Branched-chain Amino Acid Biosynthesis in the Blue-green Alga *Anabaena variabilis*

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SUMMARY

Four enzymes (threonine deaminase, α -aceto-hydroxyacid synthetase, isopropylmalate synthetase and transaminase B) involved in leucine, isoleucine and valine synthesis have been detected in extracts of the blue-green alga *Anabaena variabilis* and the control of three of the enzymes examined. A new procedure for the determination of transaminase B is described. Threonine deaminase and α -aceto-hydroxyacid synthetase are subject to feedback control by their end-products. No evidence for the repression of enzyme synthesis in the pathway has been found, although the assimilation and incorporation of exogenous valine and leucine was demonstrated.

INTRODUCTION

Previous results have indicated that several major intermediary metabolic sequences in the blue-green algae, *Anabaena variabilis* and *Anacystis nidulans*, are not regulated by the process of repression and derepression of enzyme biosynthesis (Pearce & Carr, 1967, 1969; Pearce, Leach & Carr, 1969). Blue-green algae are known to excrete large quantities of amino acids as peptides during growth. We have previously suggested that this may be a result of inability to control the synthesis of amino acids. No evidence of repression of the enzymes involved in the synthesis of arginine from glutamate by *A. variabilis* was observed when the arginine was added to the growth medium, although it was incorporated into protein (Hood & Carr, 1971). Similarly, the inclusion in the growth medium of aromatic amino acids did not reduce the level of 3-deoxy-D-arabinoheptulosonic acid-7-phosphate synthetase in *A. nidulans* (Weber & Böck, 1968).

The pathway of biosynthesis of valine, isoleucine and leucine has been elucidated in several micro-organisms (see Meister, 1957) and is unusual in that four enzymes (aceto-hydroxyacid synthetase, aceto-hydroxyacid isomeroreductase, dihydroxyacid dehydrase and transaminase B) are common to the synthesis of all three amino acids. The control of the synthesis of branched-chain amino acids, by both repression of enzyme synthesis and by feedback inhibition, has been well documented in several bacteria (see Umbarger, 1969). This communication describes some regulatory aspects of valine, leucine and isoleucine biosynthesis in obligately autotrophic blue-green algae; a preliminary account of some of the data has been presented (Hood & Carr, 1968).

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METHODS

Organisms and culture conditions. The *Anabaena variabilis* employed was the non-nitrogen fixing strain of Professor J. Myers, Department of Zoology, University of Texas, Austin. *Anacystis nidulans* was from the Culture Collection of Algae (no. 625) at Indiana University and *Escherichia coli* from the N.C.I.B. (no. 8114), Torry Research Station, Aberdeen. Blue-green algae were maintained and cultured as previously described (Pearce & Carr, 1967). The autotrophic growth medium C (Kratz & Myers, 1955) was supplemented with 0.5% NaHCO₃ and, where indicated, with L-valine, L-leucine, or L-isoleucine. Flasks (5 l) were gassed with air:CO₂ (95:5) and were placed 23 cm from 60 W warm-white fluorescent lamps and maintained at 34°C. *Escherichia coli* was grown in the mineral salt medium of Davis & Mingioli (1950) plus glucose (2 g/l) and, where indicated, with L-valine, L-leucine or L-isoleucine.

Preparation of cell-free extracts. *Anacystis nidulans* and *Escherichia coli* were harvested at room temperature at 3000 g for 15 min whilst *Anabaena variabilis* required only 1500 g for 5 min. Organisms were washed twice with 5 mM-potassium phosphate, pH 7.0, suspended (20 to 25 mg/ml) in a small volume of the same buffer and disrupted at 2°C by three 30 s bursts (separated by 1.5 min intervals to prevent overheating) in an MSE 60 W 20 kc/s sonic disintegrator. Unbroken organisms and cell debris were removed by centrifugation at 10000 g for 15 min at 0°C.

Determination of growth rate and incorporation of U-¹⁴C amino acids into blue-green algae. Cultures of blue-green algae (100 ml) were grown in 250 ml conical flasks to which side arms capable of fitting into an EEL colorimeter were annealed, in a Gallenkamp Illuminated Orbital Incubator at 34°C which was continually flushed with air:CO₂ (95:5). Light was supplied by four 60 W warm-white fluorescent lamps 30 cm above the cultures. Growth was measured turbidimetrically and dry weight was determined by reference to a standard curve. When determining the rate of incorporation of ¹⁴C amino acids, samples were removed and the organisms collected by membrane filtration (Millipore Filter, HAWP, 0.45 µ). The filters were washed 10 times with 5 mM-potassium phosphate (pH 7.0) to remove extraneous ¹⁴C acid, and then dried. Isotopic incorporation was measured, in duplicate, in a Nuclear Chicago Gas Flow Planchet Counter at an efficiency of 24%. Sufficient counts were recorded to ensure an accuracy of at least 1%. Fractionation of blue-green algae after growth on ¹⁴C amino acids was carried out by the procedure of Roberts *et al.* (1955).

Enzyme assays

β-Aspartokinase (ATP: L-aspartate 4-phosphotransferase; EC. 2.7.2.4). Reaction mixtures were prepared according to Black & Wright (1955) and the formation of the hydroxamic acid resulting from the action of hydroxylamine with acyl phosphate was determined by the method of Lipmann & Tuttle (1945).

α-Acetohydroxyacid synthetase (acetolactate synthetase). The procedure of Radhakrishnan & Snell (1960) was used, the acetolactate synthesized was converted to acetoin by heating and this was determined by the colorimetric procedure of Westerfeld (1945).

Threonine deaminase (threonine dehydrase, EC. 4.2.1.1). This enzyme was estimated by measuring the amount of α-ketobutyrate formed from threonine by the method of Friedemann & Haugen (1943). Reaction mixtures were prepared according to Yanofsky & Reissig (1953) and incubated for 1 h.

α-Isopropylmalate synthetase. The formation of free sulphydryl groups, measured by the procedure of Grunert & Phillips (1951), resulting from the condensation of acetyl CoA

and α -ketoisovalerate, formed the basis of this assay (Burns, Calvo, Margolin & Umbarger, 1966).

Transaminase B. A procedure was developed for the measurement of transaminase B activity based on the selective solubility of the 2,4-dinitrophenylhydrazones of the product and substrate keto acids. Reaction mixtures contained (μ moles): potassium phosphate buffer (pH 7.0), 200; α -ketoisovalerate, 3.3; L-glutamate sodium salt, 20; pyridoxal phosphate, 0.04; and 0 to 16 mg of algal protein in a total volume of 2.0 ml. Alanine or aspartate may replace glutamate as the amino group donor. After incubation (30 min) at 34°C, the reaction was stopped by addition of 1.0 ml of 10% trichloroacetic acid and the precipitated protein was removed by centrifugation. The deproteinized supernatants were added to 0.4 ml of a 1% solution of 2,4-dinitrophenylhydrazine in 2N-HCl and incubated for 30 min. The 2,4-dinitrophenylhydrazones of both α -ketoisovalerate and α -oxoglutarate were dissolved in 8 ml of ethyl acetate. After vigorous shaking the lower aqueous layer was removed by a pipette and 10% sodium carbonate (6 ml) added to the ethyl acetate. Only the 2,4-dinitrophenylhydrazone of the product keto acid, α -oxoglutarate (or pyruvate or oxaloacetate when alanine or aspartate were used as the amino group donors) was soluble in the aqueous layer, the 2,4-dinitrophenylhydrazone of the substrate keto acid, α -ketoisovalerate remained in the ethyl acetate. A major portion (5 ml) of the lower aqueous layer was removed and added to an equal volume of 1.5 N-NaOH. The extinction was measured at the absorption maxima for the appropriate 2,4-dinitrophenylhydrazone (410 nm for α -oxoglutarate, 445 nm for pyruvate, 450 nm for α -oxaloacetate) and compared with a standard curve of the authentic keto acid derivative. The formation of valine from α -ketoisovalerate was demonstrated by incubating the assay mixture with extract from *Anabaena variabilis* and terminating the reaction by the addition of acetic acid (0.2 ml) and 95% ethanol (0.8 ml). The deproteinized supernatant was co-chromatographed on paper with authentic valine in *n*-propanol:water (4:1, v/v) and ethanol:ammonia:water (18:1:1, by vol.). The position of the valine was located by spraying with 1% ninhydrin in acetone. The 2,4-dinitrophenylhydrazone derivatives of the keto acids resulting from *A. variabilis* transaminase B activity were identified by paper chromatography using *n*-butanol:ethanol:water:ammonia (140:20:40:1, by vol.); methanol:benzene:*n*-butanol:water (2:1:1:1, by vol.); *n*-butanol:N-sodium carbonate (1:2, v/v).

Hydrolysis of intact organisms. *Anabaena variabilis*, after growth in the presence of labelled compounds, were harvested and washed twice in the presence of the same unlabelled substrate and then hydrolysed in 6 N-HCl in a sealed pyrex tube at 108°C for 16 h. The HCl was removed from the hydrolysates by drying for 24 h under vacuum. The residue was taken up in 1 ml of warm water and filtered. The filtrates were extracted twice with equal volumes of ethyl ether and the other extracts were discarded. The aqueous layer was dried under vacuum and the residue taken up in less than 1 ml of isopropanol and water (20:80, v/v). The components were separated by two dimensional thin-layer chromatography on cellulose plates by using *n*-butanol:acetic acid:water (12:3:5, by vol.) followed by ethanol:ammonia:water (18:1:1, by vol). Autoradiograms were obtained using Kodirex X-ray film.

Chemicals. All chemicals were of analytical grade or of the purest commercial grade available and were, with the exceptions listed below, obtained from British Drug House Ltd, Poole, Dorset, England. ATP, coenzyme A and flavin adenine dinucleotide were obtained from C. F. Boehringer and Soehne GmbH., Mannheim, Germany; α -ketoisovaleric acid, α -ketoisocaproic acid, pyridoxal phosphate and thiamine pyrophosphate were obtained from Sigma Chemical Co. Ltd, London, W. 6; α -ketoisobutyric acid was obtained

Fig. 1(a). Inhibition of threonine deaminase of *Anabaena variabilis* by isoleucine, and restoration of activity by increased threonine. Each reaction mixture contained 2 mg protein; ○—○, standard assay mixture except for threonine concentration, ●—●, as above plus L-isoleucine (6.6×10^{-2} mM).

Fig. 1(b). Antagonism by valine of the isoleucine inhibition of threonine deaminase from *Anabaena variabilis*. ▲—▲, Standard reaction mixture plus L-isoleucine (10^{-4} M); ○—○, standard plus L-isoleucine (2×10^{-4} M); △—△, standard plus L-isoleucine (3×10^{-4} M); ●—●, standard plus L-isoleucine (5×10^{-4} M).

Table 1. Effect of inclusion of branched-chain amino acids in the growth medium of *Anabaena variabilis* on the activities of three enzymes involved in their biosynthesis

Enzyme	Additions to growth medium	Activity (nmol/h/mg)
Threonine deaminase	None	0.51
	+ L-Isoleucine (1.5 mM)	0.48
	L-Isoleucine	0.57
	L-Valine	
α -Acetohydroxyacid synthetase	L-Leucine	0.57
	None	
	Valine (mM)	27
	L-Valine	24
Transaminase B	L-Isoleucine	
	L-Leucine	80
	L-Isoleucine	
	None	69

from Calbiochem, Los Angeles, California, U.S.A.; [U- 14 C]-L-aspartate, [U- 14 C]-L-valine, [U- 14 C]-L-isoleucine and [U- 14 C]-L-leucine were obtained from the Radiochemical Centre, Amersham, Buckinghamshire.

RESULTS

Growth studies. Leucine, valine and isoleucine, singly or in combination, had no effect on the growth rate of *Anabaena variabilis* when they were included in the medium at concentrations between 0.5 and 6 mM; they did, however, increase the lag phase of cultures by about one third. Other amino acids were likewise without effect on the growth rate of

Fig. 2. Effect of pH on α -acetohydroxyacid synthetase in extracts of *Anabaena variabilis*. The standard reaction mixture (15 mg protein incubated for 2 h) employed sodium acetate buffer, pH 5.1 and 5.8; potassium phosphate buffer, pH 6.5 and 7.0; tris HCl, pH 8.0 and 9.0; and glycine:NaOH, pH 10.0.

Table 2. Effect of branched-chain amino acids on the activity of α -acetohydroxyacid synthetase from *Anabaena variabilis* and *Ancystis nidulans*

Addition to the assay reaction mixture	Rate (nmol/h/mg)	
	<i>A. variabilis</i>	<i>A. nidulans</i>
None	27	42
L-Valine (0.075 mM)	18	23
L-Valine (0.75 mM)	17	18
L-Leucine (0.066 mM)	25	29
L-Leucine (0.66 mM)	22	33
L-Isoleucine (0.066 mM)	22	33
L-Isoleucine (0.66 mM)	21	31
L-Valine (0.75 mM)	15	19
L-Leucine (0.66 mM)		
L-Isoleucine (0.66 mM)		

A. variabilis. However, slight stimulation (20%) of the growth rate was observed when certain amino acids, including leucine, valine and isoleucine, were added at 2 mM concentration to cultures of *Anacystis nidulans*.

Although no stimulation of growth rate by exogenous amino acids was observed, U- 14 C-labelled valine, leucine and isoleucine were readily and linearly incorporated by growing cultures of *Anabaena variabilis*. Fractionation of the organisms indicated that the major part of the [U- 14 C]valine incorporated was recovered in the protein fraction. When intact *A. variabilis* was hydrolysed and the constituent amino acids examined, only valine, and to a lesser extent leucine were radioactive.

Enzymes involved in branched-chain amino acid biosynthesis. No β -aspartokinase activity was detected in extracts of *Anabaena variabilis*, although the assay procedure employed was successful when applied to extracts of *Escherichia coli*. When [U- 14 C]aspartate (100 μ Ci,

Fig. 3. Identification of the keto acid products of transaminase B in extracts of *Anabaena variabilis*. Descending paper chromatography in *n*-butanol:ethanol:water:ammonia (140:20:40:1, by vol.). a, 2,4-Dinitrophenylhydrazones of authentic α -oxoglutarate; b, 2,4-dinitrophenylhydrazones of authentic pyruvate; c, 2,4-dinitrophenylhydrazones of authentic α -ketoisovalerate; d, 2,4-dinitrophenylhydrazones of assay mixture containing α -ketoisovalerate and glutamate before extraction; e, 2,4-dinitrophenylhydrazones of assay mixture containing α -ketoisovalerate and alanine before extraction; f, as d except after extraction; g, as e except after extraction.

Table 3. *Variation in the components of the assay procedure for transaminase B in extracts of Anabaena variabilis*

Reaction mixture	Rate (nmol/h/mg)
Complete	85
– α -Ketoisovalerate	0
– Pyridoxal phosphate	80
– Glutamate	15
– Glutamate	
+ Alanine	50
– Glutamate	
+ Aspartate	21

5 mM) was added to growth medium, it was readily incorporated, and subsequent hydrolysis and chromatography revealed radioactivity in both the aspartic family of amino acids (methionine, lysine, threonine and isoleucine) and those derived from glutamate (proline and arginine). Apparently, therefore, phosphorylation of aspartate, as the first stage in the biosynthesis of methionine, lysine, threonine and isoleucine, does occur and failure to demonstrate aspartokinase was artifactual. The presence of radioactivity in glutamate, arginine and proline suggests that some aspartate had been converted to oxaloacetate which could have given rise to α -oxoglutarate and hence to glutamate, proline and arginine. The absence of a complete tricarboxylic acid cycle in *A. variabilis* (Pearce, Leach & Carr, 1969) prevents complete randomization of the label from aspartate into other amino acids.

Threonine deaminase was readily demonstrated in extracts of *Anabaena variabilis*. When

this enzyme was assayed in the presence of low concentrations of isoleucine marked inhibition occurred (Fig. 1*a*). Threonine deaminase activity was largely restored by increasing the substrate concentration, suggesting competitive inhibition of this enzyme by isoleucine; this inhibition was relieved by valine (Fig. 1*b*). No repression of threonine deaminase was observed when *A. variabilis* was grown in the presence of isoleucine, or a combination of valine, leucine and isoleucine (Table 1).

α -Acetohydroxyacid synthetase was detected in extracts of *Anabaena variabilis* and *Anacystis nidulans* at low specific activities over a wide pH range with a minor peak of activity at pH 5.8 and maximal activity at pH 8.0 (Fig. 2). The inclusion in the assay mixture of branched-chain amino acids, either alone or in combination, was examined (Table 2). Valine was the most effective single inhibitor tested and a combination of valine, leucine and isoleucine gave a further slight increase in the inhibition of α -acetohydroxyacid synthetase from *A. variabilis*. Valine was equally inhibitory to α -acetohydroxyacid synthetase when tested at both peaks of activity, at pH 6.0 and 8.0.

Flavin adenine dinucleotide (1.6 mM) activated α -acetohydroxyacid synthetase from *Anabaena variabilis* by nearly 100%. In the presence of flavin adenine dinucleotide, valine (0.5 mM) still exerted its inhibitory effect on this enzyme. The addition of valine to the growth medium led to slightly lower activity of this enzyme in *A. variabilis* extracts, and inclusion of all three branched amino acids caused a 20% decrease in this organism and a 50% decrease in *Anacystis nidulans* (Table 1). For comparison *Escherichia coli* was grown in the presence and absence of the branched-chain amino acids, which caused a reduction in α -acetohydroxyacid synthetase of 75%.

The procedure developed for the assay of transaminase B was based upon the different solubilities of the 2,4-dinitrophenylhydrazones of substrate keto acid (α -ketoisovalerate) and the product keto acid (α -oxoglutarate). Under the conditions employed, transaminase B activity was linear with protein concentration using extracts of both *Escherichia coli* and *Anabaena variabilis*. Both the keto acid and amino acid products of transaminase B activity in extracts of *A. variabilis* were identified chromatographically. The removal of the 2,4-dinitrophenylhydrazine of the substrate keto acid (α -isoketovaleate) from that of the product keto acid (α -oxoglutarate or pyruvate) by the ethyl acetate and sodium carbonate extraction procedure is shown in Fig. 3. The formation of valine from α -ketoisovalerate (and of leucine from α -ketoisocaproate) was also demonstrated chromatographically (see Methods). Application of this assay procedure to the transamination of α -ketoisocaproate to form leucine was limited by the non-quantitative extraction of the 2,4-dinitrophenylhydrazone of α -ketoisocaproate from ethyl acetate into sodium carbonate. Omission of α -ketoisovalerate abolished activity, whilst less than 20% activity remained when the amino group donor, glutamate, was absent; alanine or aspartate were less effective as amino group donors (Table 3). Since the 2,4-dinitrophenylhydrazone derivatives of α -oxaloacetate (from aspartate), α -oxoglutarate (from glutamate), and pyruvate (from alanine) were extracted into sodium carbonate from ethyl acetate to the same extent the differences in enzyme activity shown in Table 3 were due to the specificity of the enzyme for the amino donor and not due to the differential solubility of the 2,4-dinitrophenylhydrazones of the product keto acids.

No repression of transaminase B was observed when *Anabaena variabilis* was grown in the presence of valine, leucine and isoleucine (Table 1). However, measurement of the enzyme by the same procedure in *Escherichia coli* indicated that growth in the presence of the three branched-chain amino acids led to a reduction in specific activity from 1140 to 390 nmol oxoglutarate formed/h/mg.

The conversion of valine to leucine by *Anabaena variabilis* and the findings of Hoare, Hoare & Moore (1967), who used isotopic techniques, that acetate can be converted to leucine in *Anacystis nidulans*, indicated that the enzyme α -isopropylmalate synthetase was probably present in blue-green algae. Further evidence for this was obtained with extracts of *A. variabilis* in reaction mixtures containing all the components of the α -isopropylmalate synthetase assay system were found to contain more free sulphhydryl groups at the end of the incubation than controls lacking α -ketoisovalerate. Although this was indicative of the presence of α -isopropylmalate synthetase, the first of the three enzymes specific for leucine biosynthesis, the low levels of this enzyme and the inconsistent relationship between the enzymatic activity which could be measured and the protein concentration, made it unsuitable for studies of either end-product inhibition or repression.

DISCUSSION

The enzymes observed in extracts of the blue-green algae *Anabaena variabilis* indicate that pathways of biosynthesis of valine, isoleucine and leucine are similar to those described for bacteria (Meister, 1957) and therefore α -acetohydroxyacid synthetase and transaminase B are necessary for biosynthesis of all three amino acids. The incorporation of [14 C]-leucine, valine and isoleucine by *Anabaena variabilis* indicated that the observed absence of significant repression of the branched-chain amino acid biosynthetic enzymes in this organism was not due to the impermeability of the micro-organism to these compounds. The slightly reduced level of α -acetohydroxyacid synthetase in *A. variabilis* after growth in the presence of the three branched-chain amino acids may present partial repression of the enzyme, but more probably this was due to an inhibitive effect of the exogenous amino acids. A greater reduction of the enzyme level is seen in *Escherichia coli* grown under the same conditions. The level of α -acetohydroxyacid synthetase in valine-grown *A. variabilis* was certainly no lower than in minimally grown organisms and this contrasts with those bacteria where valine represses this enzyme (Halpern & Umbarger, 1959; Radhakrishnan & Snell, 1960). Neither threonine deaminase nor transaminase B were repressed when the three branched-chain amino acids were included in the growth medium of *A. variabilis*, again contrasting with other micro-organisms examined (Freundlich, Burns & Umbarger, 1962; Dwyer & Umbarger, 1968; Bussey & Umbarger, 1969; Magee & Hereford 1969). However Barritt (1971) did not observe any repression or derepression of threonine deaminase in wild-type or in an isoleucine-valine auxotrophic *Rhodopseudomonas spheroides*, although the activity of this enzyme was inhibited by isoleucine, and valine reversed this inhibition. The extent to which feedback inhibition, in the absence of transcriptional control, permits an organism to adjust its biosynthetic activities is not known, although it may be relevant to note that mutant strains of *Salmonella typhimurium* excrete leucine into the medium when either type of control has been lost (Calvo & Calvo, 1967). It appears that repression of enzymes involved in amino acid biosynthesis does not occur in the blue-green algae examined, at least in respect of the synthesis of branched-chain amino acids, arginine (Hood, Leaver & Carr, 1969; Hood & Carr, 1971) and aromatic amino acids (Weber & Böck, 1968).

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