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Effect of Oxygen Tension on Nitrogenase and on Glutamine Synthetases I and II in Rhizobium japonicum 61A76

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Summary: When grown under aerobic conditions, Rhizobium japonicum 61A76 contains two forms of glutamine synthetase, GSI and GSII, as previously described. In contrast, cells grown under the low O_2 tensions required for nitrogenase synthesis contain only GSI. GSII activity disappears completely at O_2 levels below 0.4%. GSI activity decreases by only 50%, but the enzyme appears to become highly adenylylated under the low O_2 tensions required for nitrogenase synthesis.

Magasanik and his co-workers have shown that, in addition to its catalytic function, glutamine synthetase (GS) (EC 6.3.1.2)) has an important regulatory role in controlling the synthesis of several enzyme systems involved in nitrogen assimilation in Klebsiella aerogenes (1). Others have found evidence that GS has a similar role in controlling the synthesis of nitrogenase in K. pneumoniae (2,3).

In Rhizobium, several groups have studied the relationship between GS and nitrogenase and have reached different conclusions. Bishop et al. (4), O'Gara and Shanmugam et al. (5), and Scowcroft et al. (6) concluded that GS did not mediate the repression of nitrogenase by ammonium. On the other hand, Bergersen and Turner (7), Ludwig and Signer (8), and Kondorosi et al. (9) have presented positive evidence for a role of GS in the control of nitrogenase synthesis.

Recently, Darrow and Knotts (10) have found that each of several strains of Rhizobium contains two forms of GS, designated GSI and GSII, which were identified in crude extracts by their distinct separation during isoelectric focusing. These forms of GS differed not only in their apparent pI but in

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several other characteristics as well. GSI was heat-stable at 50°, and inhibition of the transferase activity by Mg^{++} was reversed by treatment with snake venom phosphodiesterase (SVD) suggesting that the phenomenon of adenylylation discovered in E. coli (11) and observed in other bacteria, including Klebsiella (12), also occurs in GSI. Furthermore, upon electrofocusing, extracts of E. coli or K. pneumoniae had no peak corresponding to GSII, which in Rhizobium was heat-labile at 50° and unaffected by SVD in its inhibition by Mg^{++} (10). Thus GSI appears to be directly comparable to the enzyme found in E. coli, whereas GSII is distinctly different.

Keister and Rao (16) have previously defined the cultural conditions under which R. japonicum 61A76 forms nitrogenase. In this communication, we show that growth of R. japonicum 61A76 under the low oxygen tensions which are required for nitrogenase synthesis (13) causes a) disappearance of GSII and b) a shift in GSI to a relatively high degree of adenylylation.

Materials and Methods

Stock cultures of microorganisms were maintained on solid media and cultures for inocula were grown in liquid culture in the following medium: KH_2PO_4, 7.4 mM; $MgSO_4$, 0.8 mM; $CaCl_2$, 50 µM; $FeSO_4$, 0.1 mM; yeast extract, 0.1%; gluconate 0.5% and trace metals containing Zn, Mn, Cu, Mo, Co, B, V, Se, pH adjusted to 6.5 with NaOH.

For growth under low oxygen and for nitrogenase determination the cells were washed with saline and suspended in the following medium: K_2HPO_4, 3 mM; $MgSO_4$, 0.4 mM; $NaMoO_4$, 30 µM; iron citrate, 0.1 mM; gluconate, 0.3%; and glutamate 0.1%. The medium was buffered with 50 mM MOPS, pH 7.0. The cells were grown at 28° in one liter flat-bottom bottles containing 20 ml, shaken at 170 rpm under an atmosphere of 89% Argon - 10% acetylene - 1% CO_2 and oxygen as required. The oxygen concentration was monitored by gas chromatography every 12 hrs and oxygen added as needed. Details of the techniques were described by Keister and Evans (13).

For glutamine synthetase assays, the cells were chilled rapidly in an ice bath, and cell-free extracts prepared as described by Tronick et al. (14), except that treatment with streptomycin-SO_4 was omitted. The γ-glutamyl transferase activity was assayed according to Shapiro and Stadtman (15) at 37° and the absorbance determined at 510 nm. Isoelectric focusing was performed as described by Darrow and Knotts (10).

Results

In earlier work from this laboratory, nitrogenase in R. japonicum 61A76,

Table I. Nitrogenase and Glutamine Synthetase Activities of R. japonicum 61A76

Conditions	Additions	Nitrogenase nmoles C_2H_4/mg protein/hr.	Glutamine Synthetase µmoles/mg protein/min.
Aerobic	Control		3.7
	Ammonia, 20mM		1.1
	Glutamine, 10mM		3.1
	Asparagine, 10mM		3.2
	Aspartate, 10mM		3.6
	Leucine, 10mM		3.2
0.19% O_2	Control	360	0.60
	Ammonia, 10mM	84	0.55
	Glutamine, 10mM	152	0.60
	Asparagine, 10mM	85	0.63
	Aspartate, 10mM	119	0.60
	Leucine, 10mM	119	0.56

The cells were grown in 20 ml medium in 1-liter bottles and nitrogenase activity determined as described by Keister and Evans (13). Glutamate was the nitrogen source and gluconate was the carbon source. The gas phase for the low O_2 (0.19%) grown cells was 10% C_2H_2-1% CO_2-balance Argon. In a separate experiment, ammonia at 10 mM gave essentially the same amount of repression under aerobic conditions as at 20 mM in this experiment.

was found to be repressed by ammonia and by several amino acids (16). Other workers have demonstrated that GS activity in Rhizobium may also be repressed by certain amino acids (5). In Table I, it can be seen that under aerobic conditions with gluconate as carbon source, ammonia but not amino acids caused repression of GS in R. japonicum 61A76. Under low oxygen tension, which is necessary for nitrogenase synthesis, GS activity in the control was repressed and neither ammonia nor amino acids had any additional effect.

This led us to ask whether the decrease in GS activity caused by growth under nitrogen-fixing conditions was due to a specific decrease in one or the other form of the enzyme. Figure 1a shows the analysis by isoelectric focusing of an extract from a control culture of cells grown under 0.75% O_2. At this level of O_2, no nitrogenase activity is observed. Both GSI and GSII activities are found with GSII representing about 60% of the total activity. This profile is qualitatively representative of cultures

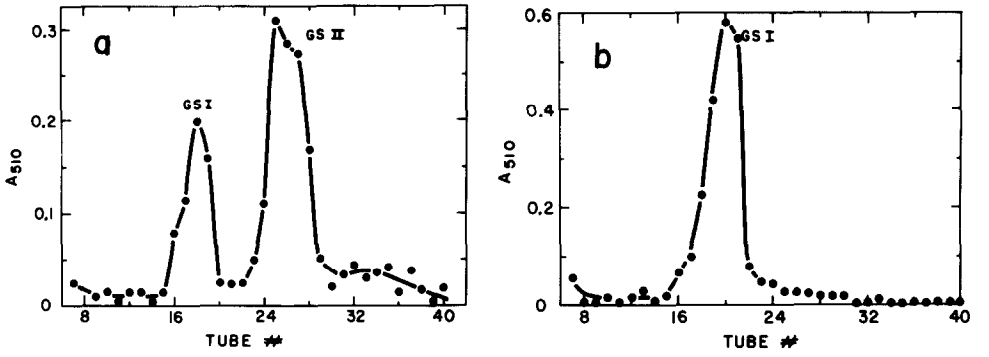

Fig. 1. Isoelectric focusing profiles of glutamine synthetase activity (γ-glutamyl transferase) in cell-free extracts of R. japonicum 61A76 grown under a) 0.75% and b) 0.16% average O_2 concentration. Details were previously described (10). The GSI peak corresponds to a pH of about 5.4 while the GSII peak is about 6.1. Both GSI and GSII require ADP for the transferase reaction (10).

grown between 0.4 and 20.9% O_2. In contrast, Fig. 1b shows a similar analysis of an extract from a culture grown under nitrogen-fixing conditions (0.16% O_2). Note that only one peak of GS activity is found and that this corresponds with GSI.

In order to determine the relative state of adenylylation of GS under nitrogen-fixing conditions, use was made of the inhibition of the transferase assay by Mg^{++} and the reversal of that inhibition by treatment with SVD, as has been described for GS from E. coli by Stadtman et al. (17). Figure 2a shows that the transferase activity of the extract from nitrogen-fixing cells (0.16% O_2) was highly inhibited by Mg^{++} and that the Mg^{++} inhibition was relieved by treatment with SVD. Note also that the pH optimum in the absence of Mg^{++} shifts from acid to alkaline after treatment with SVD, in agreement with a similar shift in pH optimum in going from adenylylated to deadenylylated GS from E. coli (17). For comparison, Fig. 2b shows a similar analysis of the transferase activity of GSI from the control culture, grown under 0.75% O_2 and lacking nitrogenase. There was much less inhibition by Mg^{++}, indicating a lesser degree of adenylylation than in the nitrogen-fixing cells.

The effect of various O_2 tensions on GS activity is shown in Table II.

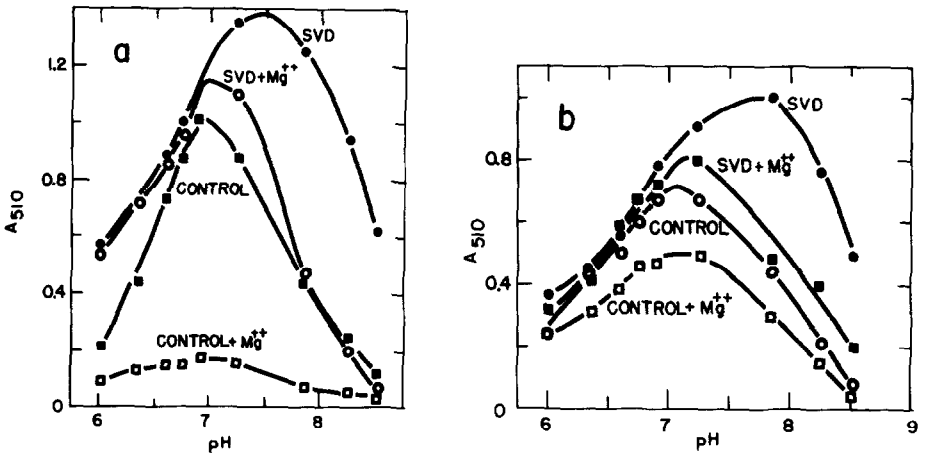

Fig. 2. Effect of $MgCl_2$ and snake venom phosphodiesterase (SVD) treatment on GSI activity of R. japonicum 61A76, grown under a) 0.16% average O_2 and b) 0.75% average O_2. a) A cell-free extract (0.56 mg protein) was incubated 60 min. at 37° at pH 9 in 0.1 M Tris·HCl, 1 mM $MgCl_2$ ± 0.05 unit SVD in 4.1 ml. Portions were then assayed for transferase activity ± 60 mM $MgCl_2$ at various pH's using a modification (10) of the unsaturated reaction mixture described by Stadtman et al. (17). b) GSI fractions from the isoelectric focusing experiment described in Fig. 1b were collected, treated with SVD and assayed as in a).

Darrow and Knotts (10) have shown that GSI is stable to heating at 50° for two hours while GSII is almost totally inactivated. Therefore we have used heat lability as an indicator of the relative proportion of GSI and GSII. Note that at O_2 levels of 0.36% O_2 and below, little or no GSII is found. At 0.4% O_2 and above, high GSII activity appears, and only slightly higher activities are found even under fully aerobic growth conditions. Thus, between 0.36 and 0.4% O_2, an abrupt change in GSII activity occurs. Also note that the level of GSI approximately doubles between growth at 0.08 and 20.9% O_2.

Discussion

Our results show that GS activity varies markedly in R. japonicum 61A76 in response to the level of O_2 under which the cells are grown. We have also observed similar results in R. japonicum 3I1b83 and Rhizobium sp. 32H1. GSII, the novel form of GS which has thus far been found only in Rhizobium,

Table II. The Effect of Oxygen Tension on Glutamine Synthetase Activity in R. japonicum 61A76

	Glutamine Synthetase (μmoles/mg protein/min.)		
Oxygen %	Total	GSI (Heated)	GSII (Total minus heated)
0.08	0.86	0.76	0.10
0.17	0.94	0.94	0
0.36	1.06	0.94	0.12
0.40	2.51	0.84	1.67
0.44	2.51	0.79	1.72
0.48	2.77	0.91	1.78
0.55	2.39	0.78	1.61
0.75	2.43	1.03	1.40
20.9	3.46	1.45	2.01

Cells were grown under the average O_2 concentration indicated for 60 hrs (0.08%), 48 hrs (0.17 - 0.75%) and 36 hrs (20.9%). For determination of GSI activity, the cell-free extract was heated at 52° for 2 hrs to destroy GSII. This was done on a diluted cell-free extract containing between 40 and 160 μg protein/ml in 10 mM imidazole - 1 mM $MnCl_2$, pH 7.0. GSII is determined by the difference in the total activity and the activity remaining after heating (GSI).

was most responsive to the level of O_2, and disappeared between 0.36 and 0.4% O_2. The sudden shift in GSII activity suggests that some signal must have occurred as a result of a change in cell metabolism at this level of O_2. It is possible that this is the level of O_2 at which the cell switches from nitrogen-limited to O_2-limited growth. Nitrogenase activity is optimal at about 0.13% O_2 in R. japonicum 61A76 (16) but small amounts of activity can be observed up to about 0.4%. Whether there is any relationship between the disappearance of GSII activity that occurs at this O_2 level and the synthesis of nitrogenase will be the subject of further investigation.

GSI, the form of GS which is like the E. coli enzyme, is also affected by the level of O_2. The total activity at low levels of O_2 decreases to about 50% of that found under aerobic conditions. The major response, however, is a marked change in the relative state of adenylylation. For cells grown under

0.75% or higher O_2, we found about 30% inhibition by Mg^{++} at pH 6.7 (Fig. 2b) whereas extracts of cells grown between 0.08 and 0.36% O_2 were inhibited 80-95% by Mg^{++}. By analogy with the E. coli enzyme this would indicate a transition to a relatively high degree of adenylylation. Further experiments are planned to determine whether this transition is gradual or abrupt, matching the disappearance of GSII.

Our results on adenylylation are not in agreement with the recent data of Bergersen and Turner (7). They found that the relative adenylylation of glutamine synthetase increased with increasing O_2 levels, with Rhizobium sp. CB756 in chemostat grown cultures. The increased adenylylation of GS was correlated with a decrease in nitrogenase activity. Because of the difference in organism and cultural conditions, it is not possible to compare their studies to ours directly. Bergersen and Turner were unaware of the possibility of the presence of two forms of GS in rhizobia, but under the low O_2 levels which they used, it appears likely that only GSI would be present.

Our results suggest that neither GSII or deadenylylated GSI has a positive regulatory function in nitrogenase synthesis, at least in our experimental system. It has been assumed that deadenylylated GS is required to activate the synthesis of nitrogenase in Rhizobium (4,7). Bender and Magasanik (18) have recently shown, however, that mutations in the structural gene for GS in K. aerogenes can lead to constitutive formation of active GS having a high degree of adenylylation, which nevertheless promotes the synthesis of ammonia-producing enzymes such as histidase and urease. These authors hypothesized that the GS, though adenylylated, is frozen in a deadenylylated conformation which is capable of functioning as a positive regulator. Something similar could easily be true of GSI in R. japonicum 61A76, explaining our observation of high adenylylation concomitant with nitrogenase production.

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