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Leucine Biosynthesis in the Blue-Green Bacterium *Anacystis nidulans*

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Leucine-requiring auxotrophs of the unicellular blue-green bacterium *Anacystis nidulans* have been isolated. Extracts of these mutants were deficient in α-isopropylmalate synthetase (EC 4.1.3.12). In wild-type cells, this enzyme was subject to feedback inhibition by leucine. However, formation of the enzymes of leucine biosynthesis was little affected by exogenous leucine in either wild-type or mutant strains. Cultures of the latter subjected to extreme leucine deprivation showed no change in specific activity of β-isopropylmalate isomerase (EC 4.2.1.33) and at most a 50% increase in the specific activity of β-isopropylmalate dehydrogenase (EC 1.1.1.85). These results are compared with others bearing on the evolution of the control of amino acid biosynthesis in blue-green bacteria.

There are now two reasons to begin to apply the biochemical genetic techniques so successful with other prokaryotes to the study of amino acid biosynthesis and its control in blue-green bacteria ("blue-green algae"). First, the recent finding (11) in blue-green bacteria of a novel mechanism for the synthesis of one amino acid (tyrosine) raises the possibility that other unusual pathways may be discovered. Second, such studies can provide a test of the suggestion of Carr et al. (4, 5) that the autotrophic blue-green bacteria control metabolic activity primarily through alterations in enzyme activity (e.g., feedback inhibition), not through alterations in enzyme level (induction or repression), and that they thus differ from the more extensively studied heterotrophic eubacteria. We here report the isolation and characterization of several leucine-requiring mutants of the obligately photoautotrophic unicellular blue-green bacterium *Anacystis nidulans* (*Synechococcus* 6301 [16]). Our results indicate that this organism produces leucine via the isopropylmalate pathway known for eubacteria but, unlike eubacteria, regulates this production primarily if not exclusively at the level of feedback inhibition.

MATERIALS AND METHODS

Organism, growth conditions, and mutant selection. Our wild-type strain of *A. nidulans* is that of J. Myers and is presumably identical to the *Synechococcus* 6301 of Stanier et al. (16). Its routine maintenance and growth on liquid and solid media at 39 C have been described (6, 7). For mutagenesis, cultures started from single colonies were exposed (under growth conditions) to 25 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml for 45 min. Cells were then washed several times in unsupplemented growth medium and suspended to 6×10^6 cells/ml in medium containing 50 μg of L-leucine per ml. After 1 to 2 days of growth, leucine was removed by washing and cells were resuspended in amino acid-free medium containing 10 U of penicillin G (Eli Lilly Co.) per ml. After 7 h of exposure to antibiotic (in the light at 30 C), cells were again washed and suspended in leucine-supplemented medium. When growth of survivors was detected, cultures were spread on plates of leucine-containing solid medium. Candidates identified by replica plating were further purified and tested for leucine auxotrophy.

Extract preparation, assays, and stability of enzymatic activity. For preparation of extracts, cultures were grown at 39 C either under 5% CO_2 in air with stirring in 1,500-ml volumes (7) or in air with shaking in 100- to 150-ml volumes. Cells were harvested by centrifugation at 4 C, suspended in cold 0.05 M potassium phosphate buffer, pH 6.5, containing 10^{-3} M $MgCl_2$ and 10^{-4} M sodium ethylenediamine-tetraacetate, and disrupted in an Aminco French pressure cell at 1.1×10^7 kg/m^2. After clarification by centrifugation at 25,000 × *g* for 25 min, extracts were placed on ice and immediately assayed for enzymatic activities. Protein was measured by the method of Lowry et al. (13).

Enzyme assays. Activity of α-isopropylmalate (αIPM) synthetase (EC 4.1.3.12) was assayed by the method of Calvo et al. (3). Assay mixtures contained, in a 1-ml volume, 250 μmol of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.5, 250 μmol of KCl, 2.25 μmol of α-ketoisovalerate, 5 μmol of acetyl phosphate, 1 U of phosphotransacetylase, and 0.125 to 0.25 μmol of acetyl coenzyme A. Reaction kinetics were linear for at least 60 min at 39 C, and the reaction was usually stopped after 30 min. The published procedure was modified

for greater sensitivity by removing the entire ether extract for analysis and by diluting only fivefold into the alkaline buffer for the fluorometric determination of 4-isopropyl-umbelliferone. Extract activity was related to production of the umbelliferone derivative by using a quinine sulfate standard (3).

The activity of β-isopropylmalate (βIPM) isomerase (EC 4.2.1.33) was followed spectrophotometrically by the method of Gross et al. (9). The reaction mixture contained, in a 1.5-ml volume, 65 μmol of potassium phosphate, pH 7.0, and 0.5 μmol of βIPM. The absorbance at 235 nm of the reaction mixture increased linearly for at least 60 min at room temperature.

The activity of βIPM dehydrogenase (EC 1.1.1.85) was measured by a modification of the method of Stieglitz and Calvo (17). The reaction mixture contained, in 0.5 ml, 150 μmol of Tris-hydrochloride, pH 8.0, 50 μmol of KCl, 0.05 μmol of $MnCl_2$, 1 μmol of nicotinamide adenine dinucleotide, and 0.5 μmol of βIPM, and was incubated at 39 C. Reaction kinetics were linear over 1 h, and the reaction was usually stopped after 30 min. Xylene was substituted for ether for sample processing, and extract activities were related to a standard curve of the reaction product α-ketoisocaproate treated in this way.

The three enzyme activities differed markedly in stability in extracts. As in other systems (2, 18), βIPM isomerase activity was especially labile. Activity was rapidly lost upon freezing whole cells before lysis and it was thus routinely measured immediately after lysis of freshly harvested cells. Activity was also unstable in assay buffer lacking substrate (80% loss in 5 h at 0 C), but this loss could be prevented by inclusion of substrate at 0.5 mM. The αIPM synthetase and βIPM dehydrogenase activities were far more stable in extracts. At pH 8 there was essentially no loss of βIPM dehydrogenase activity in extracts refrigerated overnight, and the activity was completely stable in extracts at either pH 6.5 or 8.0 for at least 5 h at room temperature. The αIPM synthetase activity was also relatively stable in extracts, retaining two-thirds of the activity after overnight refrigeration. However, desalting of crude extracts by passage through Sephadex G-25, as described below, caused 50% loss in βIPM synthetase activity and was not performed routinely.

Chemicals. The βIPM, 45% pure, was a gift from J. M. Calvo. Quinine sulfate was from Fisher Chemical Co. All other reagents were purchased from Sigma Chemical Co.

RESULTS

Characterization of leucine auxotrophs. Four independent leucine-requiring mutants were obtained by penicillin selection (see Materials and Methods) and one strain, *leu* 201, was chosen for extensive study. The frequency of prototrophic revertants in cultures of this strain was less than 2×10^{-8}. On agar, none of the leucine auxotrophs would form colonies in the absence of exogenous L-leucine. In unsupplemented liquid medium, growth of strain *leu* 201 ceased within one doubling (Fig. 1) and chlorosis (the loss of the blue biliprotein phycocyanin, which characterizes blue-green cultures starved for nitrogen, phosphorus, or certain other essential nutrients) was readily apparent. In media supplemented with 50 to 100 μg of L-leucine/ml, growth of strain *leu* 201 was indistinguishable from that of wild type.

Most, if not all, bacterial strains that can synthesize leucine use the isopropylmalate pathway (α-ketoisovalerate $\xrightarrow{[a]}$ αIPM $\xrightarrow{[b]}$ βIPM $\xrightarrow{[d]}$ α-ketoisocaproate $\xrightarrow{[c]}$ leucine [17]). The three enzymatic activities specific for leucine biosynthesis (αIPM synthetase [a]; βIPM isomerase [b]; and βIPM dehydrogenase [c]) were examined in extracts of wild type and each of the four independently selected leucine auxotrophs. Cultures were grown in medium supplemented with 100 μg of L-leucine per ml and harvested in mid-log phase. Half the collected cells of each type was retained for enzyme assays, and the other half was resuspended in leucine-free medium and incubated an additional 20 to 24 h before harvesting for enzyme assays. All three enzymatic activities required for leucine biosynthesis via the isopropylmalate pathway were present in extracts of

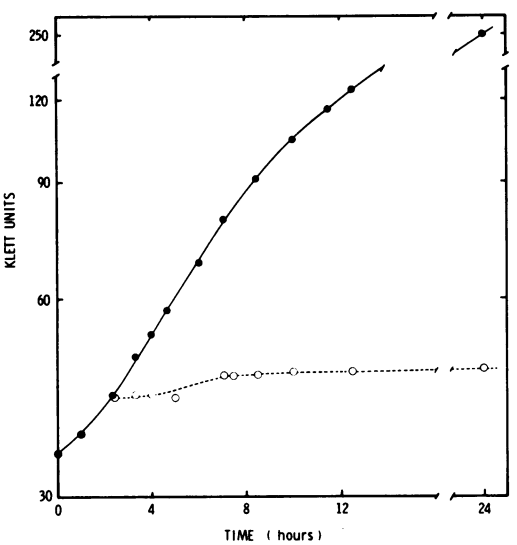

FIG. 1. *Growth of auxotroph leu 201 with and without leucine. A culture of leu 201 growing logarithmically in medium containing 100 μg of L-leucine per ml was harvested by centrifugation, washed in leucine-free medium, and suspended in medium containing either leucine at 100 μg/ml or no exogenous amino acid. Incubation was continued with illumination at 39 C under 5% CO_2 in air. Symbols: ●, Turbidity at 660 nm in culture with leucine; ○, turbidity at 660 nm in culture without leucine.*

wild-type *A. nidulans* (Table 1). Only traces of αIPM synthetase activity could be detected in extracts of three of the auxotrophic mutants, whereas the fourth showed less than 10% of wild-type activity. Specific activities of βIPM isomerase and βIPM dehydrogenase, when tested, were comparable to those of wild type. Mixing experiments with wild-type and strain *leu* 201 extracts showed that extracts of this mutant, at least, did not contain an inhibitor of αIPM synthetase activity.

Control of leucine biosynthesis. The activity of αIPM synthetase from many prokaryotes (17) is inhibited by leucine, the end product of the pathway. Feedback inhibition by leucine of αIPM synthetase from *A. nidulans* was examined in wild-type extracts after passage through Sephadex G-25 columns (1.4 by 9 cm) to remove endogenous leucine. The results presented in Fig. 2 show that αIPM synthetase is indeed inhibited by leucine, with 50% inhibition at about 2×10^{-5} M L-leucine, a concentration somewhat lower than those needed under comparable assay conditions for 50% inhibition in extracts of other bacteria (17). The related branched-chain amino acids isoleucine and valine inhibited less than 25% even when present at 10^{-3} M. L-Leucine had no effect on βIPM dehydrogenase activity in crude extracts (Fig. 2). Thus, leucine biosynthesis in *A. nidulans* is likely regulated by leucine at the level of enzyme activity, through feedback inhibition of the first pathway-specific enzyme.

Regulation at the level of enzyme synthesis, on the other hand, may be of only marginal significance in *A. nidulans*, since neither mutant nor wild-type strains appreciably altered specific activities of leucine biosynthetic enzymes in response to exogenous amino acid. This is apparent both from Table 1, where activities of leucine-supplemented and leucine-starved cultures of wild type and leucine auxotrophs are compared, and from Fig. 3, which presents results of a kinetic experiment with strain *leu* 201. For this latter experiment, a culture of strain *leu* 201 growing logarithmically (under 5% CO_2 in air) in medium containing 100 μg of L-leucine per ml was harvested

TABLE 1. *Enzymatic activities in crude extracts of wild-type and mutant strains*

Strain	Leucine concn in growth medium (μg/ml)	Sp act[a]		
		αIPM synthetase	βIPM isomerase	βIPM dehydrogenase
Wild type	100	0.26	0.45	16.2
Wild type	0	0.29	0.73	14.4
leu 10	100	0.008	ND[b]	20.9
leu 10	0	<0.0002	ND	16.4
leu 91	100	0.002	ND	22.6
leu 91	0	<0.002	0.51	14.9
leu 201	100	0.001	0.59	16.4
leu 201	0	<0.0002	0.53	19.5
leu 212	100	0.017	ND	18.1
leu 212	0	0.005	0.30	16.4

[a] Nanomoles of substrate used per minute per milligram of protein.

[b] ND, Not determined.

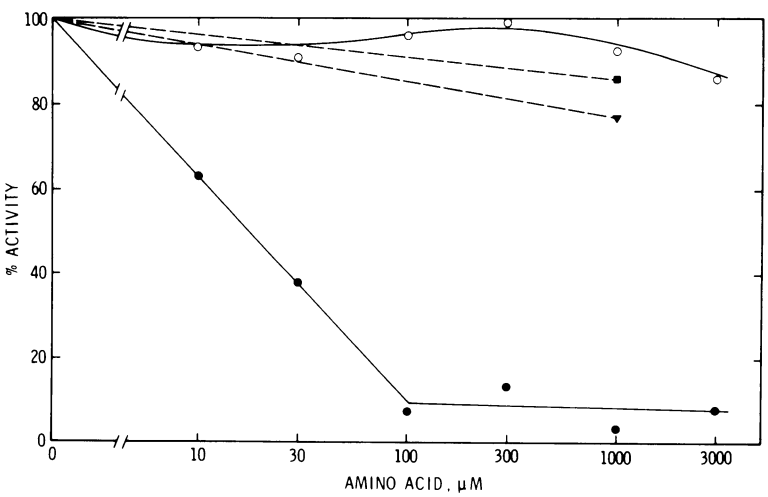

FIG. 2. *Inhibition of α-isopropylmalate synthetase and β-isopropylmalate dehydrogenase by amino acids. Activity of α-isopropylmalate synthetase was measured in extracts of wild type in the presence of either L-leucine (●), L-valine (▼), or L-isoleucine (■). Open circles are β-isopropylmalate dehydrogenase activity in the same extracts, in the presence of leucine.*

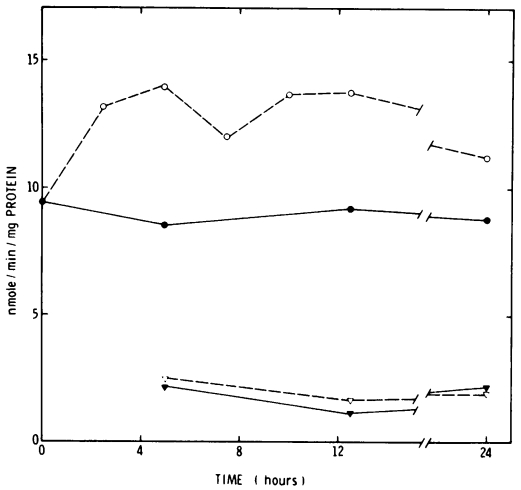

FIG. 3. *Specific activities of β-isopropylmalate isomerase and β-isopropylmalate dehydrogenase in leu 201. Samples were withdrawn at intervals from the leucine-free and leucine-containing cultures described in the legend to Fig. 1. Extracts were prepared and assayed as described in Materials and Methods. Symbols: ●, β-Isopropylmalate dehydrogenase, leucine-containing culture; ○, β-isopropylmalate dehydrogenase, leucine-free culture; ▼, β-isopropylmalate isomerase, leucine-containing culture; ▽, β-isopropylmalate isomerase, leucine-free culture.*

and washed by centrifugation, suspended in fresh medium with or without 100 μg of L-leucine per ml, and incubated under 5% CO_2 in the light for 24 h. Both cultures resumed logarithmic growth after a short lag (Fig. 1). In unsupplemented medium, however, growth ceased after only a 25% increase in turbidity and the culture became chlorotic. Activities of βIPM isomerase and βIPM dehydrogenase were assayed at intervals. Specific activities of βIPM isomerase were the same throughout the experiment in both the leucine-supplemented, growing culture and the unsupplemented, starving culture. Levels of βIPM dehydrogenase did increase after starvation, but the specific activities achieved were at no time more than 50% greater than those of the leucine-containing culture.

It could be argued that sudden, complete deprivation of exogenous leucine in the experiment illustrated in Fig. 1 and 3 reduced internal pools of this amino acid to levels too low to support de novo synthesis of any proteins. Therefore, similar experiments were performed in which cells grown in L-leucine at 100 μg/ml were washed and resuspended in medium containing only limiting concentrations of the amino acid (2 and 0.5 μg/ml). At these concentrations, growth of strain *leu* 201 ceased after increases in turbidity of approximately 150 and 50%, respectively. Specific activities of βIPM dehydrogenase in both subcultures (measured 48 h after resuspension in limiting leucine) were only 10% higher than the specific activity measured at zero time (immediately before resuspension).

Among mutants of *Bacillus subtilis* selected as resistant to the leucine analogue 4-azaleucine, a substantial proportion are found to produce high levels of leucine biosynthetic enzymes (18). Of nine independently selected 4-azaleucine-resistant mutants of *A. nidulans*, none showed levels of αIPM synthetase or βIPM dehydrogenase significantly greater than those of wild type when grown on leucine-free medium. At least six of these mutants produced an αIPM synthetase which, in crude extracts, appeared less sensitive to inhibition by leucine and/or 4-azaleucine than was the wild-type enzyme.

DISCUSSION

Our data suggest that *A. nidulans*, like *Escherichia coli* (15), *Salmonella typhimurium* (2), *B. subtilis* (18), *Hydrogenomonas* spp. (14), and 13 other diverse bacteria (17), uses the isopropylmalate pathway for leucine biosynthesis. The absolute requirement for leucine of the αIPM synthetase-deficient mutant strain *leu* 201 confirms the role of this enzyme in leucine formation. Essential and specific roles for βIPM isomerase and βIPM dehydrogenase may be inferred but not proven without mutants lacking these activities. As with other bacteria, the initial enzyme specific to leucine biosynthesis in *A. nidulans* is strongly inhibited by the end product, leucine, and not by the structurally related amino acids, valine and isoleucine. Unlike heterotrophic bacteria, however, *A. nidulans* appears to exert little control over the synthesis of leucine pathway enzymes in response to drastic alterations in leucine availability. Starvation conditions that produce at least fivefold increases in the levels of leucine biosynthetic enzymes in leucine-requiring strains of *S. typhimurium* and *B. subtilis* (8, 18) provoke in *A. nidulans* auxotrophs at most a 1.5-fold increase in βIPM dehydrogenase specific activity and no change in βIPM isomerase levels. Since such conditions can initiate degradation of phycocyanin (chlorosis), it remains possible that leucine liberated from this major biliprotein accessory pigment soon restores pools of this amino acid to repressing levels. We cannot exclude this possibility without appropriate chemostat data, but we can argue here that controls at the level of gene expression which respond to amino acid deprivation only after destruction of much of the cell's light-harvest-

ing ability can be of little functional significance or selective advantage to an obligate photoautotroph such as *A. nidulans*.

Three previous studies using auxotrophs also bear on the question of repressibility of the formation of amino acid biosynthetic enzymes in blue-green bacteria. In experiments similar to those presented here, Delaney et al. (5) found no repression by methionine of the synthesis of the first methionine pathway-specific enzyme in *A. nidulans*, and Kaney and Jhabvala (12) found no effect of exogenous phenylalanine on levels of enzymes of phenylalanine biosynthesis in the related strain *Synechococcus cedrorum*. In contrast, Ingram et al. (10) observed substantial (but noncoordinate) derepression of the formation of tryptophan biosynthetic enzymes in a tryptophan auxotroph of *Agmenellum quadruplicatum*. All but the last study are consistent with the hypothesis of Carr et al. (4, 5), who maintain that blue-green bacteria are in general incapable of controlling metabolism through induction or repression of enzyme synthesis. It is difficult, however, to visualize specific blocks to the evolution of such mechanisms, should their development have been of selective advantage in niches traditionally occupied by blue-green bacteria.

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LITERATURE CITED

1. Allen, M. M., and A. J. Smith. 1969. Nitrogen chlorosis in blue-green algae. Arch. Mikrobiol. 69:114–120.
2. Burns, R. O., J. Calvo, P. Margolin, and H. E. Umbarger. 1966. Expression of the leucine operon. J. Bacteriol. 91:1570–1576.
3. Calvo, J. M., J. C. Bartholomew, and B. I. Stieglitz. 1969. Fluorometric assay of enzymatic reactions involving acetyl coenzyme A in aldol condensations. Anal. Biochem. 28:164–181.
4. Carr, N. G. 1973. Metabolic control and autotrophic physiology, p. 39–65. *In* N. G. Carr and B. A. Whitton (ed.), The biology of the blue-green algae. Blackwell Scientific Publications, Oxford.
5. Delaney, S. F., A. Dickson, and N. G. Carr. 1973. The control of homoserine-O-transsuccinylase in a methionine requiring mutant of the blue-green alga *Anacystis nidulans*. J. Gen. Microbiol. 79:89–94.
6. Doolittle, W. F. 1973. Postmaturational cleavage of 23*s* ribosomal ribonucleic acid and its metabolic control in the blue-green alga *Anacystis nidulans*. J. Bacteriol. 113:1256–1263.
7. Doolittle, W. F., and R. A. Singer. 1974. Mutational analysis of dark endogenous metabolism in the blue-green bacterium *Anacystis nidulans*. J. Bacteriol. 119:677–683.
8. Freundlich, M., R. O. Burns, and H. E. Umbarger. 1962. Control of isoleucine, valine, and leucine biosynthesis. I. Multi-valent repression. Proc. Natl. Acad. Sci. U.S.A. 48:1804–1808.
9. Gross, S. R., R. O. Burns, and H. E. Umbarger. 1963. The biosynthesis of leucine. II. The enzymic isomerization of β-carboxy-β-hydroxyisocaproate and α-hydroxy-β-carboxyisocaproate. Biochemistry 2:1046–1052.
10. Ingram, L. O., D. Pierson, J. E. 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.
11. 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.
12. Kaney, A. R., and P. Jhabvala. 1975. Absence of repression in a phenylalanine auxotroph of *Synechococcus cedrorum*. J. Gen. Microbiol. 87:370–372.
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
14. Schlegel, H. G., and U. Eberhardt. 1972. Regulatory phenomena in the metabolism of knallgasbacteria. Adv. Microbiol. Physiol. 7:205–242.
15. Somers, J. M., A. Amzallag, and R. B. Middleton. 1973. Genetic fine structure of the leucine operon of *Escherichia coli* K-12. J. Bacteriol. 113:1268–1272.
16. Stanier, R. Y., R. Kunisawa, M. Mandel, and G. Cohen-Bazire. 1971. Purification and properties of unicellular blue-green algae (order *Chroococcales*). Bacteriol. Rev. 35:171–205.
17. Stieglitz, B. I., and J. M. Calvo. 1974. Distribution of the isopropylmalate pathway to leucine among diverse bacteria. J. Bacteriol. 118:935–941.
18. Ward, J. B., and S. A. Zahler. 1973. Regulation of leucine biosynthesis in *Bacillus subtilis*. J. Bacteriol. 116:727–735.