Vol. 93, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 13, 1980 Pages 308-314

GLUTAMATE SYNTHASE FROM BACILLUS SUBTILIS: IN VITRO RECONSTITUTION OF AN ACTIVE AMIDOTRANSFERASE

Kathryn L. Deshpande and James F. Kane*

Department of Microbiology
University of Tennessee Center for the Health Sciences
Memphis, Tennessee 38163

Received February 1,1980

SUMMARY

In Bacillus subtilis, two mutants, designated gltA and gltB, have been found to lack the enzyme glutamate synthase. When extracts from these two mutants were mixed together, an active enzyme was reconstituted. Since modification of an inactive enzyme to an activated form does not seem likely, we propose that the gltA and gltB mutations affect the two non-identical subunits which form glutamate synthase.

INTRODUCTION

The amidotransferase, glutamate synthase (GOGAT)[1] [glutamine (amide): α -ketoglutarate ($NADP^+$) amidotransferase oxidoreductase, E.C.2.6.1.53], acts in concert with GS to assimilate inorganic nitrogen into an organic form: GOGAT - gln + α kg + NADPH ---> 2 glu + $NADP^+$; GS - glu + NH_3 + ATP ---> gln + ADP + Pi.

GOGAT has been purified from Escherichia coli (1-3), Klebsiella aerogenes (4,5), and Bacillus megaterium (6). In all three instances the enzyme was a multicomponent complex composed of 2 non-identical subunits, and the properties of the subunits were obtained following denaturation of the complex. In these studies no reconstitution of an active GOGAT complex was demonstrated.

The genetic loci responsible for the synthesis of the two subunits in these organisms have not been defined. In E. coli one locus, gltB, has been found to affect GOGAT activity (7,8) but it has not been determined whether this is associated with enzyme structure. In Caulobacter crescentus, two different mutations which decrease GOGAT activity have been described (9).

---

[1]Abbreviations: GOGAT - glutamate synthase; GS - glutamine synthetase.

0006-291X/80/050308-07$01.00/0

In B. subtilis we have found that two mutations, designated gltA and gltB, affect GOGAT activity. A crude extract from either the gltA or gltB mutant lacked GOGAT activity, but an active enzyme was reconstituted when these extracts were mixed together in vitro. The absence of GOGAT activity in each mutant is attributed to a defect in each of the non-identical subunits which form this amidotransferase.

MATERIALS AND METHODS

The prototroph NP1, a derivative of the competent strain 168 of B. subtilis (10), was mutagenized with hydroxylamine (11), and a glutamate requiring mutant (gltB), designated KD2 was selected. The gltA mutant GSY292 (trpC gltA) and the citK mutant JH407 (trpC citK) were kindly provided by James A. Hoch.

The mutants were grown in 200 ml of minimal-salts glucose medium (10), and the amino acids required for growth were supplied to a final concentation of 50 μ g/ml except for glutamate which was added to a final concentration of 1 mg/ml for strain KD2 and 2 mg/ml for strain GSY292. Cultures were harvested in the late logarithmic phase of growth. Cell pellets were resuspended in an extraction buffer consisting of 50 mM potassium phosphate buffer, pH 6.8, containing 1 mM EDTA, 5 mM α - ketoglutarate, 12 mM β -mercaptoethanol and 30% glycerol (buffer A) or 40 mM potassium phosphate buffer, pH. 7.5, containing 1 mM EDTA, 12 mM β-mercaptoethanol, 5 mM histidine, 20 mM glutamine and 30% glycerol (buffer B). Cell extracts were prepared as described (10).

GOGAT was assayed as follows: enzyme was added to a reaction mixture containing 50 mM potassium phosphate, pH 7.5, 1 mM EDTA, 0.5 mM glutamine, 0.5 mM α -ketoglutarate and 0.21 mM NADPH to give a final volume of 1.0 ml at 37°. The rate of change in absorbance at 340 nm was followed continuously in a Gilford spectrophotometer. The rate of oxidation of NADPH in the absence of glutamine was subtracted from the rate observed in the presence of glutamine. The complementation assay was conducted by one of two methods. In method I, the rates of oxidation of NADPH were determined in a complete reaction mixture for various concentrations of protein from a crude extract (buffer A) of either the gltA or gltB mutant. Then, increasing concentrations of protein from the crude extract of one mutant were added to 250 μg of protein from the other mutant in the complete reaction mixture. Complementing activity was the observed rate of oxidation for the combined extracts minus the rates of the two extracts assayed individually. In method II, cell pellets were resuspended in buffer B and mixed together prior to lysis. GOGAT activity was determined with the individual extracts and the mixed cell exracts. In the absence of complementation the specific activity of GOGAT in the mixed cell extract would decrease to 1/2 that of the individual cell extract. Therefore, any activity greater than 1/2 the control rate was taken as complementation. In all cases the specific activity of GOGAT is expressed as μmoles of NADPH oxidized/hr/mg protein.

Crude extracts were prepared in buffer B, centrifuged at 100,000 x g for 1 hr. and applied to an Ultragel AcA34 column (2.5 x 87 cm) equilibrated with buffer B. Fractions (2ml) were assayed for GOGAT activity.

DNA (12) and competent cells (13) were prepared as described.

Results

Genetic analysis of the glutamate requiring mutant KD2. Since the gltA locus was linked to the citK locus (14), we used DNA from strain KD2 to trans-

TABLE I. Specific activity of GOGAT in mutants used in this study.

Collection Number	Relevant Genotype	Specific Activity of GOGAT
NP1	Prototroph	6.0
GSY292	*gltA*	0
KD2	*gltB*	0.3
GSY292+KD2	*gltA* + *gltB*	1.8

form the *citK* mutant, JH407, to prototrophy on media containing glutamate. No *citK*$^+$ transformants, however, were glutamate auxotrophs. Similarly, in a reciprocal cross, no glutamate independent transformants were found to contain the *citK* locus.

In a separate experiment the *gltA* mutant, GSY292, was transformed to *gltA*$^+$ with DNA from either strain KD2 or the prototrophic parent NP1. If KD2 were to contain a mutation in the *gltA* locus, then one would expect a difference in the number of transformants/μg of DNA in these two crosses. In each cross, approximately 1400 transformants/μg of DNA were found.

These results demonstrate that the mutation in strain KD2 was not at the *gltA* locus. Hence, the mutation has been designated *gltB*. The precise location of the *gltB* mutation has not yet been determined.

Complementation of crude extracts from the gltA and gltB mutants. Although both mutants lacked GOGAT, this activity was reconstituted when equal amounts of protein from the crude extracts of both mutants were mixed together (Table I). In order to simplify the designation of complementing activities from each mutant, the following nomenclature has been adopted. The *gltA* mutant possesses an active B subunit and the *gltB* mutant has an active A subunit. These subunits complement each other to form a functional GOGAT protomer $(AB)_n$.

The *in vitro* complementation was examined further by titrating a constant concentration of protein from a crude extract of the *gltA* or *gltB* mutant with increasing protein concentrations from a crude extract of the *gltB* or *gltA*

TABLE II. Complementing activity using two different methods.

Method	Specific Activity GOGAT
Titration with gltA extract	1.8
Titration with gltB extract	3.5
Mix cell pellets and lyse	5.3

mutant, respectively. The specific activity of GOGAT from the gltA mutant increased from an undetectable level to 3.5 at saturation, whereas the specific activity in the gltB mutant increased from 0.3 to about 1.8 (Table II). Although different levels of saturation were observed with each mutant, neither these differences nor the specific activities of the reconstituted GOGAT were consistently reproducible. Therefore, a new complementation assay, which required the mixing of cells from each mutant prior to lysis, was developed. With this improved assay method, the specific activity of the reconstituted enzyme was reproducible and equivalent to that of the prototrophic enzyme (Table II).

The optimal conditions for this latter method were explored. The highest level of complementation was observed when equal numbers of cells from the gltA and gltB mutants were mixed together. The optimal cell density to harvest the cultures is shown in Fig 1 (left). In this experiment cultures of the mutants were harvested at the same cell densities, and assayed for GOGAT. The reconstituted activity decreased as the culture density increased past an A^{600} of 1.0. This result could indicate a decreased level of complementing activity in one or both mutants. These alternatives were examined in the following experiment. Cultures of the gltA and gltB mutants were each grown to an A^{600} of 0.8. A cell pellet from 200 ml of the appropriate culture was added to a cell pellet from a 200 ml culture of the gltA and gltB mutants harvested at various cell densities and crude extracts were prepared (Fig. 1,right). We found that the activity of the gltB mutant decreased at high cell densities whereas the complementing acti-

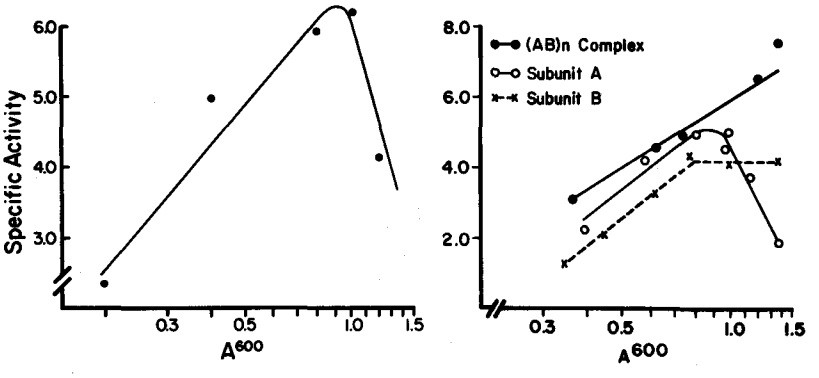

Figure 1. Complementation of subunits A and B as a function of the A^{600}. Left) Cultures of strains KD2 and GSY292 were harvested at the same A^{600}. The resulting cell pellets were resuspended together in buffer B, lysed, and assayed for GOGAT activity. Right) Cultures of strains KD2 and GSY292 were harvested at the indicated A^{600}. The resulting cell pellet from either strain KD2 or GSY292 was mixed with a cell pellet from either strain GSY292 or KD2 harvested at an A^{600} of 0.8. The pellets were resuspended in buffer B, lysed and assayed for GOGAT. The prototroph, NP1, was harvested at the indicated A^{600}, resuspended in buffer B and assayed for GOGAT.

vity of the gltA mutant did not. For comparison the response of GOGAT from the prototroph NP1 is indicated.

The possibility existed that rather than complementation of non-identical subunits, we were observing an activation or modification of an inactive GOGAT to an active state. The putative activation and/or modification process should be functional in the prototroph NP1, since it contains an active GOGAT. Thus, one would expect to get complementation (activation) when extracts were prepared by mixing NP1 with KD2 or GSY292. There was, however, no complementation found in these extracts (data not shown). This result is more consistent with the hypothesis that we are observing complementation of non-identical GOGAT subunits rather than activation of an inactive GOGAT.

Physical evidence for a reconstituted GOGAT. Extracts prepared from the prototroph NP1 and a mixture of the mutants KD2 and GSY292 were subjected to gel filtration (Fig. 2). The majority of the GOGAT activity from the prototroph NP1 was eluted as a single peak with an estimated molecular weight of 173,000 daltons. Similarly, the reconstituted GOGAT was eluted at the same Ve/Vo ratio as the prototrophic enzyme. No GOGAT activity was eluted from the column,

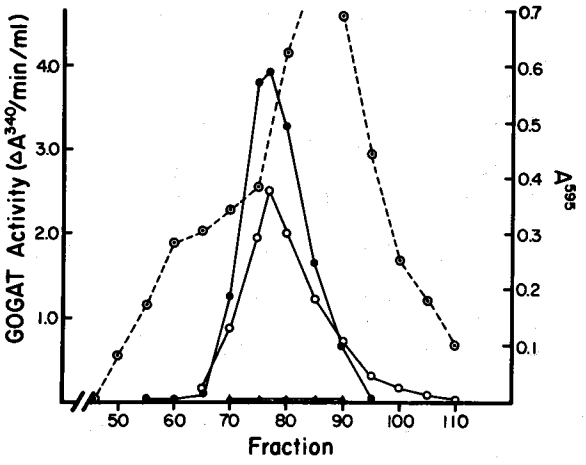

Figure 2. Elution profile of native and reconstituted GOGAT. The activity of GOGAT from NP1 (●-●), GSY292 + KD2 (o—o), GSY292 or KD2 (▲—▲) was plotted as a function of the fraction number. The reconstituted enzyme was formed by complementation method II. Protein (⊙-⊙) was determined with the BioRad protein reagent.

however, when an extract of either strain KD2 or GSY292 was used. Although we attempted to locate the putative subunits from each mutant following gel filtration, only 2% of the anticipated activity was found. This loss of complementing activity with partial purification, coupled with the high variability of the Method I assay suggest that these activities are labile in the two mutants.

We have established that an active GOGAT enzyme can be reconstituted by mixing extracts of the gltA and gltB mutants. This reconstituted enzyme was similar to the enzyme derived from the prototroph with respect to: (i) its activity and (ii) its elution profile from a gel filtration column. The unique ability of the GOGAT from B. subtilis to be reconstituted should prove most useful in the future studies on the structure and regulation of this important enzyme.

ACKNOWLEDGEMENTS

We thank Pat Moore for her excellent technical assistance. This research was supported with funds from the NIH (AM19443) and the NSF (PCM77-18960).

REFERENCES

1. Miller, R.E. and E.R. Stadtman. (1972). J. Biol. Chem. 247: 7407-7419.
2. Mantsälä, P. and H. Zalkin. (1976). J. Biol. Chem. 251: 3294-3299.
3. Mantsälä, P. and H. Zalkin. (1976). J. Biol. Chem. 251: 3300-3305.

4. Trotta, P.P., K.E.B. Platzer, R.H. Haschmeyer and A. Meister. (1974) Proc. Natl. Acad. Sci. U.S.A. 71: 4607-4611.
5. Geary, L.E. and A. Meister. (1977). J. Biol. Chem. 252:3501-3508.
6. Hemmila, I.A. and P.I. Mantsälä. (1978). Biochem. J. 173:45-52.
7 Berberich, M.A. (1972). Biochem. Biophys. Res. Commun. 47: 1498-1503.
8. Pahel, G., A.D. Zelenetz and B.M. Tyler. (1978). J. Bacteriol. 133: 139-148.
9. Ely, B., A.B.C. Amarasinghe and R.A. Bender. (1978). J. Bacteriol. 133: 225-230.
10. Kane, J.F., S.L. Stenmark, D.H. Calhoun, and R.A. Jensen. (1971). J. Biol. Chem. 246: 4306-4316.
11. Miller, J.H. (1972). Experiments in Molecular Genetics pp.138, Cold Spring Harbor Laboratory, New York.
12. Saito, H. and Miura, K. (1963). Biochem. Biophys. Acta. 72: 619-629.
13. Young, F.E. and G.A. Wilson. (1974). Bacillus subtilis, pp. 69-114. In, Handbook of Genetics, Vol. 1. (Ed. R.C. King) Plenum Publishing Corp., New York.
14. Hoch, J.A. and J. Mathews. (1972). Genetic Studies in Bacillus subtilis. In Spores V (H.O. Halvorson, R. Hanson and L.L. Campbell, eds), pp. 113-116, ASM Publication.