

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

RICHARD A. SINGER AND W. FORD DOOLITTLE*

Department of Biochemistry, Dalhousie University, Halifax, Nova Scotia, Canada

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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 μ g 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 μ g 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% CO₂ 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 MgCl₂ and 10^{-4} M sodium ethylenediamine-tetraacetate, and disrupted in an Aminco French pressure cell at 1.1×10^7 kg/m². After clarification by centrifugation at $25,000 \times g$ 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 (α IPM) 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 μ mol of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.5, 250 μ mol of KCl, 2.25 μ mol of α -ketoisovalerate, 5 μ mol of acetyl phosphate, 1 U of phosphotransacetylase, and 0.125 to 0.25 μ mol 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 (β IPM) 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 μ mol of potassium phosphate, pH 7.0, and 0.5 μ mol of β IPM. The absorbance at 235 nm of the reaction mixture increased linearly for at least 60 min at room temperature.

The activity of β IPM 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 μ mol of Tris-hydrochloride, pH 8.0, 50 μ mol of KCl, 0.05 μ mol of $MnCl_2$, 1 μ mol of nicotinamide adenine dinucleotide, and 0.5 μ mol of β IPM, 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), β IPM 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 α IPM synthetase and β IPM dehydrogenase activities were far more stable in extracts. At pH 8 there was essentially no loss of β IPM 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 α IPM 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 β IPM synthetase activity and was not performed routinely.

Chemicals. The β IPM, 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 phyco-cyanin, which characterizes blue-green cultures starved for nitrogen, phosphorus, or certain other essential nutrients) was readily apparent. In media supplemented with 50 to 100 μ g 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 $\xrightarrow{[a]}$ α IPM $\xrightarrow{[b]}$ β IPM $\xrightarrow{[d]}$ α -ketoisocaproate $\xrightarrow{[c]}$ leucine [17]). The three enzymatic activities specific for leucine biosynthesis (α IPM synthetase [a]; β IPM isomerase [b]; and β IPM 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 μ g 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

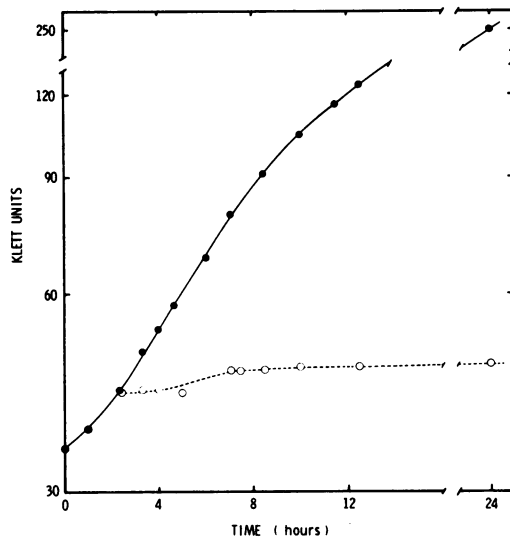


FIG. 1. Growth of auxotroph *leu* 201 with and without leucine. A culture of *leu* 201 growing logarithmically in medium containing 100 μ g of L-leucine per ml was harvested by centrifugation, washed in leucine-free medium, and suspended in medium containing either leucine at 100 μ g/ml or no exogenous 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 α IPM 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 β IPM isomerase and β IPM 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 α IPM synthetase activity.

Control of leucine biosynthesis. The activity of α IPM synthetase from many prokaryotes (17) is inhibited by leucine, the end product of the pathway. Feedback inhibition by leucine of α IPM 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 α IPM 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 β IPM 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% CO₂ in air) in medium containing 100 μ g 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 (μ g/ml)	Sp act ^a		
		α IPM synthetase	β IPM isomerase	β IPM dehydrogenase
Wild type	100	0.26	0.45	16.2
Wild type	0	0.29	0.73	14.4
<i>leu</i> 10	100	0.008	ND ^b	20.9
<i>leu</i> 10	0	<0.0002	ND	16.4
<i>leu</i> 91	100	0.002	ND	22.6
<i>leu</i> 91	0	<0.002	0.51	14.9
<i>leu</i> 201	100	0.001	0.59	16.4
<i>leu</i> 201	0	<0.0002	0.53	19.5
<i>leu</i> 212	100	0.017	ND	18.1
<i>leu</i> 212	0	0.005	0.30	16.4

^a Nanomoles of substrate used per minute per milligram of protein.

^b ND, Not determined.

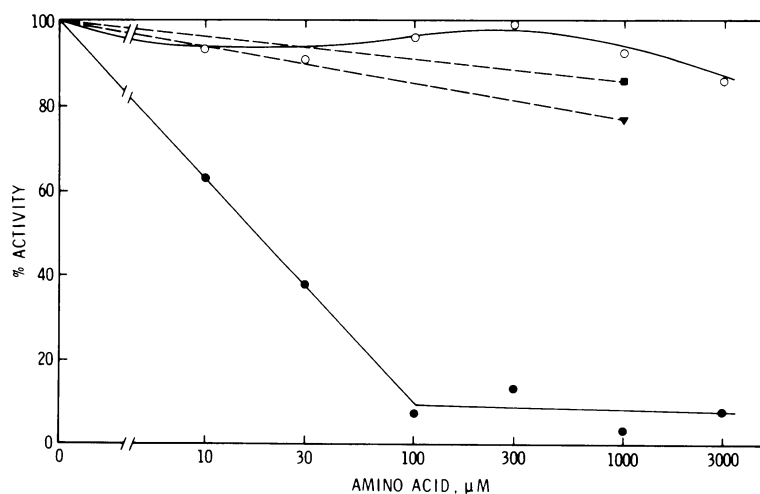


FIG. 2. Inhibition of α -isopropylmalate synthetase and β -isopropylmalate dehydrogenase by amino acids. Activity of α -isopropylmalate synthetase was measured in extracts of wild type in the presence of either L-leucine (\bullet), L-valine (\blacktriangledown), or L-isoleucine (\blacksquare). Open circles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

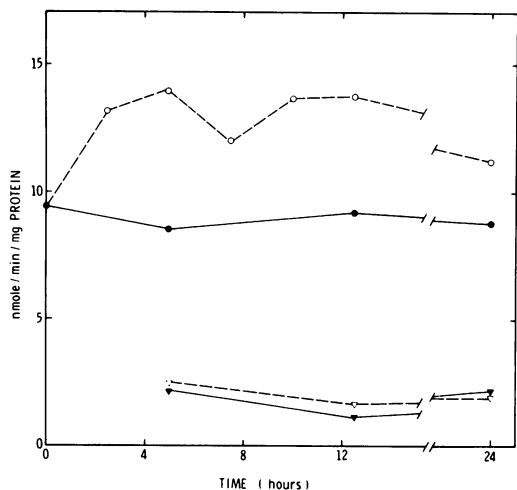


FIG. 3. Specific activities of β -isopropylmalate isomerase and β -isopropylmalate dehydrogenase in *leu 201*. Samples were withdrawn at intervals from the leucine-free and leucine-containing cultures described in the legend to Fig. 1. Extracts were prepared and assayed as described in Materials and 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 μ g of L-leucine per ml, and incubated under 5% CO₂ 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 β IPM isomerase and β IPM dehydrogenase were assayed at intervals. Specific activities of β IPM isomerase were the same throughout the experiment in both the leucine-supplemented, growing culture and the unsupplemented, starving culture. Levels of β IPM 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 μ g/ml were washed and resuspended in medium containing only limiting concentrations of the amino acid (2 and 0.5 μ g/ml). At these concentrations, growth of strain *leu 201* ceased after increases in turbidity of approximately 150 and

50%, respectively. Specific activities of β IPM 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 α IPM synthetase or β IPM dehydrogenase significantly greater than those of wild type when grown on leucine-free medium. At least six of these mutants produced an α IPM 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 α IPM synthetase-deficient mutant strain *leu 201* confirms the role of this enzyme in leucine formation. Essential and specific roles for β IPM isomerase and β IPM 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 β IPM dehydrogenase specific activity and no change in β IPM 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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