Anaplerotic reactions in *Anabaena cylindrica*

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Anaplerotic reactions occur in heterocysts and vegetative cells of *Anabaena cylindrica*. This cyanobacterium possesses phosphoenolpyruvate carboxylase and malic enzyme, but no pyruvate carboxylase and isocitrate lyase. Heterocysts contain all the enzymes for the conversion of glucose 6-phosphate to oxoglutarate and may not be dependent on a supply with glutamate from vegetative cells.

Anaplerotic reaction *Glycolysis* *Tricarboxylic acid cycle* *Heterocyst*
Vegetative cell Anabaena cylindrica

1. INTRODUCTION

Heterocysts supply vegetative cells with glutamine formed by the nitrogenase/glutamine synthetase reactions. In exchange, a disaccharide and glutamate were said to be transported from vegetative cells to heterocysts [1–3]. The latter statement is not so well substantiated by experimental findings as the former. The disaccharide has not yet been identified. The evidence for the transport of glutamate mainly stems from [4–6] that carbon catabolism proceeds via the oxidative pentose phosphate cycle and that glycolysis and the tricarboxylic acid cycle do not operate in heterocysts. However, evidence has recently been accumulated from experiments with more intact heterocyst preparations that enzymes of glycolysis and part of the tricarboxylic acid cycle occur in these cells [7]. The demonstration of the O_2-sensitive pyruvate : ferredoxin oxidoreductase [8] and glutamine : oxoglutarate amido transferase [9] as well as a rather active isocitrate dehydrogenase with complex regulatory properties [10] is particularly noteworthy. Thus heterocysts themselves may synthesize glutamate and oxoglutarate. As an enzyme catalyzing the cleavage of oxoglutarate is absent [8], heterocysts cannot regenerate oxaloacetate by the tricarboxylic acid cycle. They must therefore possess anaplerotic reactions to form oxaloacetate which also follows from two other considerations:

(i) Heterocysts form acetylcoenzyme A in the pyruvate clastic reaction. Acetylcoenzyme A can obviously not be converted to acetate for ATP-formation, as cyanobacteria do not excrete acetate in significant amounts. It is probably converted to citrate as may be deduced from the occurrence of citrate synthase [8]. This, however, requires the supply with oxaloacetate.

(ii) It has been shown [11,12] that the cyanophycin granule polymer consisting of an arginine–aspartate polypeptide is synthesized and degraded in heterocysts with markedly higher activities than in vegetative cells. The synthesis of the arginine–aspartate polypeptide requires a continuous supply with glutamate and oxaloacetate also indicating the occurrence of anaplerotic reactions in heterocysts.

Therefore, amphibolic reactions in heterocysts and vegetative cells of *Anabaena cylindrica* are investigated here.

2. MATERIALS AND METHODS

2.1. *Organism and heterocyst isolation*

Anabaena cylindrica (no. 1403-2) was obtained from the Sammlung von Algenkulturen der Universität Göttingen and grown aerobically as in

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[13]. Heterocyst isolation and preparation of extracts from heterocysts and vegetative cells have been described earlier [8,13]. Heterocysts were isolated by French press treatment and without a time-consuming lysozyme incubation step. The preparation contained <2% vegetative cells and could reduce C_2H_2 with 10–20% of the activity of intact filaments.

2.2. *Non-radioactive assays*

The methods for assaying the enzymes are given in the following: pyruvate kinase (EC 2.7.1.40) [14]; malate dehydrogenase (EC 1.1.1.37) [15]; phosphoenolpyruvate carboxylase (EC 4.1.1.31) [16]; malic enzyme (EC 1.1.1.40) [16]; glycine : oxaloacetate aminotransferase (EC 2.6.1.35) [17]; glycine : 2-oxoglutarate aminotransferase (EC 2.6.1.4) [18]; alanine : 2-oxoglutarate aminotransferase (EC 2.6.1.2) [18]; aspartate : 2-oxoglutarate aminotransferase (EC 2.6.1.1) [19]; pyruvate : ferredoxin oxidoreductase (EC 1.2.7.1) [8]; phosphotransacetylase (EC 2.3.1.8) [20]; isocitrate lyase (EC 4.1.3.1) [21]; citrate synthase (EC 4.1.3.7) [22].

2.3. *Radioactive assays*

Phosphoenolpyruvate carboxylase activity was determined by the incorporation of $^{14}CO_2$ into malate via oxaloacetate. The assays contained in 3 ml final vol.: malate dehydrogenase, 60 units; and in μmol – Tris–HCl buffer (pH 7.8) 150; $MgCl_2$, 10; NADH, 2; phosphoenolpyruvate, 15; $NaH^{14}CO_3$, 20 labeled with 0.1 μCi/μmol. After incubation in test tubes for 1 h at 28°C, the reaction was stopped by adding 200 μmol HCl, 100 μmol $NaHCO_3$ and 50 μmol malate. The non-incorporated $H^{14}CO_3^-$ was removed by bubbling with pure CO_2 gas for 2 h, and the radioactivity fixed was counted by liquid scintillation spectrometry. Radioactivity was only found in malate as identified by thin-layer chromatography [23].

Malic enzyme was assayed by the incorporation of $^{14}CO_2$ from uniformly labeled [^{14}C]malate. For malate synthesis, the assay contained in 3 ml final vol. (μmol): Tris–HCl buffer (pH 7.4) 150; $MgCl_2$, 10; NAD(P)H, 1; pyruvate, 100; $NaH^{14}CO_3$, 20 labeled with 0.1 μCi/μmol; glucose 6-phosphate, 20; glucose 6-phosphate dehydrogenase, 1.4 units (or galactose, 20 and galactose dehydrogenase, 0.2 units). After 1 hr incubation at 28°C, malate was counted and identified as in the phosphoenolpyruvate carboxylase assay. The decarboxylation of malate was performed in Warburg vessels containing in 2.5 ml (μmol): Tris–HCl buffer (pH 7.4) 150; $MgCl_2$, 10; $NAD(P)^+$, 2; L-[U-^{14}C]malate, 20; labeled with 0.1 μCi/μmol. After 1 h at 28°C, the assay was stopped by adding 200 μmol HCl, the released CO_2 was trapped into phenethylamine in the centre well and the radioactivity was counted.

2.4. *Others*

Protein was determined by the Bradford method [24]. Enzymes were from Boehringer (Mannheim) and radiochemicals from Amersham Buchler (Braunschweig). All the data are given in nmol substrate formed or utilized . min^{-1} . mg $protein^{-1}$.

3. RESULTS

Phosphoenolpyruvate carboxylase in extracts of heterocysts and vegetative cells could be demon-

Table 1

The phosphoenolpyruvate carboxylase in heterocysts and vegetative cells from *Anabaena cylindrica*

Assay condition	Photometric assay		Radioactive assay	
	Hetero-cysts	Veg. cells	Hetero-cysts	Veg. cells
1. Complete	7.2	12.0	3.7	4.4
2. – Malate dehydrogenase	7.2	10.8	3.7	0.2
3. – Phospho-enolpyruvate	4.7	10.7	0.0	0.0
4. – $NaHCO_3$	4.6	10.7	0.0	0.0
5. – $MgCl_2$	4.6	10.8	1.3	1.6
6. $MnCl_2$ instead of $MgCl_2$	8.3	13.4	4.5	5.8
7. + Avidin	7.1	11.9	3.6	4.3
8. + Acetylco-enzyme A	7.2	12.5	3.5	4.4
9. + ATP	5.1	8.7	2.3	1.5
10. + phosphate	6.5	11.3	3.5	4.1

The enzyme was assayed: (a) by the formation of oxaloacetate which was quantitatively determined by the NADH oxidation in the malate dehydrogenase reaction; (b) by the incorporation of $^{14}CO_2$ into malate via oxaloacetate. The assay conditions are described in section 2. Additions to the assay: acetylcoenzyme A, 025 μmol; avidin, 0.5 units; ATP, 10 μmol; phosphate, 10 μmol

strated photometrically and by a radioactive assay (table 1). The radioactive assay gave clear-cut results, whereas unspecific NADH oxidase activity had to be subtracted to get the NADH oxidation accounting for malate synthesis in the photometric assay. With both tests, malate synthesis via oxaloacetate was dependent on phosphoenolpyruvate and HCO_3^- and was stimulated by Mg^{2+} or Mn^{2+}. Acetylcoenzyme A or ATP did not enhance the rate of malate formation. Preincubation of the reaction mixture with avidin did not inhibit indicating that biotin is not involved.

Extracts from heterocysts and vegetative cells could also form malate from pyruvate catalyzed by malic enzyme (table 2). The enzyme was assayed either by malate synthesis of the release of $^{14}CO_2$ from labeled malate. As expected, the synthesis of malate showed lower rates than the cleavage. The formation of malate was dependent on pyruvate, NADPH, HCO_3^- and Mg^{2+}, but not on NADH or ATP. These factor dependences indicate that malate synthesis can be catalyzed by malic enzyme (table 2) in addition to the formation by phosphoenolpyruvate carboxylase/malate dehydrogenase (table 1).

Table 2

The malic enzyme in heterocysts and vegetative cells from *Anabaena cylindrica*

Assay condition	Malate synthesis		CO_2-release from malate	
	Hetero-cysts	Veg. cells	Hetero-cysts	Veg. cells
1. Complete	1.8	1.7	2.6	6.3
2. – Pyruvate	0.1	0.1	–	–
3. – $NaHCO_3$	0.0	0.0	–	–
4. – Malate	–	–	0.0	0.0
5. – NADPH or $NADP^+$	0.0	0.0	0.0	0.0
6. NADH or NAD^+ instead of NADPH or $NADP^+$	0.1	0.1	0.2	0.04
7. – $MgCl_2$	0.9	1.0	1.8	2.3
8. $MnCl_2$ instead of $MgCl_2$	1.2	1.6	2.0	2.8
9. + ATP (3.3 mM)	1.1	0.8	–	–

The enzyme was assayed by the incorporation of $^{14}CO_2$ into malate or by the release of $^{14}CO_2$ from U-^{14}C-labeled malate

Table 3

The malate dehydrogenase in heterocysts and vegetative cells from *Anabaena cylindrica*

Assay condition	Heterocysts	Veg. cells
1. Complete	47.7	21.0
2. – Oxaloacetate	2.4	16.4
3. – NADH	0.0	0.0
4. NADPH instead of NADH	8.8	19.2
5. + $MgCl_2$	54.1	23.7
Difference, oxaloacetate-dependent activity		
(a) + NADPH	6.4	2.8
(b) + NADH	45.3	4.6

Extracts from heterocysts and vegetative cells catalyzed the oxidation of malate to oxaloacetate both with NAD^+ or $NADP^+$ (table 3). NAD^+ was the preferential electron acceptor which is surprising as site I of the respiratory chain of cyanobacteria preferentially utilizes NADPH [25]. NADH is oxidized in a photosystem I-dependent reaction [26], and a NADH oxidase can be solubilized from membranes of *Nostoc muscorum* [27]

Table 4

The pyruvate kinase in heterocysts and vegetative cells from *Anabaena cylindrica*

Assay condition	Heterocysts	Veg. cells
1. Complete	3.1	16.3
2. – Phosphoenolpyruvate	0.3	5.7
3. – ADP	0.0	2.0
4. – NADH	0.0	0.0
5. – $MgCl_2$	0.1	0.1
6. $MnCl_2$ instead of $MgCl_2$	0.2	7.7
7. – KCl	3.1	15.3
8. + Citrate (3.8 mM)	1.6	7.3
9. + Fructose-6-phosphate (3.8 mM)	3.7	20.0
10. + AMP (3.8 mM)	4.5	21.3
11. ADP, + AMP (3.8 mM) + phosphate (75 mM)	0.0	0.0
12. + ATP (0.38 mM)	0.0	10.5
13. + ATP (3.8 mM)	0.0	1.3

The pyruvate formed in this assay was quantitatively determined by the NADH oxidation in the lactate dehydrogenase reaction [14]

which has similar properties as the enzyme from *Chlamydomonas* [28].

Cyanobacteria possess pyruvate kinase [8]. Table 4 gives the factor dependence for pyruvate formation from phosphoenolpyruvate catalyzed by pyruvate kinase. As with the enzyme from other organisms [29], the activity was increased by the addition of AMP and fructose-6 phosphate and decreased by citrate or ATP. AMP plus phosphate could not substitute for ADP, thus phosphoenolpyruvate synthetase is not present.

Tests for other enzymes were negative. This is true for pyruvate carboxylase, as pyruvate could not be converted to oxaloacetate in the presence of HCO_3^-, ATP and acetylcoenzyme A. Attempts to demonstrate isocitrate lyase failed, thus *Anabaena* can probably not form malate by the glyoxylic acid cycle. There were no indications for the occurrence of the phosphotransacetylase under the assay conditions employed despite the presence of the pyruvate: ferredoxin oxidoreductase [8] and presumably of the acetate thiokinase [30].

Table 5

Specific activities of several enzymes from heterocysts and vegetative cells of *Anabaena cylindrica*

Enzyme	Heterocysts	Veg. cells
1. Glutamate–oxoglutarate transamination activity	4.8	8.1
2. Glutamate–pyruvate transamination activity	2.6	0.3
3. Glutamate–glyoxylate transamination activity	0.0	0.0
4. Aspartate–glyoxylate transamination activity	3.9	0.8
5. Aspartate–pyruvate transamination activity	3.4	0.7
6. Pyruvate kinase	2.8	10.6
7. Pyruvate: ferredoxin oxidoreductase	11.9	0.4
8. Citrate synthase	1.1	0.6
9. Malate dehydrogenase ($NADP^+$)	6.4	2.8
10. Malate dehydrogenase (NAD^+)	45.3	4.6
11. Phosphotransacetylase	0.0	0.0
12. Pyruvate carboxylase (± acetylcoenzyme A)	0.0	0.0
13. Phosphoenolpyruvate synthetase	0.0	0.0
14. Phosphoenolpyruvate carboxykinase (ATP-dependent)	0.0	0.0
15. Phosphoenolpyruvate carboxylase (radioactive assay)	3.7	4.4
16. Malic enzyme (synthesis of malate)	1.8	1.7

Table 5 summarizes the specific activities of several amphibolic enzymes occurring in heterocysts and vegetative cells of *Anabaena*. Alanine and glutamate transaminases were present in both cell types. The test for a glutamate–glyoxylate transamination failed; however, *Anabaena* may form glycine by transamination from aspartate.

4. DISCUSSION

We show here and in [8–10] that heterocysts possess all the enzymes to convert a monosaccharide (glucose-6-phosphate) to oxoglutarate and glutamate. This demonstration of phosphoenolpyruvate carboxylase and of malic enzyme indicates that heterocysts can continuously generate oxaloacetate. The synthesis of this keto acid is required when the product of the pyruvate clastic reaction, acetylcoenzyme A, is to be converted to citrate. The activities of the enzymes investigated here are low (however, unequivocally demonstrable) and in the range of those of several other proteins including nitrogenase [8,31]. Studies with tracers have to show whether the activities of the enzymes are high enough in the heterocysts to meet the requirement for glutamate as the acceptor molecule for the NH_4^+ formed by N_2-fixation. It may well be that heterocysts are not dependent on a supply with glutamate from vegetative cells as believed hitherto [1,2]. The heterocyst–vegetative cell relationship may be simpler than suggested [1,2]: Heterocysts supply vegetative cells with glutamine, and fixed carbon moves from vegetative cells to heterocysts [32]. Heterocysts do not possess ribulose 1-5 bisphosphate carboxylase, but can perform CO_2-fixations by malic enzyme and by phosphoenolpyruvate carboxylase. These enzymes obviously do not substitute for ribulose 1-5 bisphosphate carboxylase but catalyze anaplerotic reactions in heterocysts.

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