Enzymes of the Calvin Cycle and Intermediary Metabolism in the Cyanobacterium *Anacystis nidulans* Grown in Chemostat Culture

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The cyanobacterium *Anacystis nidulans* grown in light-limited and CO₂-limited chemostat cultures showed varying rates of CO₂ fixation with peaks at dilution rates of 0.10 to 0.12 h⁻¹. The specific activities of a number of enzymes of the reductive and oxidative pentose phosphate and glycolytic pathways and the tricarboxylic acid and glyoxylate cycles varied significantly as a function of the growth environment (substrate limitation) and organism growth rate. Ribulose-1,5-bisphosphate carboxylase varied 15-fold under CO₂-limited conditions but did not change under light-limited conditions. With the exception of phosphoribulokinase, all enzymes which showed a change in specific activity increased with decreasing dilution rate. The specific activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, hexokinase, ribulose-1,5-bisphosphate carboxylase and malate dehydrogenase were significantly higher in organisms grown under CO₂-limited conditions than under light-limited conditions. Fructose-1,6-bisphosphate aldolase and phosphoribulokinase specific activities were similar at all growth rates and under both limitations. Isocitrate lyase was the only enzyme examined which showed higher specific activities under light-limited conditions. Thus *Anacystis nidulans* can selectively express different enzymes, possibly by transcriptional control.

INTRODUCTION

A number of previous investigations have shown that in obligately phototrophic cyanobacteria many of the enzymes involved in intermediary carbon metabolism were hardly affected by the composition of the growth environment (Pearce & Carr, 1969; Pelroy *et al.*, 1972; Carr, 1973). This has resulted in the suggestion that the inability to control enzyme activity at the transcriptional level is a characteristic feature of obligate autotrophic physiology (Carr, 1973). In particular, the presence or absence of organic compounds has little effect on the specific activities of enzymes of intermediary metabolism (Pearce & Carr, 1967; Pearce *et al.*, 1969) or amino acid biosynthesis (Hood & Carr, 1971, 1972; Weber & Böck, 1968; Delaney *et al.*, 1973). Furthermore, even facultatively phototrophic cyanobacteria such as *Aphanocapsa* strain 6714 and *Chlorogloea fritschii* showed only minor alteration in the specific activities of enzymes involved in sugar and carboxylic acid metabolism (Joset-Espardellier *et al.*, 1978). Conversely, other enzyme systems of cyanobacteria are clearly subject to regulation in response to specific environmental conditions. For example, *Anacystis nidulans* showed a 10- to 15-fold increase in alkaline phosphatase specific activity once the phosphate concentration was reduced to growth-limiting levels (Ihlenfeldt & Gibson, 1975). Similarly Bone (1971*a, b*) showed that growth conditions and specific growth rate affected the specific activities of alkaline phosphatase and nitrogenase in *Anabaena flos-aquae* grown in continuous culture.

Continuous-flow culture techniques have been extensively used to examine the influence of growth environment and growth rate on enzyme activity in heterotrophic organisms

(Hamlin *et al.*, 1967; Clarke & Lilly, 1969; Dean, 1972), particularly the balance between induction and catabolite repression. Typically, much wider ranges of enzyme specific activity may be observed in various chemostat cultures compared with the activities determined in batch culture. This paper examines the influences of CO₂-limited and light-limited growth and growth rate on selected enzymes of the reductive pentose phosphate cycle, the oxidative pentose phosphate pathway, the Embden–Meyerhof pathway, and the tricarboxylic acid and glyoxylate cycles in *Anacystis nidulans*.

METHODS

Organism and growth. *Anacystis nidulans*, strain number 625 Indiana University Culture Collection, was obtained from N. G. Carr (University of Liverpool). Stock cultures were maintained as described by Pearce & Carr (1967). The light- and CO₂-limited chemostats were the same as previously described (Slater, 1975; Karagouni & Slater, 1978).

CO₂ assimilation. CO₂ fixation was measured as previously described (Slater & Morris, 1973; Slater, 1975).

Enzyme assays. Organisms were harvested from steady-state cultures by centrifuging at 23 000 *g* for 15 min at 4 °C and washed pellets were resuspended in approximately 10 ml ice-cold 20 mM-Tris/HCl buffer pH 7.5. The organisms were disrupted by two passages through a French pressure cell at 83 MPa at 4 °C. The remaining intact organisms and cell debris were removed by centrifuging at 38 000 *g* for 45 min at 4 °C and the extracts were used immediately for enzyme assays. Cell breakage was estimated by determining the cell numbers before and after disruption. Protein was estimated by the Lowry method using bovine serum albumin as the standard.

Ribulose-1,5-bisphosphate carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing); EC 4.1.1.39] was assayed in a mixture containing, in 1.2 ml: 200 μ mol Tris/HCl buffer pH 7.8; 10 μ mol MgCl₂; 5 μ mol reduced glutathione; 25 μ mol NaH¹⁴CO₃ (specific activity 0.8 μ Ci μ mol⁻¹); 3 μ mol ribulose 1,5-bisphosphate (used to start the reaction); and crude extract (up to 2 mg protein). Samples (0.1 ml) were taken at intervals over 20 min and added to 0.2 ml ethanol/glacial acetic acid (95:5, v/v) in a scintillation vial, to terminate the reaction. After evaporating to dryness the fixed ¹⁴C was determined in a liquid scintillation counter. Ribulose-1,5-bisphosphate carboxylase specific activity was expressed as μ mol CO₂ fixed h⁻¹ (mg protein)⁻¹ or μ mol CO₂ fixed h⁻¹ (10⁸ organisms)⁻¹.

Phosphoribulokinase [ATP:D-ribulose-5-phosphate 1-phosphotransferase; EC 2.7.1.19] was measured as described by Joint *et al.* (1972). The assay mixture contained, in 1.3 ml: 200 μ mol Tris/HCl buffer pH 8.3; 20 μ mol MgCl₂; 1 μ mol ATP; 75 μ mol NaH¹⁴CO₃ (specific activity 0.8 μ Ci μ mol⁻¹); 1 μ g purified spinach ribulose-1,5-bisphosphate carboxylase; 6 μ mol ribulose 5-phosphate (used to start the reaction); and crude extract (up to 2 mg protein). Sampling, subsequent procedures and expression of enzyme specific activity were the same as for the ribulose-1,5-bisphosphate carboxylase.

6-Phosphogluconate dehydrogenase [6-phospho-D-gluconate:NADP⁺ 2-oxidoreductase (decarboxylating); EC 1.1.1.44] was assayed by the procedure of Ng & Dawes (1973). The assay mixture in silica glass cuvettes contained, in 1.0 ml: 36 μ mol Tris/HCl buffer pH 9.0; 1 μ mol NADP⁺; 12.5 μ mol MgCl₂; 1.5 μ mol 6-phosphogluconate (used to start the reaction); and crude extract (up to 0.3 mg protein). 6-Phosphogluconate was omitted from the reference cuvette, and the change in A₃₄₀ was measured. The absorption coefficient for NADH and NADPH was taken as 6.22 $\times$ 10³ l mol⁻¹ cm⁻¹. 6-Phosphogluconate dehydrogenase activity was expressed as nmol substrate converted min⁻¹ (mg protein)⁻¹ or nmol substrate converted min⁻¹ (10⁸ organisms)⁻¹.

Glucose-6-phosphate dehydrogenase [D-glucose-6-phosphate:NADP⁺ 1-oxidoreductase; EC 1.1.1.49] was assayed by the method of Ng & Dawes (1973). The assay mixture in silica glass cuvettes contained, in 1.0 ml: 36 μ mol Tris/HCl buffer pH 9.0; 1.5 μ mol NADP⁺; 12.5 μ mol MgCl₂; 6 μ mol glucose 6-phosphate (used to start the reaction); and crude extract (up to 0.3 mg protein). Glucose 6-phosphate was omitted from the reference cuvette, and the subsequent procedures and expression of results were the same as for 6-phosphogluconate dehydrogenase.

Hexokinase [ATP:D-hexose 6-phosphotransferase; EC 2.7.1.1] was assayed by the method of Ng & Dawes (1973). The assay mixture in silica glass cuvettes contained, in 1.0 ml: 36 μ mol Tris/HCl buffer pH 9.0; 12.5 μ mol MgCl₂; 10.8 μ mol ATP; 2 μ mol NADP⁺; 0.75 units yeast glucose-6-phosphate dehydrogenase; 100 μ mol D-glucose (used to start the reaction); and crude extract (up to 0.3 mg protein). The remaining procedures were as for 6-phosphogluconate dehydrogenase.

Fructose-1,6-bisphosphate aldolase [D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase; EC 4.1.2.13] was measured as described by Tabita & Lundgren (1971). The reaction mixture contained,

Fig. 1. Influence of dilution rate on the steady-state biomass concentration (A_{650}) (●) and rate of CO_2 photoassimilation (○) for light-limited (a) and CO_2 -limited (b) chemostat cultures.

in 1.0 ml: 100 μmol Tris/HCl buffer pH 8.5; 1.25 μmol fructose 1,6-bisphosphate; 10 mg α -glycerophosphate dehydrogenase from rabbit muscle; 0.2 μmol NADH; and crude extract (up to 0.4 mg protein) (used to start the reaction). The remaining procedures were as for 6-phosphogluconate dehydrogenase.

Isocitrate lyase [L -isocitrate glyoxylate-lyase; EC 4.1.3.1] was assayed according to Pearce & Carr (1967). The reaction mixture contained, in 1.0 ml: 50 μmol Tris/HCl buffer pH 6.8; 4 μmol MgCl_2 ; 16 μmol phenylhydrazine; 4 μmol cysteine; 6 μmol DL-isocitrate; and crude extract (up to 0.3 mg protein) (used to start the reaction). Enzyme activity was measured by the change in A_{340} and was expressed as nmol substrate converted min^{-1} (mg protein) $^{-1}$ or nmol substrate converted min^{-1} (10^8 organisms) $^{-1}$.

Malate dehydrogenase [L -malate:NAD $^+$ oxidoreductase; EC 1.1.1.37] was determined as described by Mehler *et al.* (1948). The assay mixture contained, in 1.0 ml: 36 μmol Tris/HCl buffer pH 6.8; 2.5 μmol NAD $^+$; 1.5 μmol oxaloacetate; and crude extract (up to 0.3 mg protein) (used to start the reaction). NAD $^+$ was omitted from the reference cuvette and the enzyme specific activity was expressed as nmol substrate converted min^{-1} (mg protein) $^{-1}$ or nmol substrate converted min^{-1} (10^8 organisms) $^{-1}$.

Chemicals. Sugars and other growth medium components were from BDH and Fisons; sugar phosphates, 6-phosphogluconate, NAD $^+$, NADH, NADP $^+$ and all assay enzymes from Sigma; ATP from Boehringer; DL-isocitrate and oxaloacetate from Calbiochem; $\text{NaH}^{14}\text{CO}_3$ from The Radiochemical Centre, Amersham.

RESULTS

CO_2 fixation and the activity of selected enzymes were examined in steady-state cultures grown under either light-limited or CO_2 (bicarbonate)-limited conditions over a range of dilution rates from 0.02 to 0.19 h^{-1} . Changing the dilution rate resulted in different patterns of steady-state biomass concentrations for the two limitations (Fig. 1a, b). Under CO_2 -limited conditions, the culture biomass, measured in terms of absorbance, dry weight or cell number, showed the normal nutrient-limited chemostat pattern with the culture density remaining relatively constant at dilution rates up to 0.10 h^{-1} , thereafter declining as the dilution rate was increased towards the critical dilution rate (Fig. 1b). Under light-limited conditions, the biomass continued to increase significantly with decreasing dilution rate. Over the range of dilution rates tested, light was the only growth-limiting factor. The patterns

Fig. 2. Influence of dilution rate on the steady-state activities of ribulose-1,5-bisphosphate carboxylase (○, ●) and phosphoribulokinase (□, ■) in CO₂-limited cultures: activities are expressed per unit protein (a) and per unit cell number (b).

of CO₂ fixation, measured in intact organisms under optimal experimental conditions, were similar, showing peaks of assimilation at dilution rates between 0.10 and 0.12 h⁻¹ (Fig. 1 a, b), although the rate of CO₂ fixation was significantly greater in organisms grown under CO₂-limited conditions than in those grown under light-limited conditions.

A number of different patterns of changing enzyme specific activity with dilution rate were observed in the cultures grown under the two different limitations. In CO₂-limited cultures, ribulose-1,5-bisphosphate carboxylase specific activity, both in terms of activity per unit protein or per unit cell number, increased 15-fold with decreasing dilution rate (Fig. 2). The specific activity was constant at dilution rates (*D*) above 0.10 h⁻¹ [lowest activity 0.40 μmol CO₂ fixed h⁻¹ (mg protein)⁻¹] and only increased when the dilution rate was below 0.10 h⁻¹. Under light-limited conditions, ribulose-1,5-bisphosphate carboxylase specific activity remained constant at all dilution rates tested at 0.45 μmol CO₂ fixed h⁻¹ (mg protein)⁻¹, i.e. similar to the lowest specific activity in high dilution rate CO₂-limited cultures. The specific activity in terms of unit cell number was also constant over the dilution rate range examined under light-limited conditions.

A second reductive pentose phosphate cycle enzyme, phosphoribulokinase, showed a different pattern with a slight increase in activity in organisms grown at higher dilution rates in CO₂-limited cultures (Fig. 2).

The two initial enzymes of the oxidative pentose phosphate pathway, 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase, increased in specific activity by factors of 2 to 4 with decreasing dilution rate (Fig. 3 a, b), although the small changes in glucose-6-phosphate dehydrogenase activity in light-limited organisms may not be significant. The minimum specific activities of glucose-6-phosphate dehydrogenase, i.e. those observed at high dilution rates, were similar under both limitations [5 to 6 nmol substrate converted min⁻¹ (mg protein)⁻¹]; the maximum activity was considerably higher in CO₂-limited cultures than in light-limited cultures at the same dilution rate. Although 6-phospho-

Fig. 3. Influence of dilution rate on the steady-state activities of 6-phosphogluconate dehydrogenase (○) and glucose-6-phosphate dehydrogenase (●) in light-limited (a) and CO₂-limited (b) chemostat cultures.

gluconate dehydrogenase activity increased approximately 2-fold in both light- and CO₂-limited cultures with decreasing dilution rate, the minimum activity in CO₂-limited cultures [25 nmol substrate converted min⁻¹ (mg protein)⁻¹] was 2.5 times greater than the lowest activity in light-limited cultures [10 nmol substrate converted min⁻¹ (mg protein)⁻¹]. The changes in enzyme activities in relation to unit number of organisms were identical to the changes in specific activities expressed on the basis of total protein.

Similarly, the activity of hexokinase, a representative enzyme of the glycolytic pathway, showed the same pattern whether the specific activities were calculated on the basis of unit cell number or unit total protein. The hexokinase activity increased 3-fold with decreasing dilution rate in light-limited cultures (Fig. 4a) whereas the activity in organisms grown under CO₂-limitation showed a sharp increase below $D = 0.10 \text{ h}^{-1}$, representing a 4-fold increase, with a peak of activity at approximately $D = 0.05 \text{ h}^{-1}$ (Fig. 4b). As in the case of 6-phosphogluconate dehydrogenase, the minimum activity observed under CO₂-limited conditions was considerably greater than that under light-limited conditions. Indeed, the maximum specific activity under light-limited conditions [24 nmol substrate converted min⁻¹ (mg protein)⁻¹] was the same as the minimum activity in organisms grown at a high dilution rate under CO₂-limited conditions.

Fructose-1,6-bisphosphate aldolase, which functions in both the reductive pentose phosphate cycle and the glycolytic pathway, had specific activities which did not vary with dilution rate and, moreover, showed similar levels [7 to 8 nmol substrate converted min⁻¹ (mg protein)⁻¹] whether the organisms were grown under light- or CO₂-limited conditions (Fig. 4a, b).

Malate dehydrogenase specific activities were approximately 2- to 3-fold higher in organisms grown under CO₂-limited conditions than in those grown under light-limited conditions (Table 1) although the pattern of changing activities as a function of dilution rate was not regular. Isocitrate lyase was the only enzyme examined which generally showed considerably higher activities in light-limited organisms [average value of 74 nmol substrate converted min⁻¹ (mg protein)⁻¹] than in CO₂-limited organisms [average value of 12 nmol substrate converted min⁻¹ (mg protein)⁻¹ for $D \geq 0.05 \text{ h}^{-1}$]. The high isocitrate lyase activity at $D = 0.02 \text{ h}^{-1}$ was the average result of four separate steady-state cultures all of which

Fig. 4. Influence of dilution rate on the steady-state activities of fructose-1,6-bisphosphate aldolase (○) and hexokinase (●) in light-limited (a) and CO₂-limited (b) chemostat cultures.

Table 1. *Changes in specific activities of malate dehydrogenase and isocitrate lyase in response to dilution rate and growth limitation*

Enzyme activities are expressed as nmol substrate converted min⁻¹ (mg protein)⁻¹.

Enzyme	Light-limited chemostat					CO ₂ -limited chemostat					
	Dilution rate (h ⁻¹)					Dilution rate (h ⁻¹)					
	0.03	0.05	0.10	0.15	0.19	0.02	0.05	0.08	0.11	0.15	0.19
Malate dehydrogenase	1.0	1.4	3.2	5.7	2.8	14.7	9.3	7.8	9.5	6.7	6.7
Isocitrate lyase	77.5	83.0	90.1	54.0	63.7	110.0	6.7	23.5	8.2	10.1	14.5

showed significantly greater activities at this dilution rate compared with other steady-state cultures (Table 1).

DISCUSSION

Our study has shown that a number of enzymes involved in intermediary metabolism in the cyanobacterium *Anacystis nidulans* were present at significantly different specific activities under appropriate growth conditions. The variations between different enzymes showed that the changes in specific activities were not associated with a general phenomenon, such as an alteration in the total protein content of the organism, since such changes might have been expected to alter all the activities in a similar fashion. Furthermore, in some cases the same enzyme behaved differently between light-limited and CO₂-limited growth conditions indicating that differences in environmental conditions, in contrast to changes in the organisms' specific growth rate only, were important in the expression of enzyme activity. These results suggest that *A. nidulans* could selectively express different enzymes at the transcriptional level, although we cannot exclude completely the possibility of regulation

at the translational level or control of enzyme levels due to activation or rates of enzyme turnover.

In one case, the change in specific activity was similar to those reported for enzymes known to be under transcriptional control (Bone, 1971 *a, b*; Ihlenfeldt & Gibson, 1975). Ribulose-1,5-bisphosphate carboxylase showed a wide range of activities under growth conditions where it might be expected to show such changes; that is, it increased in specific activity in organisms grown in environments where growth was limited by the availability of one of the enzyme's substrates, namely CO₂ (bicarbonate). The rate of CO₂ assimilation depended on the concentration of this substrate and hence in environments with low CO₂ concentrations an increase in the activity of ribulose-1,5-bisphosphate carboxylase would increase the rate of fixation. The kinetics of conventional nutrient-limited chemostat culture predicts that the CO₂ concentration would decrease with decreasing dilution rate and so account for the greater ribulose-1,5-bisphosphate carboxylase specific activity with decreasing dilution rate. The higher the ribulose-1,5-bisphosphate carboxylase activities, the greater the capacity to scavenge for and assimilate low, growth-limiting concentrations of the sole carbon source. The principle of elevated activities of an initial enzyme involved in the metabolism of a growth-limiting nutrient is well established (Dean, 1972); for example, heterotrophic bacteria have a higher β -galactosidase activity when growing on lactose as the limiting nutrient (Horiuchi *et al.*, 1962). However, ribulose-1,5-bisphosphate carboxylase (and also the other enzymes examined) did not show the wide range of activities exhibited by the catabolic enzymes of heterotrophic bacteria (for example, see Clarke & Lilly, 1969). From our results, it seems that the alteration in the activity of ribulose-1,5-bisphosphate carboxylase was in response to changes in the external CO₂ concentration and not different growth rates, since under light-limited conditions with CO₂ in excess, at all the dilution rates examined there was no change in the specific activity.

The ribulose-1,5-bisphosphate carboxylase activity *in vitro* accounted for only 10 % of the *in vivo* rate of CO₂ assimilation required for the growth of the organisms at high dilution rates and reflects the problems of correlating enzyme activity with the behaviour of the intact organisms. However, in neither light-limited nor CO₂-limited cultures did the changing pattern of CO₂ fixation by intact organisms correlate with the observed ribulose-1,5-bisphosphate carboxylase activities. This suggests that other factors than ribulose-1,5-bisphosphate carboxylase activities influenced the CO₂ fixation rate by intact organisms. For example, the influence of other carboxylases, such as phosphoenolpyruvate carboxylase (Colman *et al.*, 1976), have not been examined in this context.

The capacity of *A. nidulans* to alter enzyme specific activities with respect to changing growth rate was limited since, apart from ribulose-1,5-bisphosphate carboxylase, the enzymes with variable activities showed only 2- to 4-fold changes over the growth rate range examined. Moreover, three of the enzymes under CO₂-limited conditions and six under light-limited conditions showed no changes, substantiating the concept of little transcriptional control (Carr, 1973). With the exception of fructose-1,6-bisphosphate aldolase, phosphoribulokinase and isocitrate lyase, all the enzymes examined showed considerably higher specific activities in organisms grown under CO₂-limited conditions compared with light-limited conditions. Under CO₂-limited conditions the intracellular concentration of carbon compounds would be expected to be lower than under conditions of excess CO₂, a situation likely to result in the derepression of enzymes involved in carbon metabolism. However, the maximum differences were only 4-fold, again suggesting a limited capacity to alter enzyme levels in response to different growth environments. It is interesting that the changes observed occurred for those enzymes at the beginning of different pathways and which may be involved in the regulation of carbon flow in the pathway. Whether or not the transcription of initial enzymes is regulated more efficiently than other pathways remains to be determined by a comprehensive analysis of all the central metabolic pathway enzymes.

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