AMMONIA ASSIMILATION IN METHANOBACTERIUM FORMICIUM AND SOME PROPERTIES OF GLUTAMINE SYNTHETASE

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SUMMARY : Since Methanobacterium formicium grew in the presence of low ammonia concentration it was thought likely that GS/GOGAT system for ammonia assimilation would be present in cell extract of M. formicium. Cell free extracts of M. formicium were assayed by τ -glutamyl transferase method. The cell free extracts preparation showed significant glutamine synthetase activity. Optimum pH of glutamine synthetase was found to be 8.8. The enzyme was partially oxygen sensitive and temperature sensitive. Glutamate synthase (GOGAT) activity could not be detected in cell free extracts of M. formicium. Glutamate dehydrogenase activity was present in cells grown with high concentration (3.4 mM) of ammonia.

Key Words : Ammonia assimilation, glutamine synthetase, M. formicium.

INTRODUCTION

Glutamine synthetase is one of the key enzymes for ammonia assimilation in the Enterobacteriaceae and many other microorganisms (1, 2). When present in excess, ammonia is assimilated by glutamate dehydrogenase (GDH). Km values of containing low levels of ammonia, the high affinity 'low ammonia' pathway, the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway operates. The synthesis of 1 mole glutamate by the GS/GOGAT pathway is more energy expensive than the GDH pathway and generally GS is repressed and GDH is de-repressed when ammonia is present and excess (3). In some organisms which do not possess GDH, ammonia is assimilated via the enzyme alanine dehydrogenase (ADH) during high concentration of ammonia (4). Although ammonia assimilation in aerobes and anaerobes has been well studied, little is known about ammonia assimilation pathway in methanogenes. In this paper we report the mechanism used by M. formicium for ammonia assimilation and some properties of GS from M. formicium.

MATERIALS AND METHODS

Bacteria and growth condition

Batch cultures of M. formicium were grown on H_2CO_2 in mineral salt medium supplemented with different $(NH_4)_2SO_4$ concentrations : 3.4, 0.34 and 0.17 mM.

Preparation of cell free extracts

Batch cultures in late exponential phase of growth were harvested by centrifugation (10.000 xg at 4°C for 30 minutes). The pellets resuspended in the buffers used for the particular enzyme assay. For GDH and GOGAT assay the cells were resuspended in 50 mM anaerobic Tris HCl buffer pH 7.4 containing 2 mM DTT and 2 mM $MgCl_2$. For glutamine synthetase (GS). Cells were washed once with anaerobic solution of KCl (10 gL^{-1}) and the pellet resuspended in 10 mM imidazole buffer pH 7.2, containing 2.5 mM $MgCl_2$ and 2 mM DTT. To demonstrate the effect of O_2 the pellet was also resuspended without DTT. The washed cells were broken anaerobically by sonicating 4 ml of cell suspension held at 4°C in screw cap polycarbonate centrifuge (Backman) tubes sparged continually with oxygen free nitrogen. The cells were disrupted for 2 to 3 minutes with medium power with 30 second intervals. Cell free extracts were obtained by centrifugation of disrupted cells at 30.000 xg for 15 minutes at 4°C. The protein content of extracts was estimated by the method of Lowry *et al.* (5), using bovine serum albumin as standard.

Enzyme assays

Anaerobic conditions were maintained during all assays.

Glutamate dehydrogenase (GDH)

Glutamate dehydrogenase activity was measured by assay method of Kenealy *et al.* (6) following the oxidation of NADH at 340 nm. The reaction mixture contained Tris HCl buffer, 100 mM pH 8, NADH 0.5 mM, NH_4Cl 80 mM, sodium α-ketoglutarate 5 mM.

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Glutamine synthetase (GS) :

GS activity was measured by both the τ-glutamyl transferase assay and forward assay method of Bender *et al.* (7). The effect of oxygen was determined by performing assay under aerobic and anaerobic conditions supplemented with or not without DTT.

GOGAT was estimated by the method used by Smith *et al.* (8).

RESULTS

Enzymes of ammonia assimilation were assayed in extracts of M. formicium. GS activity was detected by both τ-glutamyl transferase exchange assay and forward assay method. The values reported in this paper was that determined by the τ-glutamyl transferase exchange assay. The data shown in Table 1 indicate that the activity was partially sensitive to oxygen. When DTT was omitted from the reaction mixture about half of GS activity was lost both in the τ-glutamyl and the forward assay. After exposure to air for 30 minutes in the absence of DTT only 0.052 and 0.02 μmol min^{-1} (mg protein)$^{-1}$ enzyme activity was detected by the τ-glutamyl transferase and forward assay method respectively. Addition of 2 mM DTT to the reaction mixture, after exposure to oxygen, caused a slight increase in GS activity. These results indicate partial sensitivity of GS to oxygen. The optimum pH for GS activity was found to be 8.8 for M. formicium GS (Figure 1). All subsequent assays were performed at this optimum pH. There was a sharp decrease in the GS activity above pH 8.8.

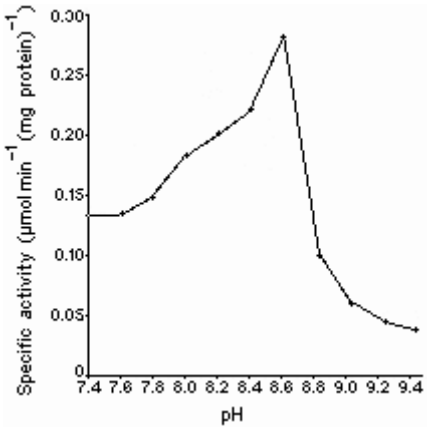
The data presented in Table 2 indicate that the GS activity of M. formicium is temperature sensitive. The specific activity was decreased from 0.096 μmol min^{-1} (mg protein)$^{-1}$ to 0.0136 μmol min^{-1} (mg protein)$^{-1}$ at 40°C after 10 minutes. Attempts to demonstrate GOGAT activity in cell extracts of M. formicium were unsuccessful.

DISCUSSION

Methanogenes contain the same mechism for ammonia assimilation as do other bacteria (9). In initial batch culture experiments with M. formicium no GS activity could be detected. However in later experiments, in which ammonia concentration was reduced from 3.4 mM to 0.17 mM GS activity was detected. So, the activity was inversely correlated with the concentration of ammonia in culture medium. The cell extracts of M. formicium from cells grown with high concentration of ammonia exhibited presence of GDH activity (data not shown).

The GS of M. formicium was partially oxygen sensitive and temperature sensitive. The pH optimum for GS using the τ-glutamyl transferase assay was 8.8. In contracts to our observations, the GS of M. ivanovii is reported to be stable at 63°C for 10 minutes and is not oxygen sensitive. The optimum pH for the τ-glutamyl assay in M. ivanovii is reported to be 8.0 (9), and appears similar to eubacterial GS (10). No GOGAT activity could be detected in cell extracts of M. formicium. This enzyme has been reported in other methanogenes, for instance extracts of M. ivanovii contain 0.02 μmol min^{-1} (mg protein)$^{-1}$ GOGAT (10). However, only low GOGAT activity <0.05 μmol min^{-1} (mg protein)$^{-1}$ in M. thermoautotrophicum and Ms. Barkeri has been reported by Keneally *et al.* (6).

Figure 1: Determination of pH optimum of glutamine synthetase in extract of M. formicium the gamma glutamyl transferase method.



There was not much increase in the specific activity of GOGAT observed in Hyphomicrobium ZV620; 0.02-0.03 μmol min^{-1} (mg protein)$^{-1}$ over a range of growth rate μ=0.02-0.14 hr^{-1} being reported (11). Failure to detect the presence of GOGAT activity in M. formicium could be due to instability of the enzyme; such instability has previously been reported in the GOGAT activity found in Cl. thermoautotrophicum (12). Another explanation could be that sufficient GS and GDH are present to maintain sufficient rates of mited growth of M. formicium; GOGAT would then be redundant. It is noteworthy that M. formicium has been isolated from the bovine rumen which is an ammonia rich environment (13). The primary route of ammonia assimilation in rumen bacteria and other species inhabiting the mammalian gastrointestinal tract is through the GDH system (14). The other methanogenes studied, in which

ammonia assimilated mainly through GS-GOGAT pathway, have been isolated from an environment containing a lower concentration of ammonia.

All results are average of three determinations. Cell extract in 1 ml imidazole buffer (10 mM) pH 7.2 with or without 2 mM DTT were kept under N_2 or exposed to air at atmospheric pressure for 30 minutes with gentle shaking.

GS activity was determined by using the τ-glutamyl tranferase and forward assay methods.

Cell extracts of M. formicium were held in imidazole buffer 50 mM pH 7.2 in side an anaerobic stoppered tube

Table 1: Effect of oxygen on glutamine synthetase activity from M. formicium.

Assay method	Specific activity (μmol min^{-1} (mg protein)$^{-1}$)			
	Aerobic		Anaerobic	
	DTT +	DTT -	DTT +	DTT -
τ-glutamyl transferase assay	0.158	0.095	0.074	0.052
Forward assay	0.069	0.038	0.032	0.020

under N_2 and were incubated at the indicated temperatures. At intervals 50 μl sample was withdrawn with an out gassed 1 ml syringe and injected into an anaerobic stoppered tube and chilled in ice. Activity was determined by τ-glutamyl transferase assay reaction. All readings are average from two assays.

Table 2: Effect of temperature on glutamine synthetase activity of *M. formicium*.

Time Min.	Specific activity (μmol min^{-1} (mg protein)$^{-1}$)		
	Temperature		
	37°C	40°C	50°C
2	88	0.096	0.068
4	88	0.038	0.028
6	88	0.022	0.028
8	88	0.0136	-
10	88	0.0136	-

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