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NITROGEN NUTRITION AND CHANGES IN AMINO ACID POOLS OF *CYANIDIUM CALDARIUM*

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Key Word Index—*Cyanidium caldarium*; Cyanophyceae; unicellular algae; amino acid pools; glutamine variation; glutamate variation.

Abstract—Cells of *Cyanidium caldarium* grown in batch under conditions of excess ammonium or nitrate contained respectively 11.2 and 11.7 μmol glutamate/ml packed cell volume (pcv). Glutamate was the dominant amino acid, and represented 40–43.3% of the total amino acid concentration; other amino acids detected, occurring at a lower level with respect to glutamate, were glutamine, aspartate, serine and alanine. Citrulline and 5-amino levulinic acid were also found. Cells grown in a chemostat under conditions of nitrogen limitation contained glutamate, aspartate, serine and alanine at concentrations equivalent to that of the above cells, but contained traces of other nitrogen compounds. Addition of ammonium or nitrate to *N*-limited cells caused a transient decrease in the concentration of glutamate, a rapid increase of glutamine and a substantial increase of other compounds. However, on adding ammonium to cells, glutamine rose abruptly during 60 min, attaining a concentration of 33 μmol/ml pcv, after which it decreased slightly; on adding nitrate to cells, by contrast, glutamine rose during a period of 30 min, attaining a value of 9.5 μmol/ml pcv, then decreased slightly to 4.3 μmol/ml pcv during 120 min; after this time, however, it remained constant around this value for the subsequent 210 min of incubation. After 48 hr of incubation under excess nitrate or ammonium, however, glutamine and other nitrogen compounds exhibited similar levels to those occurring in batch grown cells.

INTRODUCTION

It is well known that in bacteria, fungi and algae the nitrogen composition of the external medium greatly affects the activity of enzymes involved in nitrogen metabolism. In ammonium-grown cells, for instance, nitrate reductase [1, 2] and nitrogenase [3] are fully repressed, and very low levels of glutamine synthetase activity are found [2, 4]. Nitrate reductase and nitrogenase, moreover, can be reversibly inactivated by ammonium *in vivo* [5–8], and, as a general rule, supply of ammonium causes immediate inhibition of the utilization of other nitrogen compounds such as nitrate or molecular nitrogen. Nitrogen starvation, by contrast, brings about derepression of permease systems for ammonium and nitrate [9–11] as well as of nitrate reductase, nitrogenase, glutamate synthase and glutamine synthetase [2, 3, 12, 13]. *N*-Starved cells, furthermore, exhibit the ability to use ammonium both in light and darkness [14, 15], whereas *N*-normal cells utilize ammonium only in light.

In spite of the great amount of work done, however, many aspects concerning the control mechanisms operating in the pathway of nitrogen metabolism remain obscure, and very little is known about intermediates in the pathway of nitrogen assimilation and metabolites which could represent the potential effector(s). Evidence that glutamine could be an effector involved in the control of nitrate, ammonium and molecular nitrogen metabolism has been produced [16–20], but evidence that glutamine was not the sole agent involved in the regulatory phenomena was also presented [21]. Furthermore, it is well known that control phenomena operating in the pathway of nitrogen metabolism are also triggered by carbon or light conditions of the cell, and not only by the nitrogen conditions. In order to establish these control mechanisms, it is necessary to know the internal concentrations of amino acids and other metabolites, as well as variations occurring in the level of these compounds upon changes of the culture conditions.

RESULTS

Amino acid pools in cells of Cyanidium caldarium *grown in batch with excess ammonium or nitrate, or in chemostat under conditions of nitrate limitation*

The concentrations of some of the identified amino acids in cells grown under different nitrogen conditions are reported in Table 1. Glutamate was the dominant amino acid. The concentrations of glutamate in cells grown in excess ammonium or nitrate were of 11.2 and 11.7 μmol/ml pcv respectively. Glutamate, even if at a lower concentration, was significantly high also in cells grown in chemostat under conditions of nitrogen limitation, where values of 7.9 μmol/ml pcv occurred. On a percentage basis, glutamate was 43.3 and 40% of the total amino acid concentration measured in ammonium and nitrate grown cells, respectively, and was up to 70% in chemostat nitrogen limited cells.

The concentration of glutamine in all types of cell was significantly lower than that of glutamate, showing values of 1.4 μmol/ml pcv in ammonium cells, and of 1.8 μmol/ml pcv in nitrate cells, which corresponded to 5 and 4.7% only of the total amino acid concentration, respectively. In chemostat cells glutamine was, indeed,

Table 1. Amino acid concentrations in cells of *Cyanidium caldarium* growing under different nitrogen conditions

Amino acid concentrations (μmol/ml pcv)	Nitrogen conditions NH_4^+ (20 mM)	NO_3^+ (20 mM)	*N*-limited
Aspartate	1.7	2.3	0.13
Glutamate	11.2	11.7	7.9
	(43.3)	(40)	(70)
Asparagine	0.7	0.6	0.14
Serine	0.42	0.69	0.64
Glutamine	1.4	1.8	0.02
	(5)	(4.7)	
Glycine + citrulline	3.00	2.1	0.32
Arginine	1.00	0.6	0.32
5-Aminolevulinic acid	1.8	1.3	0.3
Alanine	3.2	3.3	1.3

The concentration of each amino acid was calculated from the HPLC chromatograph as described in the Experimental. The values reported are the average of eight independent determinations. Values in parenthesis are the percent of the total amino acid concentration in the extract. Under the conditions of this study, it was not possible to resolve citrulline and glycine.

present as traces. Aspartate was found at a significant level in excess ammonium or nitrate growing cells, but occurred in low concentrations in *N*-limited cells. Significant levels of alanine, tyrosine and serine were found in all types of cells.

Variation of glutamate and glutamine pools in nitrogen-limited cells upon addition of ammonium

Elution profiles of amino acids-OPA derivative HPLC in *N*-limited cells either before or 1 and 10 min after ammonium addition are shown in Fig. 1. As can be seen, upon addition, a rapid variation, in concentrations of glutamate and glutamine, occurred. Glutamate, in fact, decreased in less than a min from an initial concentration of 12.6 μmol/ml pcv to a concentration of 1.2 μmol, and increased to a value of 1.8 μmol/ml pcv after 10 min. Glutamine, after the addition of ammonium, rose from traces to concentrations of 13.9 μmol/ml pcv in 1 min, and to 16 μmol/ml pcv in 10 min. Another amino acid which appeared to increase was aspartate. Alanine, by contrast, did not vary appreciably. Both on nitrate (not shown) and ammonium there occurred an increase of a nitrogenous compound which eluted at about 60 min, which has not yet been identified.

Time dependent changes in the amino acid pools upon ammonium or nitrate addition to nitrogen-limited cells of C. caldarium

In Fig. 2A the time-dependent variation of internal concentration of glutamate and glutamine, as well as of other nitrogen compounds and amino acids such as citrulline, arginine and 5-aminolevulinic acid, occurring in *N*-limited cells of *C. caldarium* at various times from ammonium addition are presented. Glutamate, after an initial rapid decrease, (see also Fig. 1), occurred at 6.1 μmol after 60 min, which was similar to the value that occurred in the *N*-limited starting cell, and then it remained constant for the subsequent period of incubation. After 24 hr from the addition of ammonium the concentration of glutamate reached a value of 10.8 μmol, which was similar to that of batch grown cells. Glutamine attained the value of 21.5 μmol 30 min after the addition of ammonium, and up to 33 μmol after 60 min. The latter value was 18.3 to 23.6-fold higher than that occurring in batch grown cells in media containing nitrate and ammonium respectively. Then the glutamine exhibited a constant slow decrease. However, after 210 min from ammonium addition it exhibited a concentration of 6.8 μmol/ml pcv, which was 3.8 to 4.8-fold higher than that occurring in excess nitrate and excess ammonium grown cells respectively. Then glutamine further decreased, to concentrations of 1.8 μmol/ml pcv after 24 hr. This latter value is remarkably similar to that occurring in cells growing in excess nitrogen.

5-Aminolevulinic acid and arginine were other nitrogen compounds which, upon addition of ammonium to *N*-limited cells, induced a large continuous increase in concentration. There occurred also a significant citrulline and glycine peak, (under our conditions it was not possible to resolve citrulline and glycine). However, the occurrence of large amount of citrulline was supported by chemical characterization following the procedure of Oginsky [22]. The higher concentration of citrulline plus glycine, 24 μmol/ml pcv), was attained 210 min after the addition of ammonium; that of 5-aminolevulinic acid, 8.1 μmol/ml pcv, after 24 hr (not shown). The concentrations of all amino acid tested after 48 hr from ammonium addition, however, were significantly lower, (not shown) showing values similar to those occurring in cells growing in batch under conditions of excess nitrogen.

In Fig. 2B the time-dependent variation of intracellular levels of glutamate, glutamine, citrulline plus glycine, arginine and 5-aminolevulinic acid occurring upon nitrate addition to nitrogen limited cells is shown. The

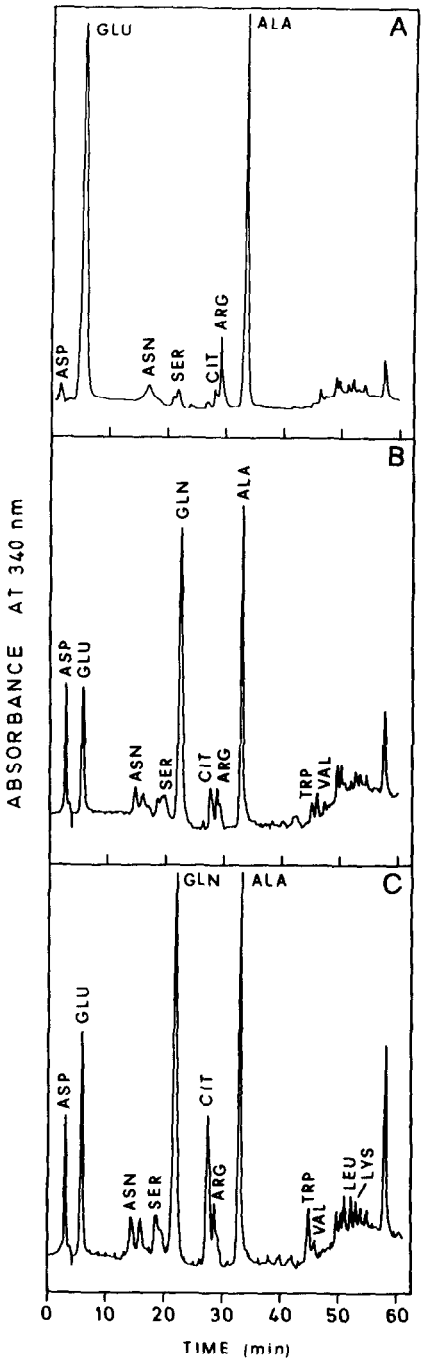

Fig. 1. HPLC elution profiles of amino acid-OPA derivatives in nitrogen-limited cells before (A) and 1 (B) or 10 (C) min after the addition of ammonium at a final 20 mM concentration. Procedure was as reported in Experimental.

addition of nitrate to *N*-limited cells of *C. caldarium* was followed by a sharp decrease in the concentration of glutamate from the initial value of 7.9 μmol/ml pcv to the value of 0.4 μmol/ml pcv after 10 min. After 120 min, indeed, the concentration of glutamate showed the higher value of 6.2 μmol/ml pcv, and for the resting period of incubation it remained constant around the slightly lower

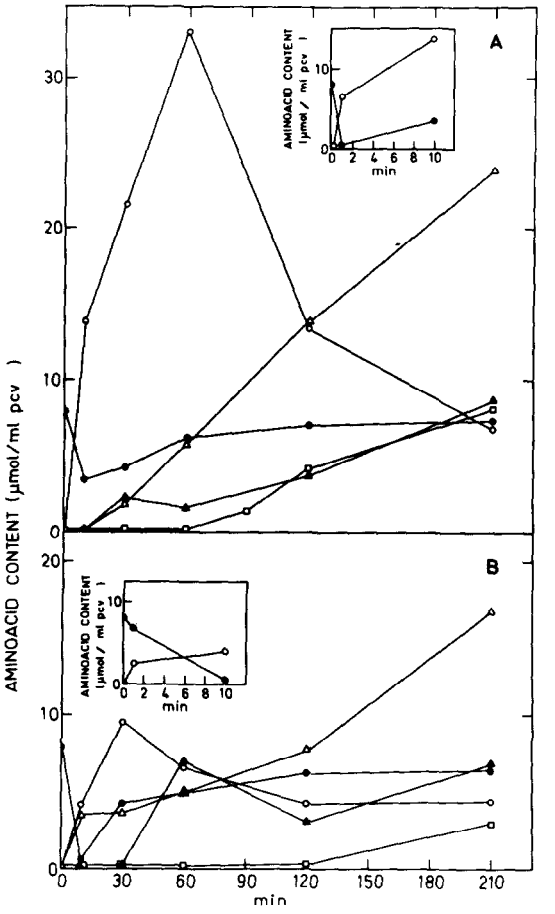

Fig. 2. Changes in the levels of (○—○) glutamine, (●—●) glutamate, (△—△) citrulline, (□—□) 5-aminolevulinic acid and (▲—▲) arginine in nitrogen limited cells of *C. caldarium* upon addition of ammonium 20 mM (A) or nitrate 20 mM (B). Procedure was as reported in Experimental.

value of 5.1 μmol. After 48 hr from nitrate addition, the concentration of glutamate showed was 9.8 μmol, which was similar to the values exhibited by cells growing in excess nitrogen.

Glutamine increased sharply, and 30 min after the addition of nitrate it exhibited a concentration of 9.5 μmol/ml pcv, which was five-to six-fold higher than in cells grown with excess nitrogen. Then the concentration of glutamine slightly decreased to the value of 6.6 μmol after 60 min, and of 4.3 after 120 min, after which no variation was found up to 210 min of incubation. It should be noted that for all the periods under observation, the concentration of glutamine, upon addition of nitrate, remained far below that attained upon addition of ammonium; moreover, in ammonium added cells glutamine exhibited a sharp increase followed by a continuous slow decrease, whereas in nitrate added cells, glutamine exhibited a steady concentration from 30 to 210 min of incubation.

As with the addition of ammonium, addition of nitrate to *N*-limited cells of *C. caldarium* also produced a significant increase in citrulline plus glycine, arginine and 5-aminolevulinic acid; however, citrulline plus glycine and

arginine attained the maximum values of 16.8 and 6.8 μmol/ml pcv, respectively, after 210 min of incubation; 5-aminolevulinic acid, by contrast, after 210 min attained 3 μmol (not shown).

DISCUSSION

Glutamate was present at high concentrations in cells of *C. caldarium* grown in batch under excess nitrate or ammonium, as well as in cells grown in chemostat under conditions of nitrogen limitation. Glutamine, on the other hand, was very low in ammonium or nitrate cells, compared to glutamate, and was present as traces in *N*-limited cells.

When ammonium or nitrate were added to chemostat cells, there occurred a transitory decrease in the concentration of glutamate, accompanied by a sharp increase in the concentration of glutamine. The pattern supports the fundamental role of glutamate in nitrogen metabolism, and the contention that in *C. caldarium*, as in other algae, ammonium is assimilated via the glutamine synthetase/-glutamate synthase pathway.

When nitrate or ammonium were added to *N*-limited cells of *C. caldarium*, the newly incorporated nitrogen was primarily distributed into glutamine and then into citrulline, 5-aminolevulinic acid, and, to a lesser extent, into other amino acids. The concentration of these substances reached values several-fold higher than those occurring in excess ammonium or excess nitrate cells, and it lasted several hr before the concentration decreased to the lower values characteristic of the high nitrogen cells. This supports the view that there exists an initial period during the transition of the cell from the low internal nitrogenous status versus a high status during which the control of synthesis of the above cited compounds undergoes control phenomena different from those which become operative in high nitrogen cells.

Citrulline could not be found in *Anabaena* [21] or *Botryochoccus* [25]. However, a glutamine dependent synthesis of citrulline was demonstrated in *E. coli* [23], and a transient accumulation of intracellular citrulline was demonstrated in cells of *Anabaena* treated with DL-7-azatryptophan [24]. A rise in the level of 5-aminolevulinic acid following ammonium addition, on the other hand, was demonstrated in the green alga *Botryococcus braunii* [25] where it was attributed to an increased synthesis of chlorophyll; in *C. caldarium* a similar increase of 5-aminolevulinic acid can be certainly attributed to an increased synthesis of chlorophyll and also of *C*-phycocyanin which, not only is an accessory photosynthetic pigment, but also represents an important storage protein [26]. Glutamine, on the basis of experiments with methionine-DL-sulphoximine, has been proposed as the possible co-repressor of nitrogenase [3, 16] and nitrate reductase [27]. However, as indicated above, intracellular concentration of glutamine in ammonium growing cells of *C. caldarium*, where nitrate reductase is fully repressed, shows similar values, or even lower values, to those occurring in nitrate growing cells where, by contrast, nitrate reductase is fully expressed [2]. This finding indicates that in *C. caldarium* glutamine is not involved in the regulation of nitrate reductase activity, or, at least, supports the contention that, beside glutamine, some other signal is needed as a regulator in this process. Similar evidence was presented for the expression of nitrogenase gene in Cyanobacteria [21].

Besides having an effect on the synthesis of nitrate reductase, ammonium given to cells which actively utilize nitrate causes immediate inhibition of nitrate utilization, which suggests that nitrate metabolism is subjected to a short-term control triggered by the nitrogenous state of the cell. On the basis of experiments with MSX [18, 28] carried out in *C. caldarium*, it was assumed that a product of ammonium assimilation, perhaps glutamine, might be the effector in this process, and that it could be, also, the signal in a so far unknown control mechanism whereby nitrate reductase is reversibly inactivated *in vivo* [28]. An involvement of glutamine in these phenomena could be supported by the fact that upon addition of ammonium to *N*-starved cells, glutamine attains a maximum of concentration of 33 μmol/ml pcv which is 23.6-fold higher than that occurring in excess ammonium growing cells, whereas upon addition of nitrate it attains a maximum concentration of 9.5 μmol which is only 6.8-fold higher. However after 120 min from ammonium addition (when the use of nitrate is even inhibited by NH_4^+), the concentration of glutamine was found to occur at the lower value of 13.4 μmol/ml pcv, which was similar to the value, 9.5 μmol/ml pcv, of glutamine concentration occurring in nitrogen limited cells upon addition of nitrate (which, indeed, are able to use nitrate linearly with time). Thus the role of glutamine in the control of nitrate utilization remains to be demonstrated, or, at least, elucidated.

Glutamine in bacteria, together with 2-ketoglutarate, represents the effector in a cascade regulatory mechanism whereby glutamine synthetase activity is modulated, and this represents one of the ways through which ammonium assimilation is controlled [29]. Glutamine was found to be involved in other control processes of ammonium utilization [30].

EXPERIMENTAL

Material. Cyanidium caldarium, strain 0206, was supplied by Prof. T. D. Brock (Wisconsin University) who isolated it from a hot acidic spring in Yellowstone National Park, U.S.A. The alga was grown at 42° either in batch culture, using media containing ammonium or nitrate in a large excess (20 mM), or in chemostat culture under conditions of nitrate limitation. The cultures were maintained under conditions of continuous light, and were continuously bubbled with air containing 5% CO_2. The composition of the medium and the culture apparatus were as previously reported [2]. The cells grown in batch were collected and directly used for amino acid analysis; those grown in chemostat were either used directly, or were added with excess nitrate or ammonium and collected at different times from the addition as indicated in the text.

10 ml of cell suspension (*ca* 95 μl pcv/ml was washed with cold water, added to 2 ml cold 80% EtOH, left for 10 min, and centrifuged. The supernatant was collected, and made up to 5 ml with 80% EtOH and, after filtration, used for amino acid analysis.

Amino acid analysis. The free amino acid pool was determined by HPLC after derivatization with *o*-phthaldialdehyde (*o*PA). The amino acid-*o*PA derivatives were separated on a reverse phase C18 ultrasphere column (250 by 4.6). Solvent A consisted of 50 mM NaOAc (pH 7) plus 1% tetrahydrofuran, and solvent B was 100% MeOH. An aliquot (50 μl) of the extract was derivatized for 1 min. A sample (20 μl) of the mixture was injected and eluted at a flow rate of 2 ml/min. The eluted amino acid-*o*Pa derivatives were detected by optical density at 340 nm with a model 163 wavelength detector. All chromatographic

equipment was from Beckman. Amino acid concns were expressed as μmol/ml pcv.

The occurrence of citrulline was determined in the corresponding eluate fraction by the procedure of Archibald as reported in ref. [22].

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