TWO FORMS OF GLUTAMINE SYNTHETASE IN FREE-LIVING ROOT-NODULE BACTERIA

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Received August 1, 1977

Summary:

Cell-free extracts of Rhizobium japonicum 61A76 contain two forms of glutamine synthetase (EC 6.3.1.2) which can be easily separated by isoelectric focusing. The more acid form (pI = 5.4), like the enzyme from E. coli, is stable at 50° and catalyses an ADP-dependent transferase reaction, whose inhibition by excess Mg^{++} can be relieved by snake venom phosphodiesterase. The more alkaline form (pI = 6.1) is labile at 50°, and catalyses an ADP-dependent transferase reaction which is strongly inhibited by Mg^{++} regardless of phosphodiesterase treatment. We have also observed the two forms of the enzyme in a nitrogenase-less mutant of 61A76, and in other strains of rhizobia, but only the acid form in E. coli W, A. vinelandii OP, and K. pneumoniae M 51A.

Introduction:

Following the pioneering work of Magasanik and his colleagues (1) on the regulatory functions of glutamine synthetase in K. aerogenes, other workers have found evidence that glutamine synthetase regulates the nitrogenase of K. pneumoniae (2,3). The case for a similar function in rhizobia and rhizobial bacteroids is less clear. Some workers have concluded that glutamine synthetase does not mediate ammonium repression of nitrogenase in rhizobia (4,5,6), and others have found positive evidence for this role (7,8).

The present study shows that R. japonicum 61A76 has two forms of glutamine synthetase, which we refer to as GSI and GSII. Although the biochemical significance of the two forms is still not clear, their presence in rhizobia must be considered in attempting to assign a regulatory role to glutamine synthetase. This is especially true when interpreting inhibition by Mg^{++} of the transferase reaction in crude extracts as an indication of adenylylation (9), since only one of the two forms (GSI) appears to be similar to the enzyme from E. coli.

* Contribution No. 592 of the Charles F. Kettering Research Laboratory.

ISSN 0006-291X

Materials and Methods:

Growth of Microorganisms. Stocks were maintained on solid media at 4°. Liquid media contained the usual salts and trace elements adjusted to pH 6.0 (10), to which were added 10 mM Na gluconate as carbon source, as well as various nitrogen sources. Transfers were made from solid media to 30 ml of liquid medium containing 0.1% yeast extract as a nitrogen source, in 125 ml conical flasks, which were shaken at 150 rpm in a rotary shaker at 30° for 3 or 4 days. The resulting cultures (1-2 ml) were used to inoculate 165 ml liquid medium, containing either 5 mM NH_4Cl or 10 mM Na glutamate as a nitrogen source, in 500 ml conical flasks, which were shaken at 150 rpm in a rotary shaker at 30°. Cells were grown to stationary phase, chilled rapidly, and crude extracts prepared according to Tronick et al. (11).

Isoelectric Focusing. Runs were performed according to the instructions supplied with the column and ampholyte provided by LKB instruments. Samples which had been dialysed as specified in the procedure of Tronick et al. (11) were added directly to the gradient components. Ampholyte was an equal mixture of pH ranges 4-6 and 5-7 at a final concentration of 1%. Runs were at 4°, 600 V, with the power supply set at constant voltage, for 22 hr. Fraction size was 2.5 ml.

Enzymes and Reagents. Snake venom phosphodiesterase (ICN) was assayed by a modification of the procedure of Koerner and Sinsheimer (12), with one unit defined as that amount hydrolysing 1 μmole of bis[p-nitrophenyl]phosphate per minute at 25°. Transferase was assayed according to Shapiro and Stadtman (13) at 37°, but absorbance was determined at 510 nm. Components for the "coupled" assay (13), which was run at 25°, were obtained from Sigma.

Results and Discussion:

Fig. 1 shows that two distinct peaks of γ-glutamyl transferase activity are obtained when crude extracts of R. japonicum 61A76 are subjected to isoelectric focusing. As shown, both peaks require ADP for transferase activity. Also shown in Fig. 1 is the transferase activity profile with 80 mM $MgCl_2$ added to the standard assay mixture, (in which Mn^{++} is the activating cation [13]). Characteristically, both GSI and GSII are inhibited by Mg^{++}, with the percentage inhibition always greater for GSII. Fig. 1 also shows that GSI and GSII focus in their original position when the peak fractions are combined, stripped of any bound ampholyte by treatment on a mixed-bed ion-exchange resin (14), and re-run separately in the same focusing system. In both the original run and the re-run, GSI has a shoulder, or skewed distribution, toward the acid side of the pH gradient, although it is less pronounced in the re-run.

We have examined additional strains of rhizobia and other bacteria in the same way, except that glutamate was substituted for NH_4^+ as the nitrogen source during growth, a condition which was found to increase the proportion of GSII to

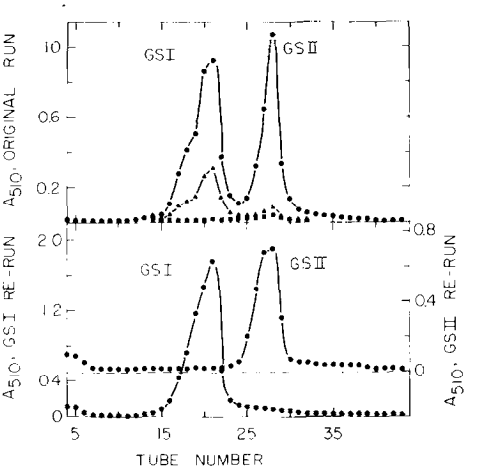

Fig. 1. Resolution of GSI and GSII by column isoelectric focusing, and re-focusing of the separated peaks. An extract of R. japonicum 61A76 (5 ml), grown to stationary phase on NH_4Cl, containing 44 units/ml of transferase and 10 mg protein per ml, was focused as described in the methods section. Samples (10 µl) were analyzed for transferase activity in the complete assay (13) (●) (30 min), in the absence of ADP (■) (120 min), and with 80 mM $MgCl_2$ added to the complete assay system (▲) (120 min). The overall yield from the column was 88%. Fractions 17-22 (GSI) and 26-29 (GSII) were combined and each reduced to 4 ml in an Amicon pressure cell (PM-30 membrane). One ml of the sample plus 0.1 ml 1% hemoglobin was then processed on a mixed-bed ion-exchanger column (Bio-Rad AG 501 x 8), 1 cm x 5 cm, as described by Baumann and Chrambach (14) for the separation of carrier ampholytes from protein. The column was pre-equilibrated and developed at 4° with 10 mM Imidazole HCl, 1 mM $MnCl_2$, pH 7.0, and 1.0 ml fractions were collected into tubes containing 0.1 ml of 0.1 M Imidazole HCl, 10 mM $MnCl_2$, pH 7.0. Fractions absorbing light at 415 nm were pooled and re-focused individually. Recovery in the second focusing of units from the combined fractions in the first run: GSI, 47%; GSII, 23%. The pH of the peak tubes was, original run/re-run: GSI: 5.43/5.40; GSII: 6.10/6.08. Similar results were obtained when treatment on the mixed-bed ion-exchanger column was omitted and the peak fractions were re-focused directly.

GSI in R. japonicum 61A76. The other strains of rhizobia, including R. japonicum 61A76 SM-5 (a nitrogenase-less mutant of 61A76 (15)), 3I1b83, 3I1b138, 3I1b110, and Rhizobium sp 32H1, gave both GSI and GSII. In contrast, only one peak was obtained from E. coli W, A. vinelandii OP, and K. pneumoniae M51A. That single peak corresponded to GSI and showed the same skewed distribution noted above for R. japonicum 61A76.

Extracts have also been prepared in the same way (11) from E. coli W which had been grown as recommended by Shapiro and Stadtman for high and for low adenylylation (13). The average degree of adenylylation of the two extracts, as

TABLE I. The effect of snake venom phosphodiesterase on the inhibition by Mg^{++} of transferase activity catalysed by GSI and GSII. GSI and GSII were obtained from the isoelectric focusing run shown in Fig. 1, and 2.4 transferase units of each, directly from the focusing column, were incubated for 60 minutes at 37° at pH 9.0, in the presence of 0.1 M Tris HCl, 1 mM $MgCl_2$, with and without 0.05 units of snake venom phosphodiesterase. The samples were chilled in ice and 20 µl assayed at 37° for 60 minutes at pH 6.7, with and without 80 mM $MgCl_2$. The reaction mixture was that described by Stadtman et al. (9) except that the buffer was 50 mM final concentration each of imidazole, bicine, and MES, added as a 5-fold concentrated mixture. Absorbance for control incubations (- Mg^{++}) at 510 nm: GSI, 0.701; GSII, 0.895.

		Percent of Control Activity	
	80 mM Mg^{++}	Untreated	Phosphodiesterase-treated
GSI	-	100	103
	+	37	89
GSII	-	100	75
	+	17	15

determined by inhibition of the transferase reaction by Mg^{++}, was $E_{\overline{9.8}}$ and $E_{\overline{3.8}}$ (13). Each extract gave only a single peak, corresponding to GSI, upon isoelectric focusing.

Treatment with snake venom phosphodiesterase has been developed by Stadtman and his colleagues as a means of estimating the degree of adenylylation of glutamine synthetase (11). For the E. coli enzyme, under certain assay conditions, inhibition of transferase activity by Mg^{++} is proportional to the extent of adenylylation (9). Treatment with phosphodiesterase causes de-adenylylation and concomitant release of the inhibition by Mg^{++}. Table I shows that only GSI exhibits the type of response shown by the E. coli enzyme when the isolated forms are treated with phosphodiesterase. We have found this true over a range of pH values and incubation times for the transferase assay, and with several sources of phosphodiesterase. We have also found that only GSI shows released inhibition by Mg^{++} when the phosphodiesterase digestion is carried out on the crude extract, before the separation of the two forms by isoelectric focusing, thus excluding any possible differential effect on the phosphodiesterase of that portion of the synthetic ampholyte which focuses around pH 6.1.

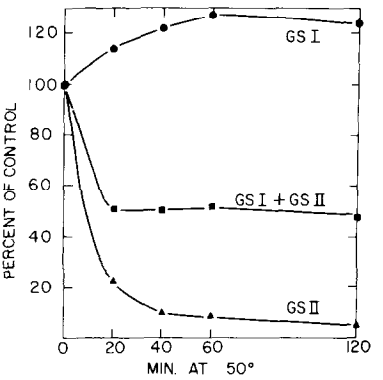

Fig. 2. Relative heat stability of GSI and GSII. GSI and GSII were obtained from an extract of glutamate-grown R. japonicum 61A76 SM-5 by isoelectric focusing, as described in the methods section. Samples of the two peaks were diluted 10-fold in 10 mM Imidazole-HCl, 1 mM $MnCl_2$, pH 7.0, and heated at 50° for the stated times, individually, and in a 1 to 1 (v/v) mixture. They were rapidly cooled in ice, and 100 µl of each was assayed by observing NADH oxidation in the coupled assay system described by Shapiro and Stadtman (13). The rates of change of absorbance at 340 nm for the unheated controls were 0.037, 0.053, and 0.046 per minute, respectively, for GSI (●), GSII (▲) and the 1 to 1 mixture (■).

We have repeatedly observed that GSII loses activity more rapidly than GSI upon storage at various temperatures. Since glutamine synthetase from E. coli is known to be stable at 50° (13), we compared the stability at this temperature of individual samples of GSI and GSII, and a mixture of the two, as shown in Fig. 2. The results shown were obtained with the "coupled" assay of the biosynthetic reaction (13), using enzyme isolated from the nitrogenase-less mutant, R. japonicum 61A76 SM-5. Similar results have been obtained with the transferase reaction, and with the wild-type organism. This difference in heat lability could also be shown in unfractionated crude extracts.

Two general questions arise from the present results. First, are GSI and GSII the products of separate genes, or do they represent stable modifications of the same peptide? Second, is there a physiological advantage, related to the ability of the rhizobia to enter into symbiotic relationships, associated with the possession of two forms of glutamine synthetase? Perhaps the catalytic and the regulatory functions of glutamine synthetase are carried out by separate proteins.

Acknowledgments: We wish to thank Dr. Winston J. Brill for providing R. japonicum 61A76 SM-5, Drs. Donald L. Keister and William R. Evans for many helpful discussions and suggestions, and Dr. Gerald A. Peters for critically reviewing the manuscript.

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