally characteristic of analog-resistant mutants of different species of cyanobacteria (11). All the phenylalanine-resistant mutants (Table 2) were regulatory mutants on the criterion of their excretion of phenylalanine and tyrosine into the medium. When a phenylalanine-resistant mutant (Phe r19), was spot inoculated onto a lawn of wild-type cells on unsupplemented medium, a zone of inhibition appeared indicating that the mutant was excreting sufficient phenylalanine to inhibit the wild-type growth. Thus, the phenylalanine-resistant mutants, by excreting the toxic substance to which they have become resistant, possess the unusual property of being self-selective.

If growth inhibition of *Synechocystis* sp. 29108 were indeed caused by starvation for L-tyrosine and L-tryptophan via unimetabolite inhibition of DAHP synthase by L-phenylalanine, then the most direct mechanism of resistance in mutants would be loss of the sensitivity of DAHP synthase to L-phenylalanine inhibition. Table 2 shows that such mutants are common. Except for mutant Phe r1 (partially desensitized), each phenylalanine-resistant mutant possessed a DAHP synthase that was completely desensitized to phenylalanine inhibition. Wild-type DAHP synthase was at least as sensitive to 4-fluorophenylalanine as to L-phenylalanine, and resistant mutants possessed DAHP synthase enzymes that were insensitive to both. Results shown in Table 2 for 4-fluorophenylalanine were similar to results (not shown) obtained with 2-fluorophenylalanine. Each mutant excreted a large excess of phenylalanine into the medium along with a smaller amount (about 10%) of tyrosine (11).

DAHP synthase activity in mutant Phe r19 was examined further with respect to the possible effect of the regulatory mutation upon the weak sensitivity to tyrosine (Table 1). Whereas the wild-type DAHP synthase was inhibited by 45% in the presence of 2 mM L-tyrosine, the activity of the mutant enzyme was unaffected, indicating the joint abolition of both sensitivities.

Prephenate dehydratase regulation and aromatic amino acid excretion. A comparison of the prephenate dehydratase activity in mutant Phe r19 with that of the wild type in the presence of varying amounts of L-phenylalanine is presented in Fig. 3. Prephenate dehydratase in Phe r19 retained its sensitivity to L-phenylalanine. Since L-tyrosine played no significant role in the regulation of its own synthesis, whereas L-phenylalanine inhibits prephenate dehydratase, it might be expected that the phenylalanine-resistant mutants would excrete larger quantities of tyrosine than phenylalanine. However, the amount of phenylalanine excreted

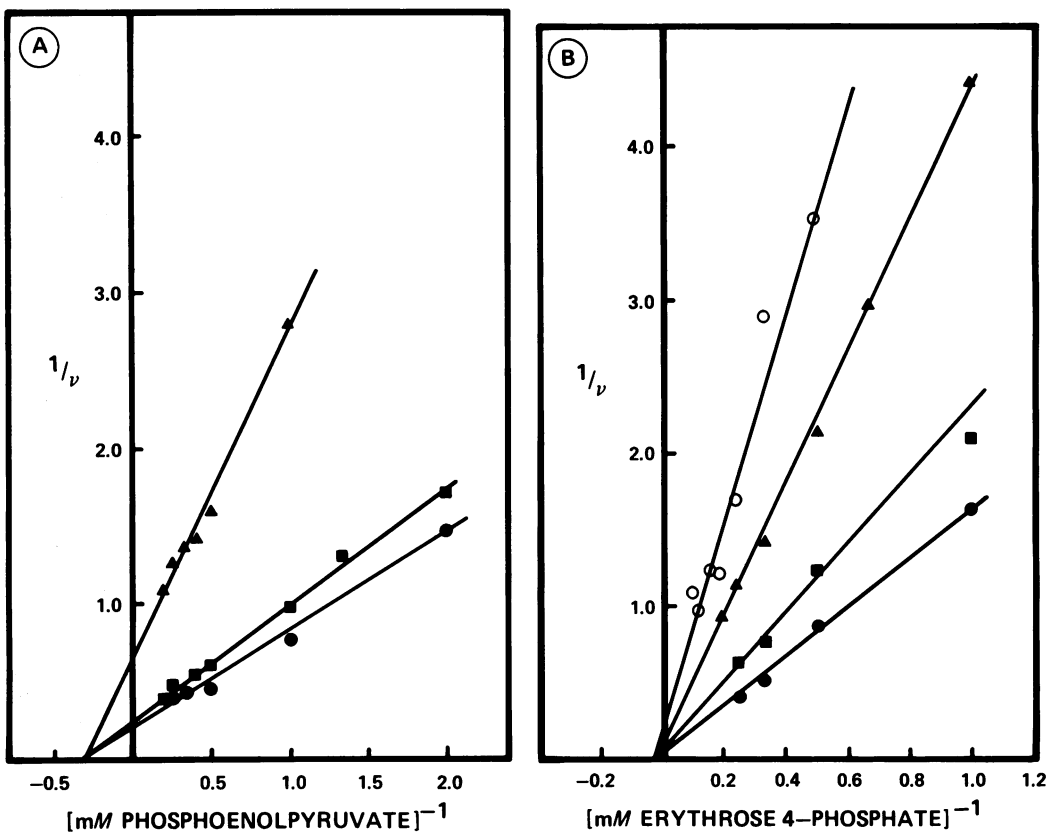

FIG. 2. *Double reciprocal plots of* L-*phenylalanine inhibition of DAHP synthase in wild-typeSynechocystis sp. 29108. Erythrose 4-phosphate was present at a fixed concentration of 4 mM (A), and phosphoenolpyruvate was present at a fixed concentration of 4 mM (B). All lines were drawn via regression analysis. A value of 1 on the ordinate scale corresponds to a specific activity of 1 nmol of DAHP formed per min per mg of protein. Symbols: ●, no* L-*phenylalanine; ■, 5 μM* L-*phenylalanine; ▲, 10 μM* L-*phenylalanine; ○, 25 μM* L-*phenylalanine.*

TABLE 2. *Deregulation of DAHP synthase and excretion of amino acids in phenylalanine or 4-fluorophenylalanine-resistant mutants of Synechocystis sp. 29108*

Organism	Sp act[a]	% Activity in presence of:		Amino acid excretion[b]	
		10 μM L-Phe	10 μM 4-fPhe	Phenylalanine	Tyrosine
Wild type	1.3	29	19	0	<1
Phe r1	2.6	71	65	109	10
Phe r4	4.1	95	98	121	14
Phe r10	1.9	104	141	34	5
Phe r19	2.6	101	100	193	16
4FP 30[c]	2.6	111	106	134	19
4FP 31[c]	2.1	108	116	133	21

[a] Expressed as nanomoles per minute per milligram of crude extract protein.

[b] Expressed as micromolar concentration per unit growth mass (optical density at 500 nm).

[c] Selected as mutants resistant to 4-fluorophenylalanine (4-fPhe), but also found to be resistant to L-phenylalanine (L-Phe).

exceeds that of tyrosine (Table 2) by factors of 6 to 12. L-Tyrosine was found to have a highly stimulatory effect upon the activity of prephenate dehydratase, i.e., a concentration of 0.125 mM in the reaction mixture increased the activity sixfold (Table 1). The addition of an equal amount of L-phenylalanine decreased the activating effect of L-tyrosine, but the residual activity still substantially exceeded the activity obtained in the absence of any effector (Table 1). The relatively high excretion of phenylalanine by the resistant mutants indicates that L-tyrosine activation of prephenate dehydratase predominates over the inhibition by L-phenylalanine in vivo.

Elevated DAHP synthase activity in mutant Phe r19. Several investigators have reported (12, 22) that in amino acid auxotrophs of cyanobacteria starved for the required nutrients, no derepression of the corresponding biosynthetic enzymes was found and that wild-type strains exposed to high exogenous concentra-

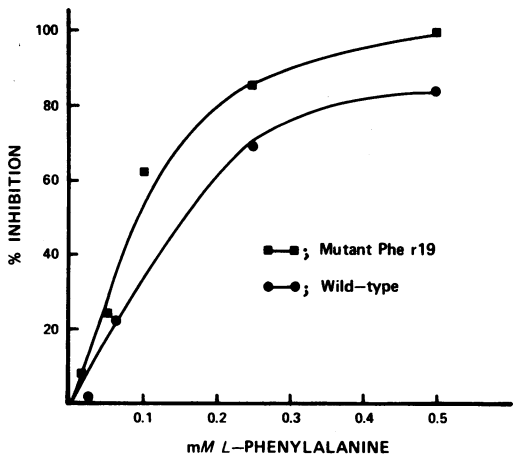

FIG. 3. *Inhibition of prephenate dehydratase by L-phenylalanine in wild-type Synechocystis sp. 29108 and mutant Phe r19.*

tions of metabolites did not contain altered levels of enzymes. The enzyme levels of wild-type *Synechocystis* sp. 29108 were compared with those of the mutant Phe r19. Triplicate cultures of each strain were grown simultaneously and extracts were prepared under identical conditions. The results of the assays are presented in Table 3. L-Malate dehydrogenase activity was included as an extra-pathway enzyme. The specific activities of shikimate dehydrogenase, chorismate mutase, prephenate dehydratase, and arogenate dehydrogenase were essentially the same in both the wild type and mutant Phe r19. However, the specific activity of DAHP synthase in mutant Phe r19 was 2.9 times that of the wild-type activity. The specific activities of the other phenylalanine-resistant mutants were also higher, ranging from 1.5 to 3.3 times that found in the wild type (Table 2). Growth of mutant Phe r19 in the presence of 1 mM each of L-tyrosine and L-tryptophan did not alter the specific activities. Two possible explanations exist for the elevated DAHP synthase activities in the mutants: (i) the mutation that results in loss of sensitivity also results in a protein with enhanced catalytic activity, or (ii) the mechanisms of repression and feedback inhibition are linked, e.g., autogenous regulation (3). Kinetic parameters of DAHP synthase were compared in mutant Phe r19 with those of the wild type. Alteration in apparent K_m for substrate would not be consistent with derepression. The apparent K_m values with respect to phosphoenolpyruvate or erythrose 4-phosphate were similar in both organisms. The apparent V_{max} for wild-type DAHP synthase was 4.2 nmol/min per mg of protein compared with a value of 15.5 for mutant Phe r19. The latter result is consistent with

TABLE 3. *Specific activities of enzymes in extracts of wild-type Synechocystis sp. 29108 and mutant Phe r19*

Enzyme	Sp act[a]	
	Wild type	Phe r19
DAHP synthase	1.24 ± 0.12	3.57 ± 0.29
Shikimate dehydrogenase	4.67 ± 0.50	4.55 ± 0.18
Chorismate mutase	1.40 ± 0.17	1.29 ± 0.10
Prephenate dehydratase	2.49 ± 0.27	2.30 ± 0.23
Arogenate dehydrogenase	8.28 ± 0.40	7.37 ± 1.90
Malate dehydrogenase	0.83 ± 0.09	0.69 ± 0.07

[a] Specific activities are expressed as nanomoles of product formed per minute per milligram of protein. All values represent the average of three extracts prepared from independent, but simultaneously grown, cultures. The Phe r19 culture filtrates contained 850 ± 60 μM phenylalanine, 40 ± 2 μM tyrosine, and 25 ± 8 μM tryptophan. Extracts were prepared in the presence of 0.1 mM L-tyrosine to stabilize prephenate dehydratase.

either of the possible explanations. On the other hand, growth of the wild type in the presence of 0.5 mM L-tryptophan and 1.0 mM each L-phenylalanine and L-tyrosine did not depress the activity of DAHP synthase. When the phenylalanine auxotroph *Synechococcus* 602 *pheA1* was cultured under conditions of limiting L-phenylalanine (10 μM) and nonlimiting L-phenylalanine (500 μM), the DAHP synthase specific activities were essentially the same (2.33 and 2.20 nmol/min per mg of protein, respectively).

DISCUSSION

Growth inhibition by L-phenylalanine. The growth inhibition of *Synechocystis* sp. 29108 by L-phenylalanine has been shown to result from hyperinhibition of DAHP synthase by L-phenylalanine, which then leads to starvation for precursors of L-tyrosine and L-tryptophan. Accordingly, phenylalanine inhibition was reversed by the presence of L-tyrosine and L-tryptophan in the growth medium. Several phenylalanine-resistant mutants were isolated and contained an altered species of DAHP synthase no longer sensitive to L-phenylalanine. As a result of desensitization of DAHP synthase, L-phenylalanine and L-tyrosine were excreted into the medium. It seems doubtful that the very weak inhibition of DAHP synthase by tyrosine has any appreciable physiological significance. The same allosteric site which binds phenylalanine also appears to recognize tyrosine as a poor analog mimic. Thus, the kinetics of inhibition by phenylalanine and tyrosine are similar (both are V_{max} effectors), and desensitized mutants always exhibited joint loss of DAHP synthase sensitivity to inhibition by both molecules.

Since allosteric regulation patterns in the aromatic pathway have been shown by Jensen et

al. (16) to be a highly conserved generic characteristic, it is expected that all species of *Synechocystis* will exhibit the retro-Phe pattern of unimetabolite allostery for DAHP synthase and that this pattern of control will probably be accompanied by L-phenylalanine growth inhibition.

Overall regulatory role of L-tyrosine. The major role played by L-tyrosine in the regulation of the aromatic pathway is the activation of prephenate dehydratase, the first enzyme committed to L-phenylalanine synthesis. It was observed that prephenate dehydratase in *Synechocystis* sp. 29108 is extremely labile, 50% of the activity being lost within 6 to 7 h after extract preparation. The presence of L-tyrosine stabilized the enzyme activity for at least 24 h. A similar activation/stabilization pattern has been found in species of coryneform bacteria (9).

L-Tyrosine plays no direct feedback role within the tyrosine branchlet of the aromatic pathway, neither inhibiting nor repressing arogenate dehydrogenase. Again, a similar situation exists in species of coryneform bacteria (8). In the light of hypotheses proposing that ancestral counterparts of modern cyanobacteria were progenitors of chloroplasts (29), it is interesting that both prephenate and arogenate dehydrogenase activities were isolated from mung bean seedlings and that the prephenate dehydrogenase activity was inhibited by L-tyrosine, whereas arogenate dehydrogenase activity was unaffected (28). It was suggested that the arogenate dehydrogenase activity might be localized within the chloroplast.

It is often difficult to assess the in vivo importance of data acquired in vitro, but excretion patterns of regulatory mutants provide invaluable clues. The relatively high excretion of phenylalanine, as compared with tyrosine, by the phenylalanine-resistant mutants provides in vivo evidence for relative domination of L-tyrosine activation over L-phenylalanine inhibition of prephenate dehydratase. Since an excess of cellular L-tyrosine diverts prephenate so efficiently from the L-tyrosine biosynthetic branchlet into the L-phenylalanine branchlet, production of large amounts of L-tyrosine could be accomplished only in regulatory mutants isolated in a genetic background of phenylalanine auxotrophy (i.e., lacking prephenate dehydratase).

Dilemma of unimetabolite-controlled DAHP synthase in nature. A balanced responsivity of a single branch-point enzyme to multiple end products generated is the simplest regulation scheme to envision. However, with surprising frequency this is not found in nature (19). Even in *E. coli*, where multiple regulatory isoenzymes are usually regarded as a model example of balanced responsivity to multiple end products, a phenylalanine-sensitive isoenzyme is dominant (90 to 95%) in expression (15). When end products must be synthesized endogenously, it is possible to relate unimetabolite control of DAHP synthase to the gestalt of regulatory features elsewhere in the aromatic pathway to come to overall interpretations consistent with economy of metabolite flow. For example, the retro-Tyr pattern of DAHP synthase control in *Pseudomonas aeruginosa* has been considered in the context of a channel-shuttle mechanism (4) that favors prephenate utilization by the phenylalanine branchlet as well as the further complexity of dual enzymatic routes to L-tyrosine and L-phenylalanine (26).

If the dominating regulatory influence of a single end product is sufficiently great, then the exogenous presence of that end product may inhibit growth. It has been pointed out that (7, 18) selection against such instances of metabolite inhibition may not occur in nature where growth conditions may rarely resemble those provided in the laboratory. An examination of the basis for growth inhibition in many autotrophic organisms by amino acids may reveal other one-effector patterns of regulation in common branch-point enzymes.

Starvation-induced chlorosis. In their study of phycobilisome turnover during nitrogen starvation in *Synechococcus* sp. 6301, Yamanaka and Glazer (35) found that starvation of a methionine auxotroph for methionine did not induce phycocyanin depletion. The authors postulated that one of the metabolites of nitrogen metabolism, e.g., glutamine, acts as a signal for the induction of phycobilisome degradation. In this context, the chlorosis of *Synechocystis* sp. 29108 upon starvation for L-tyrosine, and the chlorosis of a phenylalanine auxotroph, *Synechococcus* 602 *pheA1*, when starved for L-phenylalanine (Hall and Jensen, unpublished data), would indicate some metabolic interrelationship between the aromatic pathway and the signal for phycobilisome degradation. The use of methionine, however, as a limiting amino acid is complicated by the fact that methionine is not only the initiating residue in the synthesis of procaryotic proteins but is also an important source of methyl groups, and the degradation of phycobilisomes is attributed to the de novo synthesis of a protease(s), since chloramphenicol treatment of nitrogen-starved cultures prevented phycobilisome degradation (33). It is likely that, in general, starvation for amino acids (with the exception of methionine) results in chlorosis and that a mechanism for maintaining amino acid balance is involved in phycobilisome

degradation.

Is DAHP synthase a repressible enzyme? The extent of transcriptional/translational control in cyanobacterial organisms is uncertain (12), particularly with biosynthetic pathways. In a phenylalanine auxotroph of *Synechococcus cedrorum*, several pathway enzymes were not derepressed on starvation for phenylalanine, but DAHP synthase assays were not included in the study (22). In the unicellular cyanobacterium *Agmenellum quadruplicatum*, enzymes of the tryptophan pathway were derepressed threefold when a tryptophan auxotroph was starved for tryptophan (13). Each of the regulatory mutants in our study had a greater specific activity than the wild type (usually two- to threefold) in addition to the loss of allosteric sensitivity to L-phenylalanine. This may suggest a mechanism of autogenous regulation (3) linking the mechanisms of feedback inhibition and repression. Alternatively, modification of the allosteric site may indirectly affect the intrinsic rate of catalysis. If DAHP synthase is a repressible enzyme, then it must be present in a maximally repressed state in the wild type, since the activity of the wild-type DAHP synthase was not altered when the wild type was grown in the presence of the three aromatic amino acids. The absence of derepressed DAHP synthase after phenylalanine starvation of *Synechococcus* 602 *pheA1* suggests the absence of a mechanism of enzyme repression at the level of DAHP synthase. Caution is advisable in making general conclusions, however, in view of species differences so far known. For example, DAHP synthase of *Synechocystis* sp. 29108 is strongly feedback inhibited by phenylalanine, whereas that of *Synechococcus* 602 *pheA1* is only weakly inhibited by tyrosine. It is of interest that one class of mutant described in *Anacystis nidulans* (27) was also pleiotropically feedback resistant and (apparently) derepressed threefold with respect to DAHP synthase. Amino acid overproduction was not evaluated in the latter study.

Since both DAHP synthase and prephenate dehydratase are feedback inhibited by L-phenylalanine, possibilities of a multifunctional protein or of a common regulatory subunit were considered. The first possibility is ruled out by separation of the two enzymes during partial purification. The existence of a common regulatory subunit seems unlikely in consideration of the maintenance of a feedback-sensitive prephenate dehydratase in mutant Phe r19 which acquired a feedback-resistant DAHP synthase.

ACKNOWLEDGMENTS

This investigation was supported in part by Public Health Service research grant AM-19447 from the National Institutes of Health and in part by funds from the Center for Somatic-Cell Genetics and Biochemistry.

We gratefully acknowledge the help of Joan Markiewicz with the illustrative art work.

LITERATURE CITED

1. **Allen, M. M., and A. J. Smith.** 1969. Nitrogen chlorosis in blue-green algae. Arch. Mikrobiol. **69**:114-120.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248-254.
3. **Calhoun, D. H., and G. W. Hatfield.** 1975. Autoregulation of gene expression. Annu. Rev. Microbiol. **29**:275-299.
4. **Calhoun, D. H., D. L. Pierso, and R. A. Jensen.** 1973. Channel-shuttle mechanism for the regulation of phenylalanine and tyrosine synthesis at a metabolic branch point in *Pseudomomas aeruginosa*. J. Bacteriol. **113**: 241-251.
5. **Conrad, R. S., J. R. Sokatch, and R. A. Jensen.** 1976. Relationship of metabolite inhibition of growth to flow-of-carbon patterns in nature. (MINIREVIEW). Life Sci. **19**:229-320.
6. **Dayan, J., and D. B. Sprinson.** 1970. Preparation of prephenic acid. Methods Enzymol. **17A**:559-561.
7. **DeFelice, M., M. Levinthal, M. Iaccarino, and J. Guardiola.** 1979. Growth inhibition as a consequence of antagonism between related amino acids: effects of valine in *Escherichia coli* K-12. Microbiol. Rev. **43**:42-58.
8. **Fazel, A. M., and R. A. Jensen.** 1979. Obligatory biosynthesis of L-tyrosine via the pretyrosine branchlet in coryneform bacteria. J. Bacteriol. **138**:805-815.
9. **Fazel, A. M., and R. A. Jensen.** 1980. Regulation of prephenate dehydratase in coryneform species of bacteria by *L*-phenylalanine and by remote effectors. Arch. Biochem. Biophys. **200**:165-176.
10. **Gibson, F.** 1964. Chorismic acid purification and some chemical and physical studies. Biochem. J. **90**:256-261.
11. **Hall, G., M. B. Flick, and R. A. Jensen.** 1980. An approach to the recognition of regulatory mutants in cyanobacteria. J. Bacteriol. **143**:981-988.
12. **Hood, W., and N. G. Carr.** 1971. Apparent lack of control by repression of arginine metabolism in blue-green algae. J. Bacteriol. **107**:365-367.
13. **Ingram, L. O., D. Pierson, J. F. Kane, C. Van Baalen, and R. A. Jensen.** 1972. Documentation of auxotrophic mutation in blue-green bacteria: characterization of a tryptophan auxotroph in *Agmenellum quadruplicatum*. J. Bacteriol. **111**:112-118.
14. **Ingram, L. O., and R. A. Jensen.** 1973. Growth inhibition by *L*-phenylalanine in *Agmenellum quadruplicatum*: a clue to some amino acid interrelationships. Arch. Mikrobiol. **91**:221-233.
15. **Jensen, R. A., and D. S. Nasser.** 1968. Comparative regulation of isoenzymic DAHP synthetases in microorganisms. J. Bacteriol. **95**:188-196.
16. **Jensen, R. A., D. S. Nasser, and E. W. Nester.** 1967. Comparative control of a branch-point enzyme in microorganisms. J. Bacteriol. **94**:1582-1593.
17. **Jensen, R. A., and E. W. Nester.** 1966. Regulatory enzymes of aromatic amino acid biosynthesis in *Bacillus subtilis*. I. Purification and properties of DAHP synthetase. J. Biol. Chem. **241**:3365-3372.
18. **Jensen, R. A., and D. L. Pierson.** 1975. Evolutionary implications of different types of microbial enzymology for *L*-tyrosine biosynthesis. Nature (London) **254**:667-671.
19. **Jensen, R. A., and J. L. Rebello.** 1970. Comparative allostery of microbial enzymes at metabolic branch-points: evolutionary implications. Dev. Ind. Microbiol. **11**:105-121.

20. **Jensen, R. A., S. Stenmark-Cox, and L. O. Ingram.** 1974. Mis-regulation of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase does not account for growth inhibition by phenylalanine in *Agmenellum quadruplicatum*. J. Bacteriol. **120**:1124–1132.
21. **Jensen, R. A., L. Zamir, M. St. Pierre, N. Patel, and D. L. Pierson.** 1977. Isolation and preparation of pretyrosine, accumulated as a dead-end metabolite by *Neurospora crassa*. J. Bacteriol. **132**:896–903.
22. **Kaney, A. R., and P. Jhabvala.** 1975. Absence of repression in a phenylalanine auxotroph of *Synechococcus cedrorum*. J. Gen. Microbiol. **87**:370–372.
23. **Kelly, D. P.** 1969. Regulation of chemoautotrophic metabolism. I. Toxicity of phenylalanine to thiobacilli. Arch. Mikrobiol. **69**:330–342.
24. **Kelly, D. P.** 1969. Regulation of chemoautotrophic metabolism. III. DAHP synthetase in *Thiobacillus neapolitanus*. Arch. Mikrobiol. **69**:360–369.
25. **Leavitt, R. I., and H. E. Umbarger.** 1962. Isoleucine and valine metabolism in *Escherichia coli*. XI. Valine inhibition of the growth of *Escherichia coli* K-12. J. Bacteriol. **83**:624–630.
26. **Patel, N., D. L. Pierson, and R. A. Jensen.** 1977. Dual enzymatic routes to L-tyrosine and L-phenylalanine via pretyrosine in *Pseudomonas aeruginosa*. J. Biol. Chem. **252**:5839–5846.
27. **Phares, W., and L. F. Chapman.** 1975. *Anacystis nidulans* mutants resistant to aromatic amino acid analogues. J. Bacteriol. **122**:943–948.
28. **Rubin, J. L., and R. A. Jensen.** 1979. The enzymology of *L*-tyrosine biosynthesis in mung bean [*Vigna radiata* (L). Wilczek]. Plant Physiol. **64**:727–734.
29. **Sagan (Margulis), L.** 1967. On the origin of mitosing cells. J. Theoret. Biol. **14**:225–274.
30. **Smith, A. J., and D. S. Hoare.** 1977. Specialist phototrophs, lithotrophs and methylotrophs: a unity among a diversity of procaryotes? Bacteriol. Rev. **41**:419–448.
31. **Srinivasan, P. R., and D. B. Sprinson.** 1959. α-Keto-3-deoxy-D-*arabo*-heptonic acid 7-phosphate synthetase. J. Biol. Chem. **234**:716–722.
32. **Van Baalen, C.** 1967. Further observations on growth of single cells of coccoid blue-green algae. J. Phycol. **3**: 154–157.
33. **Van Baalen, C.** 1965. Quantitative surface plating of coccoid blue-green algae. J. Phycol. **1**:19–22.
34. **Weber, H. L., and A. Böck.** 1968. Comparative studies on the regulation of DAHP synthetase activity in blue-green and green algae. Arch. Mikrobiol. **61**:159–168.
35. **Yamanaka, G., and A. N. Glazer.** 1980. Dynamic aspects of phycobilisome structure. Arch. Microbiol. **124**: 39–47.
36. **Zamir, L. O., R. A. Jensen, B. H. Arison, A. W. Douglas, G. Albers-Schönberg. and J. R. Bowen.** 1980. Structure of arogenate (pretyrosine), an amino acid intermediate of aromatic biosynthesis. J. Am. Chem. Soc. **102**:4499–4504.