Regulation of Glutamate Dehydrogenase in *Bacillus subtilis*

JAMES F. KANE,[1][†]* JUBRAN WAKIM,[2] AND RANDY S. FISCHER[1][‡]

*Department of Microbiology and Immunology, University of Tennessee Center of the Health Sciences,
Memphis, Tennessee 38163,[1] and Department of Chemistry, University of Tennessee at Martin,
Martin, Tennessee 38238[2]*

The activity of the nicotinamide adenine dinucleotide-dependent glutamate dehydrogenase in *Bacillus subtilis* was influenced by the carbon source, but not the nitrogen source, in the growth medium. The highest specific activity for this enzyme was found when *B. subtilis* was grown in a minimal or rich medium that contained glutamate as the carbon source. It is proposed that glutamate dehydrogenase serves a catabolic function in the metabolism of glutamate, is induced by glutamate, and is subject to catabolite repression.

Glutamate dehydrogenases catalyze the oxidative amination of α-ketoglutarate to glutamate and the reductive deamination of glutamate. This enzyme, therefore, can function in either ammonia assimilation or glutamate catabolism (1-4, 6, 7, 11, 12, 14, 15, 17, 18). Although glutamate dehydrogenase has been found in many species of *Bacillus* (1, 10, 18), there have been conflicting reports on the presence of a glutamate dehydrogenase in *Bacillus subtilis* (1, 9, 12, 14, 17, 18). Kimura et al. (14) reported that strains 168 and PCI219 of *B. subtilis* possessed a glutamate dehydrogenase with dual specificity for NAD and NADP, and these investigators purified this enzyme from strain PCI219 and described its properties. In this report, we describe the properties and regulation of an NAD-dependent glutamate dehydrogenase purified from *B. subtilis* strain 168.

Cells were grown in a minimal salts (12), nutrient broth, Penassay broth, or Trypticase soy broth medium (BBL Microbiology Systems) at 37°C. Cell growth was monitored by measuring the turbidity at 600 nm, and the cultures were harvested at an absorbance of between 0.6 and 0.8. The cell pellets were suspended in either 40 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 12 mM β-mercaptoethanol, 12 mM glutamine, 5 mM histidine, 1 mM NaN$_3$, 1 mM phenylmethylsulfonyl fluoride, and 30% glycerol (buffer A); or 50 mM potassium phosphate buffer, pH 6.5, containing 1 mM EDTA, 6 mM β-mercaptoethanol, 5 mM α-ketoglutarate, and 30% glycerol (buffer B). Buffer B was used in the purification of glutamate

† Present address: Bethesda Research Laboratories, Inc., Gaithersburg, MD 20760.
‡ Present address: Department of Biochemistry, Georgetown University Medical Center, Washington, DC 20007.

dehydrogenase. Crude extracts, prepared in either buffer A or B as previously described (8), contained identical activities for glutamate dehydrogenase.

Glutamate dehydrogenase was assayed at 37°C by monitoring the oxidation of NADH with a Gilford recording spectrophotometer. The reaction mixture contained 90 μmol of Tris buffer, pH 7.25, 10 μmol of α-ketoglutarate, 0.26 μmol of NADH, and 200 μmol of (NH$_4$)$_2$SO$_4$ in a final volume of 1.0 ml. The reaction was initiated by the addition of enzyme (50 μg of crude protein or 1 μg of purified protein in 10 μl), and the decrease in absorbance at 340 nm was monitored for 2 min. A blank consisted of the oxidation of NADH in the absence of (NH$_4$)$_2$SO$_4$. Alanine dehydrogenase was assayed as described previously (21), except that the NAD analog, *N*-acetylpyridine adenine dinucleotide, was used as the cofactor. The specific activity was expressed as micromoles of cofactor oxidized per minute per milligram of protein.

Strain B15 was isolated as follows. The *mtr* mutant NP100 (a strain 168 derivative [12]) that possesses a derepressed tryptophan biosynthetic pathway and a high level of glutamate dehydrogenase was inoculated onto a minimal glucose plate containing tryptophan (100 μg/ml) and glutamate (800 μg/ml). Since strain NP100 does not grow on this medium, we isolated a number of spontaneous mutant derivatives of NP100. Strain B15 does not express the tryptophan biosynthetic enzymes constitutively, but does possess the same activity for glutamate dehydrogenase.

The glutamate dehydrogenase from *B. subtilis* strain B15 was purified approximately 200-fold (Table 1). A 10-liter amount of cells was grown aerobically in a fermentor until an ab-

TABLE 1. *Purification of glutamate dehydrogenase from B. subtilis mutant B15, a derivative of strain 168[a]*

Step[b]	Total protein (mg)	Total U	Sp act	% Yield
Crude extract	1,102	153	0.14	100
Sephacryl S-200	456	103	0.23	67
DEAE-Sephacel	20	85	4.30	56
Hydroxylapatite	2	52	26.00	34

[a] Mutant B15 was grown in a minimal glucose medium and contained a 10-fold-greater specific activity for glutamate dehydrogenase than the parent strain 168 (NP19). Similar results were found, however, with the *trpC* mutant NP19 grown in nutrient broth, except the starting specific activity was greater than that of mutant B15.

[b] Buffer B was used in this purification scheme.

sorbance of 0.6 at 600 nm was reached. At this time, the cells were pelleted, suspended in a minimal volume of buffer B, treated with lysozyme (100 µg/ml) and DNase (10 µg/ml), and clarified by centrifugation at 100,000 × *g* for 90 min. The crude extract was passed through a Sephacryl S-200 (Pharmacia Fine Chemicals, Inc.) column (5.0 by 80 cm) equilibrated with buffer B. Fractions comprising the peak of glutamate dehydrogenase activity were recovered, pooled, and applied to a DEAE-Sephacel (Pharmacia Fine Chemicals, Inc.) column (2.5 by 11.0 cm). The glutamate dehydrogenase activity was eluted with a KCl gradient from 0.03 to 0.35 M. Fractions containing glutamate dehydrogenase were combined and dialyzed against buffer B before this pool was passed through a hydroxylapatite column (1.0 by 14.0 cm) consisting of Bio-Gel HTP (Bio-Rad Laboratories). This column was developed by a linear gradient of buffer B containing 50 to 300 mM potassium phosphate. Before storage, selected fractions or appropriate combinations of fractions were concentrated on an Amicon XM-50 membrane and dialyzed against buffer B. This preparation was used to determine some of the kinetic properties of the enzyme. At the pH optima of 7.25 and 8.0, respectively, the amination reaction was about 10-fold more active than the deamination reaction. Substrate inhibition was found with NH_4^+ and α-ketoglutarate, and NADH was 50-fold more active than NADPH at pH 7.25. This slight activity with NADPH was consistent with the dual specificity of this enzyme reported by Kimura et al. (14). Neither glutamine nor asparagine could replace NH_4^+ as a substrate. These properties were very similar to those of the glutamate dehydrogenase purified from *B. subtilis* strain PCI219 (14).

Although the kinetic results are consistent

with a biosynthetic function, physiological studies suggest a catabolic role for glutamate dehydrogenase (Table 2). The activity of this enzyme is low in minimal medium containing ammonium salts, glutamate, or ammonium plus glutamate as the nitrogen source. Thus, glutamate dehydrogenase is not responsible for generating ammonia from glutamate. The highest activity for this enzyme, however, is found in cells grown in nutrient broth. Since Trypticase soy broth and Penassay broth both contain glucose (0.25% and 0.1%, respectively) but nutrient broth does not, we tested the possibility that glutamate dehydrogenase is repressed when a readily metabolizable carbon source is in the medium. The specific activity for glutamate dehydrogenase decreased 60-fold when the *trpC* mutant NP19 (13) was grown in nutrient broth containing more than 0.01% glucose. Furthermore, it appears that glucose affects the synthesis of glutamate dehydrogenase rather than the activity since the addition of 0.5% glucose to strain NP19 growing in nutrient broth decreased the specific activity approximately twofold after one cell doubling. This observation was more consistent with the inhibition of transcription and dilution of enzyme activity than with the modification of an active to an inactive enzyme mediated by glucose or some catabolite of glucose. Since 0.5% mannose, fructose, or glycerol had a similar repressive effect (specific activities, 3.4, 1.5, and 1.5, respectively), we propose that some common

TABLE 2. *Specific activity of glutamate dehydrogenase in B. subtilis trpC mutant NP19 as a function of the growth medium*

Medium[a]	Sp act[b]
Minimal + ammonium + glucose	1.1
Minimal + glutamate + glucose	0.9
Minimal + ammonium + glutamate + glucose	1.2
Trypticase soy broth + yeast extract	1.3
Penassay broth	2.0
Nutrient broth	61.0

[a] The final concentrations of glucose, glutamate, and ammonium in the various minimal media were 0.5, 0.2, and 0.2%, respectively. Trypticase soy broth, Penassay broth, and nutrient broth were rehydrated according to the manufacturers directions. Yeast extract was added to a final concentration of 0.6%. Yeast extract was obtained from Difco Laboratories, Detroit, Michigan. Trypticase soy broth, Penassay broth, and nutrient broth were obtained from BBL Microbiology Systems, Cockeysville, Md. The glucose concentrations in Trypticase soy broth and Penassay broth which are discussed in the text are cited according to the manufacturers specifications.

[b] These specific activities represent the average of 17, 5, 3, 2, 3, and 5 separate experiments, respectively.

catabolite is the effector molecule controlling the expression of glutamate dehydrogenase.

We compared the response of glutamate dehydrogenase to that of alanine dehydrogenase, a known catabolic enzyme in *B. subtilis*. Alanine dehydrogenase, an enzyme involved in spore germination (9), was induced 15- to 18-fold in cells, using alanine as carbon or nitrogen source (Table 3), and glucose or citrate had no repressive effect on enzyme activity. Glutamate dehydrogenase, on the other hand, was low in cells grown in a defined medium, except when glutamate was the carbon source (Table 3). In this latter case, the activity of this enzyme increased almost 50-fold, although the activity did not reach that found in cells grown on nutrient broth. This difference may reflect the fivefold difference between the growth rates of strain NP19 in these two media. Thus, the activities of both enzymes were increased in cells metabolizing glutamate or alanine as a carbon or carbon-nitrogen source, respectively. As expected, the activity of both enzymes was increased in cells grown in nutrient broth (Table 3), and glutamate dehydrogenase was increased further when glutamate was added to the medium (Table 3). The addition of glucose to this medium resulted in decreased activity for both enzymes.

Since glutamate plays a role in sporulation (5), we examined the possible role of glutamate dehydrogenase as a sporulation specific enzyme in the following experiments. First, we grew a *guaB* mutant (20) in nutrient broth containing glucose plus guanine or guanine alone. In the former case, glutamate dehydrogenase was low (1.0 μmol h^{-1} mg^{-1} of protein), and in the latter case, activity was high (58 μmol h^{-1} mg^{-1} of protein). Freese and co-workers report that guanine starvation induces sporulation even in the presence of glucose (16, 20). Therefore, we starved the *guaB* mutant for guanine in nutrient broth containing glucose. Although the sporulation process begins as a result of this growth regimen, glutamate dehydrogenase was not increased even after 3 h of guanine starvation.

Second, a culture of the *trpC* mutant NP19 was grown in minimal glucose medium and shifted to the sporulation medium described by Sterlini and Mandelstam (19). Again, no increase in glutamate dehydrogenase activity was observed for 3 h after this shift. These results suggest that glutamate dehydrogenase does not function as a sporulation enzyme.

We propose that glutamate dehydrogenase in *B. subtilis* has a catabolic function, is induced by glutamate, and is subject to catabolite repression. The basis for the increased activity of glutamate dehydrogenase in some mutants (12) grown in a medium containing glucose and cit-

TABLE 3. *Comparison of glutamate and alanine dehydrogenases from strain NP19 grown in different media*

Medium[a]	Carbon source	Nitrogen source	Sp act	
			Glutamate dehydrogenase	Alanine dehydrogenase
MS	Glucose + citrate	(NH$_4$)$_2$SO$_4$	1.1	1.1
MS	Glucose + citrate	Alanine	0.8	18.0
MS	Alanine	(NH$_4$)$_2$SO$_4$	3.5	15.0
MS	Glutamate	(NH$_4$)$_2$SO$_4$	38.0	1.6
MS	Glucose + citrate	Glutamate	0.8	1.0
NB	Amino acids	Amino acids	61.0	8.6
NB	Amino acids + glutamate	Amino acids + glutamate	97.0	8.5
NB	Glucose	Amino acids	0.8	2.5

[a] NB, Nutrient broth; MS, minimal salts mixture. Glutamate was added to NB to a final concentration of 0.2%. The carbon sources were added to give the following final concentrations: glucose, 0.5%; citrate, 0.4%; glutamate, 0.4%. The nitrogen sources in MS were all added to a final concentration of 0.2%.

rate is unknown at this time. These mutants may contain a defect in catabolite repression in general or a specific defect in the putative repressor protein for the locus that specifies glutamate dehydrogenase. Experiments are in progress to distinguish these possibilities.

LITERATURE CITED

1. **Aronson, J. N.** 1976. Ammonia assimilation and glutamate catabolism by *Bacillus thuringiensis*, p. 444–449. *In* D. Schlessinger (ed.), Microbiology—1976. American Society for Microbiology, Washington, D.C.
2. **Bender, R. A., A. Macaluso, and B. Magasanik.** 1976. Glutamate dehydrogenase genetic mapping and isolation of regulatory mutants of *Klebsiella aerogenes*. J. Bacteriol. **127:**141–148.
3. **Brenchley, J. E., C. A. Baker, and L. G. Patil.** 1975. Regulation of the ammonia assimilatory enzymes in *Salmonella typhimurium*. J. Bacteriol. **124:**182–189.
4. **Brenchley, J. E., and B. Magasanik.** 1974. Mutants of *Klebsiella aerogenes* lacking glutamate dehydrogenase. J. Bacteriol. **117:**544–550.
5. **Charba, J. F., and H. M. Nakata.** 1977. Role of glutamate in the sporogenesis of *Bacillus cereus*. J. Bacteriol. **130:**242–248.
6. **Dendinger, S. M., and J. E. Brenchley.** 1980. Temperature sensitive glutamate dehydrogenase mutants of *Salmonella typhimurium*. J. Bacteriol. **144:**1043–1047.
7. **Dendinger, S. M., L. G. Patil, and J. E. Brenchley.** 1980. *Salmonella typhimurium* mutants with altered glutamate dehydrogenase and glutamate synthase activities. J. Bacteriol. **141:**190–198.
8. **Deshpande, K. L., J. R. Katze, and J. F. Kane.** 1981. Effect of glutamine on enzymes of nitrogen metabolism in *Bacillus subtilis*. J. Bacteriol. **145:**768–774.
9. **Freese, E., S. W. Park, and M. Cashel.** 1964. The

developmental significance of alanine dehydrogenase in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **51**:1164-1172.

10. **Hemmilä, I. A., and P. I. Mäntsälä.** 1978. Purification and properties of glutamate synthase and glutamate dehydrogenase from *Bacillus megaterium*. Biochem. J. **173**:45-52.

11. **Hemmings, B. A., and A. P. Sims.** 1977. The regulation of glutamate metabolism in *Candida utilis*: evidence for two interconvertible forms of NAD-dependent glutamate dehydrogenase. Eur. J. Biochem. **80**:143-151.

12. **Kane, J. F., and K. L. Deshpande.** 1979. Properties of glutamate dehydrogenase from *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **88**:761-767.

13. **Kane, J. F., S. L. Stenmark, D. H. Calhoun, and R. A. Jensen.** 1971. Metabolic interlock: the role of the subordinate type of enzyme in the regulation of a complex pathway. J. Biol. Chem. **246**:4306-4316.

14. **Kimura, K., A. Miyakawa, T. Imai, and T. Sasakawa.** 1977. Glutamate dehydrogenase from *Bacillus subtilis* PCI219. I. Purification and properties. J. Biochem. **81**:467-476.

15. **Kinghorn, J. R., and J. A. Pateman.** 1973. NAD and NADP L-glutamate dehydrogenase activity and ammonium regulation in *Asperigillus nidulans*. J. Gen. Microbiol. **78**:39-46.

16. **Lopez, J. M., C. L. Marks, and E. Freese.** 1979. The decrease of guanine nucleotides initiates sporulation of *Bacillus subtilis*. Biochem. Biophys. Acta **587**:238-252.

17. **Pateman, J. A.** 1969. Regulation of synthesis of glutamate dehydrogenase and glutamine synthetase in micro-organisms. J. Biochem. **115**:769-775.

18. **Phibbs, P. V., and R. W. Bernlohr.** 1971. Purification, properties, and regulation of glutamic dehydrogenase of *Bacillus licheniformis*. J. Bacteriol. **106**:375-385.

19. **Sterlini, J. M., and J. Mandelstam.** 1969. Commitment to sporulation in *Bacillus subtilis* and its relationship to development of actinomycin resistance. Biochem. J. **113**:29-37.

20. **Vasantha, N., and E. Freese.** 1980. Enzyme changes during *Bacillus subtilis* sporulation caused by deprivation of guanine nucleotides. J. Bacteriol. **144**:1119-1125.

21. **Yoshida, A., and E. Freese.** 1970. L-Alanine dehydrogenase (*Bacillus subtilis*). Methods Enzymol. **17**:176-181.