SYNTHESIS OF GLUTAMATE BY A GLUTAMINE : 2-OXO-GLUTARATE AMIDOTRANSFERASE (NADP OXIDOREDUCTASE) IN BACILLUS MEGATERIUM.

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In B.megaterium, grown in glucose ammonia minimal medium, glutamate is synthesized by the coupled functioning of a glutamine synthetase (GlnS) and of a glutamine : 2-oxo-glutarate amidotransferase (NADP oxidoreductase) (GGAT). The properties of GGAT and the physiological study of mutants deficient in GGAT or GlnS activity suggest that these coupled reactions represent the major if not the only pathway of glutamate synthesis.

In many bacteria glutamate is synthesized with the aid of a glutamate dehydrogenase (GluDH), but in Bacilli, GluDH activity has been reported only for B.antracoïdes (1) and for a mutant of B.subtilis (2). Alanine dehydrogenase (AlaDH) on the other hand has been found in all species studied (1, 2) and, according to Shen et al. (3) is the enzyme responsible for ammonia assimilation. Freeze et al. (4) however observed that AlaDH$^-$ mutants of B.subtilis could grow normally in a minimal medium that contained glucose and ammonium ions. These authors concluded that in B.subtilis ammonia is assimilated through the action of aspartase and that glutamate and alanine are synthesized by transamination.

In Bacilli, glutamate is required for both growth and sporulation. Its concentration in the cells increases at the beginning of sporulation (5, 6, 7) then decreases as glutamate is metabolized (6). Moreover, repression of sporulation requires the simultaneous presence of a carbon and a nitrogen source (8) even under non-growing conditions (9).

These observations led us to reexamine the nitrogen

metabolism in Bacilli and particularly the pathway of glutamate synthesis. Results reported here show that in B.megaterium one main route of ammonia assimilation involves the glutamine synthetase (GlnS) and that glutamate is synthesized by a new enzyme recently discovered in A.aerogenes (10). This enzyme which catalyzes the reaction :

Glutamine + 2-oxo-glutarate + NADPH $\rightleftharpoons$ 2 Glutamate + NADP (I)

has been termed Glutamine : 2-oxo-glutarate amidotransferase (NADP oxidoreductase) (GGAT).

RESULTS

1) Characterization of GGAT

The uracil requiring strain MA22 of B.megaterium was grown at 30° C in a minimal medium (11) that contained 1 mg glucose/ml and 0.038 M ammonium chloride. Bacteria were centrifuged before the end of growth phase and suspended at a concentration of 10 mg dry weight/ml in a buffer that contained 0.1 M potassium phosphate, pH 7.2, and 10^{-3}M mercaptoethanol. Extract was prepared by sonication for 2 minutes with a Branson Sonifier Model B-12 and centrifugation for 20 minutes at 15.000 g.

Oxidation of NADPH by the crude extract was followed spectrophotometrically. Results reported in Table I show that this oxydation increased significantly when the reaction mixture contained both glutamine and 2-oxo-glutarate. There was no activity when NADH was substituted for NADPH.

2) Identification and assay of the reaction products

Partial purification of the enzyme was achieved by 1) protamine sulfate treatment (15 % by weight of total protein), 2) ammonium sulfate fractionation (50 % to 80 % saturation), 3) column chromatography on DEAE cellulose previously equilibrated with a 0.1 M potassium phosphate buffer, pH 7.2 that contained 10^{-3}M mercaptoethanol. Elution was performed by a NaCl linear gradient in the same buffer. GGAT activity was eluted in an almost symmetrical peak in

Table I. Characterization of GGAT activity in crude extract of B.megaterium MA22 grown in minimal medium.

Substrates	Activity (units/mg. protein)
NADPH (1.2×10^{-4}M)	0.008
NADPH + 2-oxo-glutarate (5×10^{-3}M)	0.008
NADPH + L-glutamine (5×10^{-3}M)	0.008
NADPH + 2-oxo-glutarate + L-glutamine	0.12

The reaction mixtures consisted of 0.1 ml extract and various substrates in 3 ml 0.1 M potassium buffer, pH 7.2. One unit of activity was defined as one µMole NADPH oxidized per minute at 30°C.

the fractions around 0.2 M NaCl. A 20 fold purification was achieved with a yield of 70 %.

50 µl (0.05 unit of activity) of the enzyme solution were reacted in 0.1 M potassium phosphate buffer, pH 7.2, with 2×10^{-3}M L-Glutamine-U-C^{14} (New England Nuclear), and either none, one or both of the two other reagents NADPH and 2-oxo-glutarate at 2×10^{-3}M concentration. Reaction mixtures, total volume 250 µl, were incubated for 30 minutes at 37°C. 50 µl of each mixture were then applied to Whatman 3 MM paper strips (17 x 4 cm) previously washed with water then dried. An electrophoresis was carried out by subjecting the strips to 7 v/cm for 1.5 hr. in 0.1 M potassium phosphate buffer, pH 7.2.

Following autoradiography, a spot different from that of glutamine appeared only when the enzyme and all other reaction components were present. The radioactive compound was identified as glutamate by chromatographic fingerprinting.

In the reaction mixture the specific radioactivity of glutamine was 2.9 µC/µMole. After elution from paper of the radioactive spots, the specific radioactivity of not

metabolized glutamine was found 3.3 μC/μMole and that of glutamic acid 1.35 μC/μMole (mean of two experiments).

These results justify the stoechiometric relationships in reaction (I). Relative concentrations of glutamine and glutamate at the end of the experiment showed that the reaction equilibrium strongly favors glutamate synthesis.

3) GGAT and GlnS biosynthesis

For determination of GlnS activity, bacteria were washed once with 0.1 M imidazole buffer, pH 7.0 and submitted to sonication in the same buffer that contained 10^{-3}M mercaptoethanol. GlnS was determined by measuring the amount of inorganic phosphate liberated in the presence of 6×10^{-2}M glutamate, 5×10^{-2}M NH_4^+, 10^{-3}M Mn^{++} and 7.5×10^{-3}M ATP in 0.1 M imidazole buffer, pH 7.0 at 37° C.

GGAT and GlnS contents were determined in bacteria grown in various media. From the results reported in Table II it can be seen that during growth GGAT activity was either low or absent when the medium contained yeast extract or glutamate. After growth was completed, i.e. during sporulation, GGAT activity was present, whatever the composition of the medium. This suggests that glutamate represses GGAT synthesis.

Contrary to GGAT, GlnS activity was found in all media, both during growth and sporulation.

4) Mutants $GGAT^-$ and $GlnS^-$

MA22 spores were irradiated with UV light to 10^{-1} survivors. After enrichment by the azaguanine method (11), mutant Glu^-11 was isolated by its capacity to grow on glucose glutamate agar and its inability to replicate on glucose ammonia agar. Mutant Gln^-26 was isolated by its capacity to grow on glucose glutamate glutamine agar and its inability to replicate on glucose glutamate agar.

Mutant Glu^-11 was $GGAT^-$ and required for normal growth in minimal medium high concentrations of glutamate. Glutamate could be replaced by glutamine. This suggested the

Table II. Specific activity of GGAT and GlnS in B.megaterium MA22 grown in various media.

Media (with 2 mg/ml glucose)	μ (div./hr.)	GGAT (units/mg. protein)	GlnS (units/mg. protein)
+ 0.038 M NH_4^+	0.65	0.112	0.40
+ NH_4^+ + 4 mg/ml Yeast Extract	1.8	0.010	0.39
+ 1 mg/ml glutamate	0.75	<0.001	0.50
+ 1 mg/ml glutamine	0.70	0.045	0.15
+ 1 mg/ml aspartate	0.75	0.038	0.30

Activity was determined in crude extracts of bacteria in exponential growth for 10 to 12 generations in each medium. One unit of activity was defined as one micromole substrate metabolized per minute (see text).

existence of a glutaminase.

Mutant Gln^-26 was $GlnS^-$ and specifically required for normal growth in minimal medium high concentrations of glutamine.

Mutant Glu^-11 was $GlnS^+$ and mutant Gln^-26 was $GGAT^+$. Both were $AlaDH^+$.

DISCUSSION

In A.aerogenes, GGAT is synthesized only when the supply of ammonia is limited. Under normal conditions glutamate is synthesized by a GluDH (10).

In B.megaterium, which lacks GluDH, GGAT is synthsized in the presence of high concentrations of ammonium ions in the growth medium. Several observations reported here suggest however that, in vivo, the main if not the only route of glutamate synthesis is catalysed by GGAT. These are : 1) GGAT seems to be specifically repressible by glutamate. This supports the hypothesis that the physiological role of the enzyme is to synthesize glutamate and not glutamine. 2) Mutant Glu^-11, $GGAT^-$, requires high concentrations of glutamate, as though there existed in the cells no other major pathway of

glutamate synthesis. 3) Mutant Gln^-26, $GlnS^-$, requires high concentrations of glutamine, as though glutamine was the only precursor of glutamate.

Using the method described above we have also characterized in B.subtilis Marburg a GGAT activity specific for NADPH. When bacteria were grown in the minimal G medium (4) the specific activity of the enzyme in the crude extract was very close to that found in MA22 extract. It is therefore possible that the coupled enzymes GlnS-GGAT represent the pathway of glutamate synthesis in those bacteria which, like the Bacilli, do not possess GluDH.

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