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Effect of the C/N balance on the regulation of nitrogen fixation in *Rhodobacter capsulatus* E1F1

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In *Rhodobacter capsulatus* E1F1, nitrogenase synthesis was repressed by L-glutamate, L-glutamine, L-alanine, L-arginine, L-aspartate, L-asparagine, L-cysteine and L-serine when used in the light as the sole nitrogen and carbon sources. With either D,L-malate or pyruvate as carbon source, nitrogenase repression by these L-amino acids was alleviated and in some cases prevented. Nitrogenase derepression was also observed at high (3 or more) D,L-malate to L-glutamine external ratios. Ammonium and urea, but not methylammonium, repressed nitrogenase. L-Methionine-D,L-sulfoximine (MSX) prevented nitrogenase repression by ammonium or urea, whereas repression by L-glutamine did occur in the presence of this specific inhibitor of glutamine synthesis. The role of ammonium and L-glutamine in nitrogenase regulation was assessed by comparing half-life values of nitrogenase with and without MSX. We conclude that nitrogenase synthesis in *Rb. capsulatus* E1F1 is not regulated by ammonium per se or L-glutamine alone but rather by the intracellular C/N balance.

Introduction

In *Rhodospirillaceae*, nitrogenase in vivo is repressed [1–3] and inactivated by ammonium [4–7]. Almost all the information hitherto indicates that nitrogenase regulation by ammonium is directly related with the GS/GOGAT ammonium assimilation pathway of bacterial cells. Thus, *Rhodobacter capsulatus* mutant auxotrophs for glutamine exhibited nitrogenase activity in the presence of ammonium [8]. In this bacterium, regulation of nitrogenase transcription depended on the activity of *gln*A, the GS structural gene [2]. In several other strains of *Rhodospirillaceae*, a lack of effect of ammonium on nitrogenase activity in the presence of MSX, a powerful inhibitor of GS, has been reported [5,9,10]. In consequence, L-glutamine has been proposed as the metabolite responsible for regulation of nitrogen fixation in these bacteria [5,10–13].

In this paper we present evidence that in *Rb. capsulatus* E1F1 nitrogenase expression is regulated by the current C/N intracellular balance: neither ammonium per se nor glutamine alone is responsible for nitrogenase repression which is triggered when the C/N internal ratio is shifted in favor of N whose excess appears in the medium as ammonium.

Materials and Methods

Organisms and growth conditions. *Rhodobacter capsulatus* E1F1 was cultured anaerobically, at 30 °C under saturating light conditions (40 $W \cdot m^{-2}$), in the RCV medium described by Weaver et al. [14]. *Rhodopseudomonas palustris* DSM 123, *Rhodobacter sphaeroides* DSM 158, *Rhodomicrobium vannielii* DSM 162 and *Rhodospirillum rubrum* DSM 467 were cultured as previously described [15].

Enzymatic activities. Cells were cultured up to the mid-exponential phase of growth and then harvested by centrifugation at 20000 × *g*, 15 min, and, after washing with fresh growth medium, resuspended in 50 mM Tris-HCl buffer (pH 7.5). Nitrogenase activity was measured in washed cells by following acetylene reduction in a gas chromatograph as described by Zumft and Castillo [1]. Transferase activity of GS was determined by measuring spectrophotometrically at 500 nm the

Abbreviations: ESR, electron spin resonance; GOGAT, glutamate synthase (EC 1.4.1.13); GS, glutamine synthetase (EC 6.3.1.2); HPLC, high-performance liquid chromatography; MSX, L-methionine-D,L-sulfoximine; MTA, mixed alkyltrimethylammonium bromide.

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production of γ-glutamylhydroxamate [16]. The adenylylation state ($\bar{n}$) of GS was determined in the presence of MTA (0.1 mg per ml), by measuring the transferase activity either with or without 60 mM $MgCl_2$ [17,18].

One unit of enzymatic activity is defined as the amount of enzyme which catalyzed the transformation of 1 μmol of substrate per min under optimal assay conditions.

ESR spectra. ESR spectra of whole cells were recorded in a Bruker ER 200 tt spectrometer at 10 K. Samples were prepared as previously described [1].

Analytical determinations. Ammonium was determined colorimetrically by the Conway microdiffusion technique [19]. Protein was estimated spectrophotometrically according to the Lowry procedure [20], using bovine serum albumin as a protein standard.

Results are mean values of at least three independent experiments.

Results and Discussion

L-Glutamine has been proposed to be involved in the expression of *nif* genes in *Rhodospirillaceae* [8,13], although recently it has been shown that L-glutamine is not the sole compound that regulates expression of these genes [21].

A possible role for L-glutamine on nitrogenase regulation in *Rb. capsulatus* E1F1 was tested by studying nitrogenase repression by L-glutamine either in presence or absence of an additional carbon source. Nitrogenase was repressed by L-glutamine only in the absence of D,L-malate (Table I). Nitrogenase was also repressed when several L-amino acids (L-glutamate, L-alanine, L-arginine, L-aspartate, L-asparagine, L-cysteine and L-serine) were used as the sole source of both carbon and nitrogen, under which conditions significant concentrations of ammonium were detected in culture media. When either D,L-malate (Table I) or pyruvate (results not shown) was used as carbon source and L-amino acids as nitrogen source, neither nitrogenase repression nor a significant ammonium concentration in the medium was observed. This suggests that L-glutamine alone is not responsible for nitrogenase regulation but rather the actual intracellular C/N balance, since L-glutamine alone as well as other L-amino acids are incapable of repressing nitrogenase if D,L-malate or pyruvate is present. Besides, when several C/N external ratios were tested, nitrogenase derepression was observed at high (3 or more) D,L-malate to L-glutamine ratios (Fig. 1). This reinforces the concept that the actual C/N ratio is responsible for nitrogenase repression when the balance is shifted in favor of nitrogen, when its excess appears as ammonium in the culture medium (Table I). Addition of D,L-malate and other organic acids derepresses nitrogenase of *R. rubrum* cells

TABLE I

Effect of carbon and nitrogen source on induction of nitrogenase of Rb capsulatus E1F1

Cells were cultured with the indicated C and N sources, harvested and assayed for nitrogenase activity after 30 h growth. Ammonia was determined in the supernatant and growth was estimated turbidimetrically at 680 nm (+ + +, $A_{680} > 1.0$; + +, $A_{680} > 0.5$; +, $A_{680} > 0.1$ and −, $A_{680} < 0.1$). 100% of nitrogenase activity corresponded to 8[illegible] mU per mg protein.

N source	C source	Growth	NH_4^+ in the medium (mM)	Nitrogenase (%)
L-Glutamate	D,L-malate	+ + +	0.2	90
L-Glutamate	L-glutamate	+ + +	2.1	0
L-Glutamine	D,L-malate	+ + +	0.3	62
L-Glutamine	L-glutamine	+	21.7	0
L-Alanine	D,L-malate	+ + +	0.3	85
L-Alanine	L-alanine	+ + +	10.6	0
L-Arginine	D,L-malate	+ + +	0.5	32
L-Arginine	L-arginine	+	2.6	0
L-Aspartate	D,L-malate	+ + +	0.5	93
L-Aspartate	L-aspartate	+ +	2.1	0
L-Asparagine	D,L-malate	+ + +	0.4	73
L-Asparagine	L-asparagine	+ + +	14.7	0
L-Cysteine	D,L-malate	+ + +	0.5	55
L-Cysteine	L-cysteine	+ + +	5.9	0
L-Histidine	D,L-malate	+	0.3	61
L-Histidine	L-histidine	−	0.7	0
L-Methionine	D,L-malate	+ +	0.5	67
L-Methionine	L-methionine	−	1.7	0
L-Serine	D,L-malate	+ + +	0.4	100
L-Serine	L-serine	+ + +	8.1	0
L-Threonine	D,L-malate	+ +	0.3	40
L-Threonine	L-threonine	−	1.8	0

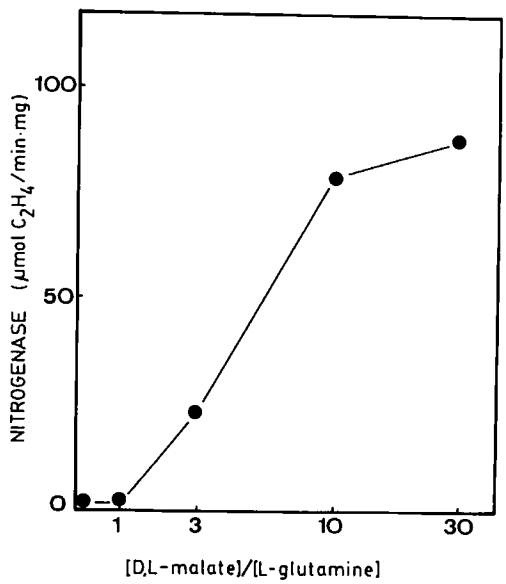

Fig. 1. Effect of the external C/N ratio on derepression of nitrogenase of *Rb. capsulatus* E1F1. Cells cultured phototrophically with ammonium as N source and D,L-malate as C source were transferred to media containing different D,L-malate to L-glutamine ratios. After 30 h growth, nitrogenase activity was determined in whole cells.

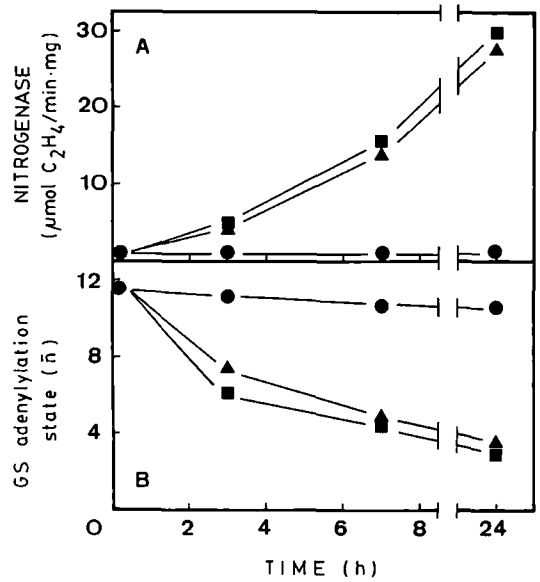

Fig. 2. Induction of nitrogenase of *Rb. capsulatus* E1F1 in the presence of methylammonium. Cells were cultured phototrophically with ammonium as nitrogen source, washed and resuspended in RCV medium without nitrogen (▲), with either 10 mM methylammonium (■) or ammonium (●). Nitrogenase activity (A) and GS adenylylation state (B) were determined in washed cells at the indicated times.

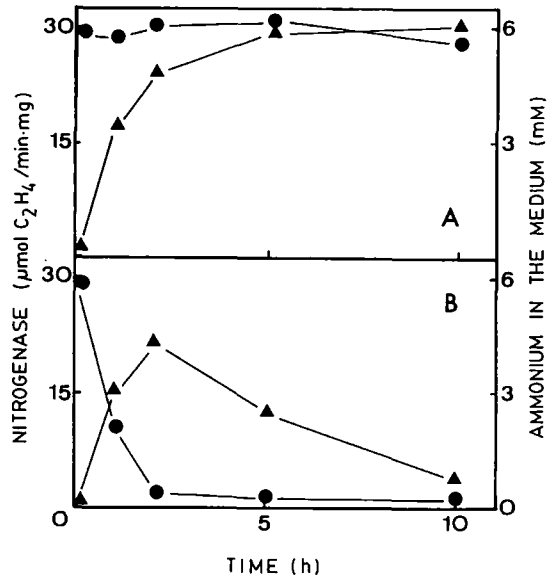

Fig. 3. Effect of urea on nitrogenase activity of *Rb. capsulatus* E1F1 in the presence (A) or in the absence (B) of MSX. Cells were cultured phototrophically with L-glutamate and D,L-malate and, after washing, they were resuspended in RCV media containing 3 mM urea (B) or 3 mM urea + 2 mM MSX (A). Nitrogenase (●) and ammonium (▲) were measured as described in Materials and Methods. In (A), GS was fully inactivated; in (B), GS was 65% inactivated.

grown on L-glutamate alone [3], and a high C/N ratio seems to be required for nitrogenase induction in ammonium-assimilating cultures of certain non-photosynthetic bacteria [22,23].

In *Rb. capsulatus* E1F1, methylammonium, a structural analog of ammonium, did not repress nitrogenase (Fig. 2A), which indicates that ammonium per se is not the regulatory metabolite in the nitrogenase control in vivo. The existence of common regulatory elements for *gln* and *nif* genes in *Rhodospirillaceae* is established from the isolation of glutamine auxotrophs lacking GS and derepressed for nitrogenase in the presence of ammonium [8]. In *Rb. capsulatus* E1F1, induction of nitrogenase, under conditions which prevented repression by ammonium, was accompanied by a parallel decrease in the adenylylation state of GS activity (Fig. 2B), which agrees with the presence of common regulatory effectors, possibly the 2-oxoglutarate-to-L-glutamine ratio, for nitrogenase and GS.

MSX prevented nitrogenase repression by ammonium (results not shown) or urea (Fig. 3), whereas repression by L-glutamine did occur in the presence of this specific inhibitor of glutamine synthesis (results not shown). MSX effect on nitrogenase repression by ammonium or L-glutamine was corroborated by the different nitrogenase half-life values found in the presence and in the absence of this specific GS inhibitor. Half-life values of nitrogenase in the presence of ammonium and L-glutamine are nearly identical (2–3 h) in *Rb. capsulatus* E1F1. *Rb. sphaeroides* and *R. rubrum*, whereas values of 25–30 h and 2–3 h were found in *Rps.*

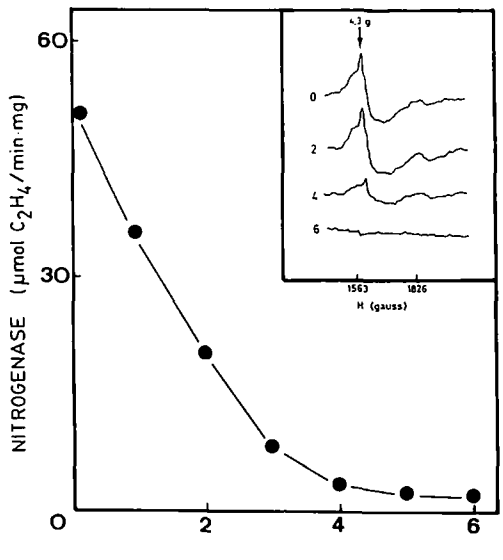

Fig. 4. Correlation between ESR spectra and nitrogenase activity of *Rb. capsulatus* E1F1 cells in the presence of ammonia. Cells were cultured phototrophically with D,L-malate as carbon source and L-glutamate as nitrogen source, washed and resuspended in RCV media containing 5 mM NH_4Cl. At the indicated times, cells were harvested, assayed for nitrogenase activity in the presence of 2 mM MSX and frozen in liquid nitrogen. Inset: In vivo ESR spectra in the 4.3 g region taken at 0, 2, 4 and 6 h after cell resuspension in ammonium media. Spectrometer settings: temperature, 11 K; microwave intensity, 15 dB; modulation amplitude, 10 G; frequency, 9.4 GHz.

palustris and *Rm. vannielii* with ammonium and L-glutamine, respectively. However, when MSX was added simultaneously with ammonium, nitrogenase was not repressed and half-life values between 60 and 100 h were calculated for all strains studied. In contrast, addition of MSX had not effect on the half-life values for nitrogenase in the presence of L-glutamine (2–4 h). Half-life of *Rb. capsulatus* E1F1 was confirmed by following the disappearance of the characteristic signal at 4.3 g of the MoFe protein in the ESR spectrum of whole cells in the presence of ammonium (Fig. 4).

MSX inhibition of ammonium transport is another possible explanation of the presence of nitrogenase in cultures with ammonium and MSX. This possibility was tested by using urea as ammonium source for bacteria. Urea inhibited nitrogenase when MSX was absent, but not when present (Fig. 3). This indicates that the GS/GOGAT system is required to repress nitrogenase and that the effector molecule is not ammonium, since in the presence of MSX and urea, when GS is fully inhibited, nitrogenase was not repressed, and nitrogen as ammonium was excreted and not used by the cells (Fig. 3A). However, in the absence of MSX, GS was only 65% inactivated and the excreted ammonium was subsequently consumed (Fig. 3B). In this respect, it has been recently shown that MSX does not inhibit ammonium transport in *Rb. sphaeroides* [24].

Our results indicate that in *Rb. capsulatus* (i) ammonium per se is not the regulatory metabolite for nitrogenase; (ii) L-glutamine is not the sole agent that regulates nitrogenase synthesis; and (iii) the C/N internal ratio is probably the factor that controls nitrogenase expression. Nitrogenase of *Klebsiella pneumoniae* seems to be regulated by the intracellular L-glutamine to 2-oxoglutarate ratio. An uridylyltransferase (GlnD), which directly responds to the intracellular L-glutamine to 2-oxoglutarate ratio, uridylates PII protein (GlnB) at high C/N ratios. When GlnB is uridylylated, NtrB protein activates by phosphorylation the NtrC protein which, in combination with NtrA (sigma factor), activates *nif* genes transcription [25]. A similar conclusion (regulation by L-glutamine in combination with other metabolite(s), e.g., 2-oxoglutarate) has been suggested for *nif* expression in *R. rubrum* [21], and, on the basis of our results, can be also suggested for *Rb. capsulatus* E1F1 *nif* genes expression.

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