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Regulation of Glutamate Synthase from Bacillus subtilis by Glutamine

by

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Summary. Glutamate synthase, an important enzyme in the assimilation of ammonia, was measured in cultures of Bacillus subtilis grown with different nitrogen sources. An attempt was made to correlate the specific activity to the intracellular levels of five metabolites of glutamate metabolism: aspartate, glutamate, glutamine, alanine and NH_4^+. An inverse relationship was found between the activity of glutamate synthase and the pool level of glutamine. We propose that the intracellular concentration of glutamine is an important element in controlling the level of glutamate synthase.

INTRODUCTION

In Bacillus subtilis, the primary route for the assimilation of inorganic nitrogen into an organic form is through the concerted action of glutamine synthetase (GS, E.C.6.3.1.2) and glutamate synthase (GOGAT; E.C.2.5.1.53).

a) Glutamine Synthetase

NH_3 + glutamate + ATP ---> glutamine + ADP + P_i

b) Glutamate Synthase

glutamine + α-ketoglutarate + NADPH ---> 2 glutamate + NADP

In several gram negative bacteria GOGAT activity was repressed with glutamate as the nitrogen source (1-3); however, when NH_4^+ was also present in the growth medium the activity of this enzyme was increased. Similarly, GOGAT from several species of Bacillus was maximally repressed with glutamate as a nitrogen source (4-8), was alleviated from this repression by the addition of NH_4^+ to the growth medium, and was at its highest level with NH_4^+ as the nitrogen source (4-8). The effect of NH_4^+ in eliminating the repressive action of glutamate, as well as the partially repressive effects of other nitrogen sources, has made it

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difficult to identify the specific effector(s) that control GOGAT activity.

We have examined in B. subtilis the activity of GOGAT and the intracellular pool levels of ammonia, aspartate, glutamate, glutamine and alanine as a function of the nitrogen source. Evidence is presented which supports the hypothesis that GOGAT is controlled by the intracellular concentration of glutamine.

MATERIALS AND METHODS

Bacterial mutants. All strains used in this study were derivatives of the competent strain 168 of B. subtilis (9): NP19 - trpC mutant; NP1 - a prototrophic derivative of the trpC mutant; NP100 - an mtr mutant (defective in the presumed aporepressor of the tryptophan biosynthetic pathway); I9 - an mtr gat mutant (10) that was a spontaneous derivative of mutant NP100.

Growth of cultures and preparation of extracts. Cells were routinely grown at 37°C in a medium containing 14 g K_2HPO_4, 6 g KH_2PO_4, 4 g sodium citrate, 0.1 mg $ZnSO_4$, 0.02 mg $FeCl_3 \cdot 6H_2O$, 0.01 mg $CuSO_4 \cdot 5H_2O$, 0.07 mg $MnCl_2 \cdot 4H_2O$, 0.04 mg $(NH_4)_2 Mo_7O_{24} \cdot 4H_2O$, 0.09 mg $Na_2B_4O_7 \cdot 10H_2O$, 0.5 mg $CaCl_2$, and 0.02 mg $CoCl_2 \cdot 6H_2O$ per liter. The nitrogen source was supplied to a final concentration of 0.2%, and glucose (0.5%) was used throughout this study as the carbon source.

Cultures were grown in 200 ml of medium to an absorbance of 0.5 to 0.6 at 600 nm. Each culture was divided into two and treated as follows. A 100 ml sample was centrifuged at 6000 x g for 20 min at 4°C. The cell pellet was resuspended in a potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 1mM dithiothreitol, 20mM glutamine, 5 mM histidine, 0.1 mM phenylmethyl sulfonyl fluoride and 30% glycerol. The cell suspension was incubated at 37°C with lysozyme (100 µg/ml) and DNase (50 µg/ml) for 30 min, and clarified by centrifugation at 12,000 x g for 20 min. This crude extract was used directly in the assay of GOGAT (11) since it was found that passing the extract through a Sephadex G25 column did not affect the relative enzymatic activities.

The second 100 ml sample was filtered through a 0.45 µ Millipore filter, 90 mm in diameter. The filter was washed with 75 ml of minimal media lacking glucose and a nitrogen source, and placed in 15 ml of ice cold 0.3M perchloric acid. After 20 minutes the solution and filter were placed in a Waring blender for 1 minute. The suspension was clarified by centrifugation (8000 x g) at 4°C. The acid was precipitated by the addition of 8 ml of 0.2 M K_2CO_3, to give a final pH of 6.5 to 7.0. The $KClO_3$ was removed by centrifugation in a clinical serofuge and the supernatant was assayed for the amino acids and NH_4^+ as described below.

Assay of amino acids and NH_4^+. The amino acids were assayed by a modification of methods previously described (12,13). A 100 µl sample was added to 100 µl of borate buffer pH, 10.5. The reaction was initiated by the addition of 50 µl of a solution of o-pthalaldehyde and β-mercaptoethanol (10). After 1.5 minutes at room temperature 10 µl of the reaction mixture was separated on a reverse-phase column (Waters µBondapak C18) eluted with a 100 ml linear gradient of 8% to 50% methanol in 12.5 mM potassium phosphate buffer, pH 7.2, at a flow rate of 1.5 ml/minute. The derivatized amino acids were detected with an Aminco filter fluorometer (American Inst. Co.) equipped with a 70 µl flow cell, Corning 7-51 primary filter, and Wratten No. 8 secondary filter. Amino acid concentrations were calculated by comparing sample peak heights with those from a standard mixture of amino acids (20 pmoles each) and NH_4Cl (2 nmoles). In most cases only the first eight peaks were monitored since the amino acids aspartate, glutamate, asparagine, glutamine, and alanine eluted during this time. The NH_4^+ was assayed as previously described (14). In some cases the concentration of NH_4^+ was also determined on the Bondapak C18 column.

The intracellular concentration of amino acids was calculated as follows. A standard curve relating the culture absorbance at 600 nm to the cell volume was constructed, by assuming that 1 mg of cellular protein represented 8 µl of cytoplasm (15). Protein concentrations were measured with the Bio Rad Protein Reagent using bovine serum albumin as a standard. It was assumed that the cell shape and volume were constant under all growth conditions used in this study.

RESULTS AND DISCUSSION

The activity of GOGAT as a function of the nitrogen source was measured in four different mutants of the B. subtilis competent strain 168 (Table 1). In each isolate the enzyme activity is derepressed with NH_4^+ as the nitrogen source, and repressed with either glutamate or proline as the nitrogen source. The response of the mutants differed from one another, however, when aspartate or glutamine served as the nitrogen source. Aspartate repressed GOGAT 5-fold in the trpC mutant, NP19, but only 1.3-fold in the prototroph, NP1, whereas glutamine repressed GOGAT 8-fold in the prototroph, NP1, but only 1.6-fold in the Trp^- mutant, NP19. Addition of NH_4^+ to the medium eliminated the repressive effects of these amino acids in both the prototroph and the Trp^- mutant (data not shown).

The varying activities for GOGAT in different mutants plus the changes in activity as a function of the nitrogen source have made it difficult to define the regulatory metabolite(s) that controls the activity of this enzyme. We have

Table 1. Specific activities of GOGAT as a function of the nitrogen source

Isolate	Nitrogen Source[b]	Specific Activities of GOGAT[a]				
		NH_4^+	Asp	Glu	Gln	Pro
NP1		5.6	3.4	0.5	0.7	0.5
NP19		3.2	0.4	0.7	2.0	0.1
NP100		5.1	-	0.5	-	-
I9		7.1	-	0.3	-	-

[a]Specific activities are expressed as µmol of NADPH oxidized/mg protein/hr.

[b]Nitrogen sources were supplied to a final concentration of 0.2%: Asp-aspartate; Glu-glutamate; Gln-glutamine; Pro-proline. Media was supplemented with tryptophan (50 µg/ml) for growth of the Trp^- mutant NP19, and phenylalanine (50 µg/ml) for the mtr mutant NP100.

Table 2. Intracellular concentrations of five metabolites as a function of nitrogen source.

Isolate	Nitrogen Source	Intracellular Concentrations (mM) Asp	Glu	Gln	Ala	NH_4^+
NP1	NH_4^+	0.6	22.0	0.3	0.6	6.2
	Asp	4.6	48.0	1.5	1.0	11.0
	Glu	6.6	69.0	2.6	0.5	6.8
	Gln	2.5	67.0	6.7	0.6	12.0
	Pro	4.3	64.0	4.3	1.4	20.0
NP19	NH_4^+	0.9	58.0	1.0	0.6	5.3
	Asp	7.0	58.0	6.1	0.4	4.3
	Glu	3.5	55.0	6.7	0.3	3.1
	Gln	4.0	70.0	1.1	0.8	3.7
	Pro	1.9	43.0	5.9	0.9	8.7
NP100	NH_4^+	1.9	63.0	0.5	0.8	8.1
I9	NH_4^+	2.0	58.0	0.1	0.9	9.3

compared enzyme levels with the pool levels of five important components of glutamate metabolism, namely: aspartate, glutamate, glutamine, alanine and NH_4^+. The results of these analyses are shown in Table 2.

In the prototroph, NP1, all of the pool levels changed in response to a change in the nitrogen source, although there was no pattern to the change in NH_4^+ or alanine. With NH_4^+ as a nitrogen source glutamate, aspartate, and glutamine were at their lowest concentrations, and these concentrations increased when an amino acid replaced NH_4^+ as the source of nitrogen. In the trpC mutant, NP19, the levels of glutamate, alanine, and NH_4^+ were essentially the same regardless of the nitrogen source. The concentrations of aspartate and glutamine, however, varied when an amino acid replaced NH_4^+. In the mtr mutants, NP100 and I9, the pool level of glutamine was very low but the concentrations of both aspartate and glutamate were greater than those of the prototroph, NP1.

The greatest change was in the glutamine pool. Although the intracellular pool level of glutamine in different species of Bacillus had been reported to be undetectable (16) or could not be separated from threonine during the analysis (5), we have found that the level of this amino acid is easily detectable, with a range varying 67-fold depending upon the mutant and nitrogen source used.

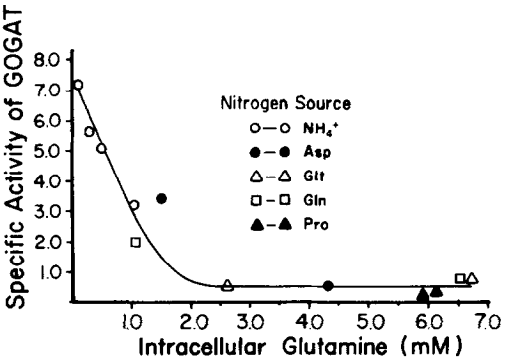

Figure 1. Activity of GOGAT as a function of the intracellular concentration of glutamine. The enzyme activities from Table 1 and the concentrations of glutamine from Table 2 were used to construct this figure.

These results, therefore, represent the first description of significant changes in the glutamine pool level in Bacillus.

When the activity of GOGAT was compared to the pool levels of these metabolites, an inverse relationship was found between the concentration of glutamine and the enyzme activity. This relationship is shown in Fig. 1. The GOGAT activity was highest in mutant I9, which had the lowest pool level of glutamine (0.1 mM). As the intracellular concentration of glutamine increased, enzyme activity decreased, and GOGAT was completely repressed at a glutamine concentration of about 2.5 mM.

There are two possible explanations for these data. First, glutamine could repress GOGAT. Second, the glutamine pool could reflect the activity of GOGAT and increase when GOGAT activity decreases. We favor the first hypothesis because changes in the glutamine pool have been found to precede the decrease in GOGAT activity (Deshpande, Katze and Kane, manuscript in preparation).

We propose, therefore, that GOGAT synthesis is controlled by the pool level of glutamine. The mechanism which in turn controls the glutamine concentration is unknown; however, it is notable that the highest concentrations of glutamine were found in cells with a derepressed GS [e.g., following growth with glutamate as a nitrogen source (16)]. The regulatory signals which affect the glutamine pool as well as GS and GOGAT activity are being investigated.

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