The regulation of some enzymes involved in ammonia assimilation by *Rhizobium meliloti*

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Summary. The activities of glutamate dehydrogenase, glutamine synthetase and glutamate synthase were influenced by nitrogen and carbon sources in the culture medium. These changes were accompanied by corresponding changes in the substrate affinities. In succinate grown culture, the regulation of these enzymes may be mediated through the intracellular levels of ammonia and α-ketoglutarate.

Ammonia is the first stable product of nitrogen fixation, and glutamate is the primary end product[2]. The assimilation of ammonia by bacteria proceeds either via glutamate dehydrogenase (GDH) or via the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway, depending on the organism and the ammonia concentration of the environment[3-5]. In the present study, the activities of GDH, GS and GOGAT were measured in *R. meliloti* grown on different nitrogen and carbon sources. The possibility of the regulation of these enzymes by ammonia and α-ketoglutarate is discussed.

The strain of *R. meliloti* (isolate No. 13) used in the present study was selected as described earlier[6]. The culture was grown for 24 h in the medium described by Kurz and La Rue[7]. The cells were harvested by centrifugation for 15 min at 10,000 × g and 0-4 °C and were washed twice with 0.05 M Tris-HCl buffer (pH 7.2). The washed cells were then broken by ultrasonic treatment, and a 10% (w/v) cell homogenate was prepared in 0.05 M Tris-HCl buffer (pH 7.2). The cell homogenate thus obtained was centrifuged for 20 min at 15,000 × g and 0-4 °C. The supernatant thus obtained was applied to a column of Sephadex G-15 precalibrated and eluted with buffer (Tris-HCl, 0.05 M, pH 7.2) as described earlier[6], before assaying for enzyme activities. GS, GDH and GOGAT were assayed by the methods of Elliot[8], Thomulka and Moat[9] and Roon et al.[10] respectively. The unit of GS was defined as the amount of enzyme necessary for the formation of 1.0 µmole of product (hydroxamic acid) per 30 min at 30 °C. Hydroxamic acid formed in the reaction was estimated by the method of Lipmann and Tuttle[11]. The unit for both GDH and GOGAT was defined as the amount of enzyme which brings about a change of 0.01 OD units in the optical density at 340 nm, per min at 30 °C. The activities of all the enzymes are expressed in terms of specific activities (units/mg protein). The protein was estimated by the method of Lowry et al.[12] using bovine serum albumin as standard. The intracellular concentration of α-ketoglutarate and ammonia were estimated by the methods of Theodore and Friedman[13] and Fawcett and Scott[14] respectively.

The activity of GDH was found to be higher and that of GS/GOGAT lower in ammonium sulphate-grown cultures than with potassium nitrate (table 1). This may indicate that under conditions of ammonia excess assimilation of ammonia proceeds via GDH, as has been postulated in other cases[15,16]. It is worthwhile to note that Brown and Dilworth[5] have observed a similar phenomenon; in the case of *R. japonicum*, *R. leguminosarum* and *R. trifolii*. With an excess of ammonia, in a chemostat, the assimilation of ammonia was found to proceed via GDH, while under conditions of ammonia limitation the GS/GOGAT pathway was used.

The changes in the enzyme activities were found to be associated with changes in the substrate affinities (table 2). *R. meliloti* grown on potassium nitrate showed increased apparent K_m-values of GDH, and lowered apparent K_m-values of GS for their corresponding substrates, in comparison to enzymes from ammonium sulphate-grown cells.

Table 1. Ammonia assimilatory enzymes in *Rhizobium meliloti* grown under different conditions

C/N Source	Glutamate dehydrogenase	Glutamine synthetase	Glutamate synthase
1. Ammonium sulphate (0.1%) Glucose (1%)	22.7 ± 1.34	1.69 ± 0.14	ND
2. Potassium nitrate (0.1%) Glucose (1%)	4.2 ± 0.39	24.32 ± 1.96	8.31 ± 0.96

Results expressed as mean ± SD for 4 determinations.
ND: Not detectable.

Table 2. Substrate affinities (K_m (mM)) of ammonia assimilatory enzymes of *Rhizobium meliloti* grown under different conditions

System	C/N Source	
	Potassium nitrate (0.1%) Glucose (1%)	Ammonium sulphate (0.1%) Glucose (1%)
1. Glutamate dehydrogenase		
a) α-Ketoglutarate	1.4	0.34
b) Ammonium sulphate	7.57	1.69
2. Glutamine synthetase		
a) Glutamate	0.55	1.11
b) Hydroxylamine (ammonia donor)	0.36	0.6

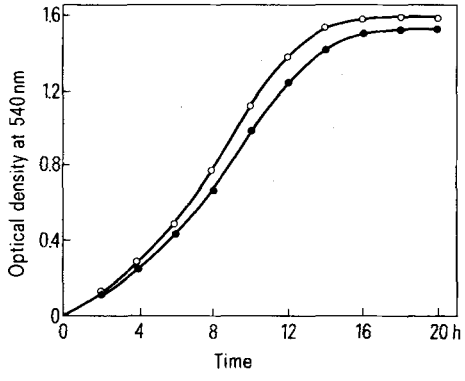
Table 3. Ammonia assimilatory enzymes and levels of α-ketoglutarate and ammonia in *Rhizobium meliloti* grown under different conditions

C/N Source	Glutamate dehydrogenase	Glutamine synthetase	Glutamate synthase	Intracellular levels (mmoles)	
				α-Keto-glutarate	Ammonia
1. Potassium nitrate (0.1%) Sucrose (1%)	3.89 ± 0.42	21.05 ± 2.66	6.6 ± 0.35	0.102 ± 0.017	28.76 ± 3.98
2. Potassium nitrate (0.1%) Sodium succinate (1%)	10.0 ± 1.46	9.54 ± 1.73	2.54 ± 0.19	0.041 ± 0.006	104.54 ± 9.64

Results expressed as mean ± SD for 4 determinations.

Since GS has a low K_m for ammonia, it can, together with GOGAT provide an efficient ammonia assimilatory pathway when the ammonia concentration in the environment is low.

The effect of sucrose and succinate on the activities of the enzymes was studied, as these carbon sources were found to be essential for the asymbiotic fixation of nitrogen[7,17,18]. GDH activity was found to be considerably higher and that of GS/GOGAT lower when succinate was used as carbon source instead of sucrose (table 3). Sucrose-grown cultures also showed higher levels of α-ketoglutarate and lower levels of ammonia than succinate-grown cultures (table 3). Growth rates were nearly identical on both carbon sources (doubling time was about 2 h). α-Ketoglutarate and ammonia are known to regulate GS activity in *Escherichia coli*[19–22]. Thus a higher activity of GS in sucrose grown cultures as compared with succinate grown cells may be due to the increased concentration of α-ketoglutarate. The comparatively low activity for GDH in sucrose-grown cultures could be due to the reduced levels of ammonia.



The growth curve of *Rhizobium meliloti* grown in the presence of succinate (○) and sucrose (●).

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A comment on the design of experiments to estimate the Michaelis-Menten parameters of enzyme-catalysed reactions

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Summary. Michaelis-Menten parameters can be estimated by measuring initial velocities (v) in replicate at 2 concentrations of substrate, one much lower than K_m and the other much higher than it. Analysis of simulated experimental data suggests that this design will probably give more precise estimates of K_m and V than the conventional design in which v is measured at several different concentrations of substrate.

The biochemical literature is full of papers in which the Michaelis-Menten equation is fitted to initial velocity data. In some of them the object of the experiment was solely to estimate K_m and V, and not to find out if the equation fitted the data: for example, in the search for kinetic variants of an enzyme. In these instances, one might intuitively expect that the most efficient experimental design would be to measure the initial velocity (v) in replicate at only 2 concentrations of substrate (s), the 1st much lower than a provisional estimate of K_m and the 2nd much higher than it. (In terms of a Hanes or Lineweaver-Burk plot, this is equivalent to drawing a straight line between 2 points spaced as far apart as possible.) In this note we examine the validity of the expectation, by analysing simulated experimental data.

Methods. Two experimental designs were compared. In the 2-substrate concentration design A v was measured 6 times at each s and in the conventional design B it was measured once at each of 12 concentrations. Thus in both cases an experiment comprised 12 points.

Simulations were carried out as previously described[1]. A set of perfect (i.e. error-free) data was formed by setting $K_m = V = 1$ and calculating v at chosen values of s: 0.2 and 3.0 for design A, and between 0.2 and 4.0 inclusive for