The Control of Homoserine-*O*-transsuccinylase in a Methionine-requiring Mutant of the Blue-green Alga *Anacystis nidulans*

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SUMMARY

The regulation of the first step in methionine biosynthesis, homoserine-*O*-transsuccinylase, has been examined in a methionine-requiring mutant of the blue-green alga *Anacystis nidulans*. Using an improved assay for homoserine-*O*-transsuccinylase, no evidence of derepression of the biosynthesis of this enzyme was found, even under conditions of acute methionine starvation. End-product inhibition of the enzyme by homoserine, cystathionine and methionine was demonstrated, and shown in the latter case to be of an allosteric nature. The lack of transcription control of this enzyme is discussed as an example of a general phenomenon in this group of micro-organisms.

INTRODUCTION

It has been suggested that obligately phototrophic blue-green algae do not control their intermediary metabolism by repression and derepression of enzyme biosynthesis and that this lack of transcriptional regulation is the characteristic, and causative, feature of obligate autotrophic physiology (see Carr, 1973*a*). The inclusion of amino acids in the growth medium, and demonstration of their assimilation into cell material, was not accompanied by reduction in levels of amino-acid biosynthetic enzymes. This has been shown in detail for arginine and the branched chain amino acids in *Anabaena variabilis* (Hood & Carr, 1971; 1972) and for 3-deoxy-D-arabinoheptulosonic-7-phosphate synthetase when *Anacystis nidulans* was grown in the presence of aromatic amino acids (Weber & Böck, 1968). For example, the inclusion in the growth medium of valine or isoleucine, singly or in combination, did not alter the activities of threonine deaminase, α -acetoxyacid synthetase or transaminase B in *A. variabilis* (Hood & Carr, 1972). End-product interaction with existing enzymes was, however, readily shown. It is possible that the observations indicating lack of repression of biosynthetic enzymes following the addition of exogenous amino acids are a consequence of endogenous repression, that is repression by endogenously synthesized compounds available in excess amounts during normal growth. This idea has been examined in some detail with respect to arginine biosynthesis in *Chlamydomonas reinhardtii* by Holden & Morris (1970).

The isolation of a mutant of *Anacystis nidulans* which requires methionine for growth has permitted us to exclude the possibility of endogenous repression, at least in the case of methionine biosynthesis. This paper describes the measurement of homoserine-*O*-transsuccinylase, the enzyme necessary for the first stage in methionine synthesis, in cultures under conditions of methionine adequacy and depletion.

METHODS

Organisms and growth conditions. A methionine-requiring mutant (1173) was derived from the parent *Anacystis nidulans* (no. 625, Collection of Algae and Protozoa, University of Indiana, Bloomington, Indiana, U.S.A.) by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment and penicillin enrichment (Herdman & Carr, 1972). Stock cultures were maintained on mineral salt agar (Allen, 1968) supplemented with 0.1 mM L-methionine. Cultures for experimental purposes were grown on the mineral-salt medium C of Kratz & Myers (1955), plus 0.05 % NaHCO₃ and supplemented with methionine as described. Flasks (5 l) were incubated at 34 °C 9 inches in front of three 40 W warm-white fluorescent tubes and gassed with air plus carbon dioxide (95:5, v/v). Wild-type *A. nidulans* was grown in the absence of methionine under otherwise identical conditions.

Preparation of extracts. Cultures were harvested by centrifugation at room temperature at approx. 2500 g. After resuspension in 10 mM-potassium phosphate (pH 7.6) the organisms were disrupted by three 1-min bursts of ultrasound (MSE 100 W Sonic Disintegrator), the containing vessel being held in ice and allowed to cool for 1 min between bursts. Unbroken cells and debris were removed by centrifugation at 15000 g for 10 min; a chlorophyll-containing particulate fraction was sedimented after 1 h at 100000 g and the supernatant from this centrifugation used for enzyme assay. The passage of the extract through a Sephadex G-25 column was necessary for reliable results (see Results).

Homoserine-O-transsuccinylase assay. The condensation of succinyl-CoA and homoserine results in the formation of *O*-succinyl homoserine and free CoA. The latter may be reacted with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) to produce the thionitrophenylate ion which may be measured spectrophotometrically at 412 nm. The procedure employed was that of Lawrence (1972) with the exception that the colour development, for which one should allow 30 min, was carried out at 0 °C. This prevented a high blank resulting from breakdown of unreacted succinyl-CoA at room temperature in the presence of DTNB. The enzymatic reaction mixture contained (in μ M) potassium phosphate (pH 7.6), 5; KCl, 15; MgCl₂, 0.2; succinyl-CoA, 0.6; L-homoserine (omitted from the blank), 2.5. The reaction (in a total vol. of 0.5 ml) was started by the addition of extract containing up to 0.5 mg protein and incubated at 34 °C for 10 min, when it was ended by the addition of 1.5 ml ethanol at 0 °C. Precipitated protein was removed by centrifugation at 0 °C and a 1 ml sample of the supernatant was added to 2 ml 0.1 mM-DTNB in phosphate buffer, pH 7.0. Colour development was as above, and the extinction at 412 nm read against a water blank in a Unicam SP 600 spectrophotometer. The extinction produced in the absence of L-homoserine was subtracted from experimental values, and the amount of free CoA calculated from a calibration curve.

Preparation of succinyl-CoA. The reaction between succinic anhydride and CoA was conducted as described by Lawrence (1972).

Protein estimation. After decolorization of the extract by acidified ethanol, protein concentration was measured by the Biuret reaction.

Chemicals. The purest commercial grade available was employed and usually obtained from BDH Ltd, Poole, Dorset. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and DTNB were supplied by the Adrich Chemical Co.; CoA was obtained from the Boehringer Corp. (London) Ltd.

Fig. 1. Growth rates of *Anacystis nidulans* 1173 on different concentrations of methionine.

RESULTS AND DISCUSSION

Growth of Anacystis nidulans 1173. The requirement for methionine shown by this mutant was not fulfilled by any other amino acid. Vitamin B₁₂ was not required, and neither cystathionine nor homocysteine (intermediates between *O*-succinyl homoserine and methionine) satisfied the methionine requirement. It was concluded, therefore, that the metabolic lesion occurred in the terminal stages of methionine synthesis. The methionine requirement of this mutant was satisfied by 10 μ M concentration, and this permitted growth at a rate equal to that of wild-type under the conditions employed. Lower concentrations produced markedly reduced yields of organisms (Fig. 1).

Homoserine-O-transsuccinylase in extracts of Anacystis nidulans. The enzyme in extracts exhibited a broad optimum pH from 7.6 to 8.4. The specific activities of extracts prepared from methionine-limited cultures of *Anacystis nidulans* 1173 were considerably (6 to 10-fold) more active than those of extracts from cultures with adequate methionine supply. However, after dialysis overnight in 50 mM-potassium phosphate (pH 7.6) both extracts had similar activities although this procedure did lead to irregular loss of activity. The conclusion that extracts from cultures grown in the presence of excess methionine contained an inhibitor of homoserine-*O*-transsuccinylase was strengthened by the observation that the relationship of activity to amount of protein added was not linear (Fig. 2), considerable inhibition occurring at increased enzyme concentrations. Furthermore no end-product inhibition of the enzyme by methionine was demonstrated in the untreated extracts, which contrasted with the results of Lawrence (1972) using *Salmonella typhimurium*.

Low molecular weight material was removed from the extract by passage through a Sephadex G-25 column (1 × 6 cm). This resulted in an enzyme preparation that gave consistent, linear kinetics (Fig. 2) and was inhibited by the addition of methionine to the

Fig. 2

Fig. 3

Fig. 2. Relationship between homoserine-*O*-transsuccinylase activity and protein concentration in Sephadex G-25-treated extracts of *Anacystis nidulans* (○—○) and in extracts before gel-filtration (●—●).

Fig. 3. Inhibition by methionine of homoserine-*O*-transsuccinylase in Sephadex G-25-treated extracts of *Anacystis nidulans* 1173.

Table 1. Levels of homoserine-*O*-transsuccinylase in Sephadex G-25-treated extracts of *Anacystis nidulans*

Activities are the mean of separate cultures and determinations, the range and number of which are given in brackets

Strain	Methionine in growth medium (M)	Rate (nm/min/mg)
Wild-type	Nil	22 (17.0-37; 2)
1173	4×10^{-6}	21 (18-24; 3)
1173	10^{-5}	26 (25-28; 3)
1173	10^{-4}	17 (14-23; 3)

assay system (Fig. 3). The activity of homoserine-*O*-transsuccinylase, in Sephadex G-25-treated extracts, was not significantly different in extracts of *Anacystis nidulans* 1173 grown under conditions of methionine limitation or excess (Table 1). A similar level of enzymatic activity was found in wild-type *A. nidulans* after photoautotrophic growth.

The inhibition of homoserine-*O*-transsuccinylase by methionine was shown to be reversible by increasing the homoserine level. The sigmoidal nature of the relationship between enzyma-

Fig. 4. The reversal of methionine (5×10^{-3} M) inhibition of homoserine-*O*-transsuccinylase of *Anacystis nidulans* by increasing concentrations of homoserine added to the incubation mixture.

Table 2. Effect of inclusion of amino acids in the assay procedure of homoserine-*O*-transsuccinylase in Sephadex G-25-treated extracts of *Anacystis nidulans* 1173

Amino acid added*	Enzymic activity remaining (%)
Nil	100
Methionine	34
<i>S</i> -adenosyl methionine	105
Cystathionine	0
Homocysteine	0
Glutamate	91
Glycine	100
Isoleucine	76
Proline	76
Serine	68
Threonine	92
Tyrosine	92

* All additions were at 5 mM.

tic activity and substrate (homoserine) concentration in the presence of a fixed amount of inhibitor (methionine) (Fig. 4) is characteristic of an allosteric interaction of the inhibitor and enzyme. Two other intermediates on the path of methionine biosynthesis, homocysteine and cystathionine, inhibited the enzyme to a greater degree than did methionine, whilst other amino acids, including *S*-adenosyl methionine, were less inhibitory or without effect (Table 2).

The results presented are in contrast to the repression of the homoserine-*O*-transsuccinylase formation in *Salmonella typhimurium* and *Escherichia coli* (Rowbury, 1964; Lawrence,

1972). The allosteric inhibition by methionine was not unexpected from knowledge of the bacterial enzyme, although the failure of *S*-adenosyl methionine to inhibit was unusual. The isolation of a stable mutant has permitted, for the first time, the demonstration that a key biosynthetic enzyme was not under repressive control by the product of its biosynthetic pathway. We take this as strong support for the idea that the numerous examples of lack of repressive control in *Anacystis nidulans* and other blue-green algae (Carr, 1973*a*) are in fact examples of lack of transcriptional control. Some implications of this conclusion have been discussed elsewhere (Carr, 1973*b*).

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