A Feedback-resistant Mutant of *Bacillus subtilis* Glutamine Synthetase with Pleiotropic Defects in Nitrogen-regulated Gene Expression*

Received for publication, May 5, 2005, and in revised form, July 1, 2005 Published, JBC Papers in Press, July 29, 2005, DOI 10.1074/jbc.M504957200

Lewis V. Wray, Jr. and Susan H. Fisher

From the Department of Microbiology, Boston University School of Medicine, Boston, Massachusetts 02118-2526

The *Bacillus subtilis* TnrA transcription factor regulates gene expression during nitrogen-limited growth. When cells are grown with excess nitrogen, feedback-inhibited glutamine synthetase forms a protein-protein complex with TnrA and prevents TnrA from binding to DNA. A mutation in glutamine synthetase with a phenylalanine replacement at the Ser-186 residue (S186F) was isolated by screening for *B. subtilis* mutants with constitutive TnrA activity. Although S186F glutamine synthetase has kinetic properties that are similar to the wild-type protein, the S186F enzyme is resistant to feedback inhibition by glutamine and AMP. Ligand binding experiments revealed that the S186F protein had a lower affinity for glutamine and AMP than the wild-type enzyme. S186F glutamine synthetase was defective in its ability to block DNA binding by TnrA in vitro. The properties of the feedback-resistant S186F mutant support the model in which the feedback-inhibited form of glutamine synthetase regulates TnrA activity in vivo.

Glutamine, a key component in microbial nitrogen metabolism, is synthesized *de novo* from glutamate and ammonium in an ATP-dependent reaction catalyzed by glutamine synthetase (GS)1 (1, 2). The synthesis and activity of GS is highly regulated to ensure that cells contain adequate levels of glutamine under all nutritional conditions. In enteric bacteria, glutamine is the metabolic signal for the Ntr regulatory system (3). During nitrogen-limited growth conditions, where glutamine levels are low, the NR transcription factor activates gene expression. Under nitrogen excess condition, where glutamine levels are high and NR is inactive, other components of the Ntr system posttranslationally inactivate GS by adenylylation. The adenyllylated form of *Escherichia coli* GS is susceptible to cumulative feedback inhibition by nine different nitrogen-containing compounds (4).

The low G + C Gram-positive bacterium *Bacillus subtilis* utilizes two transcriptional factors, GlnR and TnrA, to control the expression of nitrogen-regulated genes (5). The GlnR protein is active during growth with excess nitrogen (5–8). Although genetic experiments have shown that GS is required for GlnR-dependent gene regulation, the precise role of GS in GlnR regulation has yet to be determined (7, 9). The TnrA protein regulates gene expression during nitrogen-limited growth conditions (5). GS directly controls TnrA activity (5, 10–12). When nitrogen is in excess, feedback-inhibited GS forms a protein-protein complex with TnrA and inhibits DNA binding by TnrA (13). In the *in vitro* interaction between GS and TnrA is most strongly promoted by the feedback inhibitor glutamine (13).

GS is a metalloenzyme that requires either Mg2+ or Mn2+ for catalytic activity (4). Distinct groups of compounds inhibit the different *in vitro* reactions catalyzed by *B. subtilis* GS (14, 15). Although the Mg2+ dependent biosynthetic reaction can be completely inhibited by glutamine or AMP, the Mn2+ dependent biosynthetic activity is only partially inhibited by alanine, serine, or glycine. The γ-glutamyltransferase reverse reaction is inhibited by AMP, alanine, glycine, serine, tryptophan, and histidine (16). Posttranslational modification of *B. subtilis* GS has not been observed (17, 18).

The crystal structures of the GS proteins from *B. subtilis*, *Salmonella typhimurium*, and *Mycobacterium tuberculosis* have been determined (20, 21). These enzymes contain 12 identical subunits that are arranged as two hexameric rings stacked on top of one another. The active sites are located at the interface of adjacent subunits within the hexamer rings and have a cylindrical structure that is constricted in the middle. The substrates ATP and glutamate enter and bind at opposite ends of this cylinder. The structural models of the *B. subtilis* and *S. typhimurium* enzymes revealed that glutamine and AMP bind within the active site at the glutamate and ATP substrate sites, respectively (22, 23).

We have previously isolated mutations in GS that have enzymatic properties similar to the wild-type enzyme but are unable to regulate TnrA (24). The mutated amino acid residues were found to be located near the glutamate entrance to the active site, indicating that this region of GS is critical for the interaction with TnrA. This study describes the isolation and characterization of a *B. subtilis* mutant encoding a GS that is both resistant to feedback inhibition and defective in regulating TnrA DNA binding activity.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth**—The glnA186 mutation was isolated using SF402S cells (amyE::[amtB-lacZ]402 cat) tnrA(spc) trpC2 mutagenized with N-methyl-N’-nitro-N-nitrosoguanidine (13). Strains for analysis of β-galactosidase levels were constructed by using glnA186 mutant chromosomal DNA to transform strain SF416G (amyE::[amtB-lacZ]416 neo) glnA14::spc trpC2 or SF17GT (amyE::[glnRA-lacZ]17 neo) glnA14::spc tnrA62::Tn917 trpC2 with selection for Gln1 on glucose minimal medium plates with glutamate as the nitrogen source. Bacteria were grown in minimal medium liquid cultures using methods reported previously (25). The minimal medium described by Neidhardt et al. (26) was used for the growth of liquid cultures. BSS minimal medium agar plates were prepared as described previously (27). The carbon source was 0.5% glucose, whereas the final concentra-

---

*This work was supported by National Institutes of Health Grant GM51127 (to S. H. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Dept. of Microbiology, Boston University School of Medicine, 715 Albany St., Boston, MA 02118-2526. Tel: 617-638-5498; Fax: 617-638-4286; E-mail: shfisher@bu.edu.

2 The abbreviations used are: GS, glutamine synthetase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactosidase.

3 D. S. Murray, L. V. Wray, Jr., S. H. Fisher, and R. G. Brennan, manuscript submitted for publication.
tion of all of the nitrogen sources was 0.2%. Casamino acids, an acid hydrolysate of casein, was obtained from Difco Laboratories. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added to agar plates to give a final concentration of 40 μg/ml.

**DNA and Protein Methods**—DNA sequencing of the glnA186 mutation and construction of the S186F GS overexpression plasmid were performed as described previously (24). Purification of TnRA and GS was performed according to published procedures (13, 28). The concentrations of TnRA and GS were determined by measuring their absorbance at 280 nm. The molar absorption coefficients of the proteins were calculated from their amino acid sequences (29). Gel mobility shift experiments to examine the ability of wild-type and S186F GS to inhibit the DNA binding by TnRA were performed as described previously (13).

Dried gels were exposed to a PhosphorImager storage screen (Amer sham Biosciences), and the band intensities were quantitated with the ImageQuant software by using the volume measurement function. The fraction of bound DNA was calculated as the volume of the band for the TnRA-bound DNA divided by the sum of the volumes of the bound and free bands.

**Enzyme Assays**—The enzymatic activities of glutamine synthetase were measured by the production of γ-glutamyl hydroxamate as described previously (24). The K<sub>v</sub> values for glutamate, glutamine, and hydroxylamine were determined by fitting data to the Michaelis-Menten equation with the Prism 3.0 software (GraphPad Software, Inc.). The ATP saturation curves for the Mg<sup>2+</sup>-dependent biosynthetic reaction had a slightly sigmoidal shape and were fit to the Hill equation, \( v = \frac{V_{max}S}{S + K_{app}} \), where S is the substrate concentration, K<sub>app</sub> is the substrate concentration at half-maximal velocity, and h is the Hill coefficient (30). Comparison of the fits of the ATP data to the Michaelis-Menten and Hill equations with an F test indicated that the Hill equation provided a better fit. Sigmoidal ATP velocity curves do not necessarily indicate cooperative binding and can occur as a consequence of the interaction of Mg<sup>2+</sup> ions with both the enzyme and ATP (31). The data for glutamate saturation curves in the presence of glutamine were also fit to the Hill equation.

The inhibition constant for L-methionine-S-sulfoximine was determined from glutamate saturation curves using inhibitor concentrations of 15, 50, and 150 μM. The data from these experiments were globally fit to the Hill equation, \( v = \frac{V_{max}S}{S + K_{app}} \), where I is the inhibitor concentration and K<sub>i</sub> is the inhibition constant. The IC<sub>50</sub> values for glutamine and AMP were determined with the Mg<sup>2+</sup>-dependent biosynthetic reaction with glutamate and ATP concentrations of 150 and 18 mM, respectively. Nonlinear regression analysis was used to fit the data to the equation, \( B = \frac{(nL)/(L + K_p)} \), where B is the amount of ligand bound per subunit, n is the number of binding sites per subunit, L is the free ligand concentration, and K<sub>p</sub> is the equilibrium dissociation binding constant. To reliably measure ligand binding, this method requires that the amount of bound ligand must be a significant fraction of the total ligand and thus is limited to the detection of binding constants that are similar to the concentration of the protein binding sites used in the experiment. Glutamine binding was determined with a GS subunit concentration of 0.5 mM. The binding of AMP to the wild-type and S186F proteins was determined with GS subunit concentrations of 15 and 100 μM, respectively. The measurement of glutamine binding to wild-type GS in the presence of TnRA was not attempted because it was not practical to isolate the large amounts of TnRA that would be required for this experiment.

**RESULTS**

**Isolation of a Novel glnA Mutation**—Transcriptional activation and expression of the amtB (formerly nrgA) promoter is dependent upon TnRA (10, 25). *B. subtilis* mutants that constitutively express TnRA-regulated genes can be identified by utilizing a strain that contains an amtB-lacZ transcriptional fusion to examine gene expression. Mutants with this constitutive phenotype were identified previously as blue colonies on glucose minimal medium agar plates containing glutamate and ammonium chloride as the nitrogen sources and incubated at 37 °C for 24 h.

![FIGURE 1. Cross-feeding of a ΔglnA mutant by the glnA186 strain. Strains were streaked onto glucose minimal media plates containing glutamate and ammonium chloride as the nitrogen sources and incubated at 37 °C for 24 h.](http://www.jbc.org)
mine, indicating that this strain is sensitive to glutamine. Impaired growth of the glnA186 mutant is observed on glucose minimal media containing either glutamate plus ammonium or proline plus ammonium as the nitrogen source. When cells are shifted from minimal media with proline as the nitrogen source to minimal media with proline plus ammonium as the nitrogen source as the nitrogen source but not when casamino acids plus glutamate plus ammonium as the nitrogen source, the use of the latter medium in the mutant-screening protocol facilitated isolation of the glnA186 mutant.

The effect of the glnA186 mutation on TnrA-dependent gene regulation was determined by measuring γ-galactosidase levels in cells containing an amtB-lacZ fusion. Glutamate plus casamino acids was used as the excess nitrogen source in these experiments because growth of glnA186 mutant cells is inhibited in liquid media containing the excess nitrogen sources such as glutamine or glutamate plus ammonium. In the wild-type strain, amtB expression is 110-fold higher in cells grown with limiting nitrogen than in cells grown with excess nitrogen (TABLE ONE). In cells grown with excess nitrogen, the glnA186 mutant had levels of β-galactosidase that were 80-fold higher than wild-type cells, and amtB expression only increased 1.6-fold during nitrogen limitation. These results indicate that cells containing the glnA186 mutation are defective in their ability to regulate TnrA activity in cells grown with excess nitrogen.

Because the DNA binding activity of GlnR is controlled by GS, the effect of the glnA186 mutation on the GlnR-dependent repression of a glnRA-lacZ fusion was also examined. Because glnRA expression is weakly repressed by TnrA (10), these experiments were performed with a tnrA− strain so that the TnrA-dependent regulation of this promoter was eliminated. Although glnRA expression was regulated 4-fold in the wild-type strain under these growth conditions, constitutive expression was observed in the glnA186 mutant (TABLE ONE). Although it is not known how GS controls GlnR activity, this result demonstrates that S186F GS is unable to activate GlnR-dependent repression.

**Enzymatic Properties of S186F GS**—To characterize its kinetic and feedback inhibition attributes, the S186F enzyme was overexpressed and purified to homogeneity. The data in TABLE TWO summarize the effects of the S186F mutation on the enzymatic properties of the mutant enzyme. The kinetic constants for the Mg2+−dependent biosynthetic activity for the wild-type and S186F enzymes were essentially identical. One difference between the two enzymes is that the Mn2+−dependent biosynthetic activity of the S186F protein was 1.8-fold higher than the wild-type enzyme. When the reverse enzymatic activity of the enzymes was examined using the transferase assay (16), no dramatic differences were observed between the wild-type and S186F enzymes.

The most significant alteration of the S186F enzyme was its insensitivity to feedback inhibition. Although wild-type GS has a glutamine

| Relevant genotype | TnrA-dependent regulation | GlnR-dependent regulation |
|-------------------|---------------------------|---------------------------|
|                   | β-Galactosidase activity (nmol/min/mg) from an amtB-lacZ fusion strain | β-Galactosidase activity (nmol/min/mg) from a glnRA-lacZ fusion strain |
|                   | Excess nitrogen | Limiting nitrogen | Excess nitrogen | Limiting nitrogen |
| Wild type         | 1.5          | 170         | 13          | 55          |
| glnA186           | 120          | 190         | 71          | 57          |

| Assay               | Wild type | S186F |
|---------------------|-----------|-------|
| Mg2+−dependent biosynthetic |           |       |
| Km glutamate (mM)   | 27 ± 2.2  | 29 ± 2.6  |
| Km ATP (mM)         | 2.4 ± 0.1 | 2.5 ± 0.1 |
| vmax (μmol/min/mg)  | 3.7 ± 0.2 | 4.3 ± 0.2 |
| Mn2+−dependent biosynthetic |           |       |
| Specific activity (μmol/min/mg) | 1.6 ± 0.1 | 2.7 ± 0.1 |
| Transferase         |           |       |
| Km glutamine (mM)   | 13 ± 0.6  | 14 ± 0.4  |
| Km hydroxylamine (mM)| 2.9 ± 0.2 | 3.4 ± 0.4  |
| vmax (μmol/min/mg)  | 120 ± 3   | 140 ± 2   |

**FIGURE 2. Inhibition of the Mg2+−dependent biosynthetic activity of wild-type and S186F glutamine synthetases by glutamine and AMP.** The wild-type (○) and S186F (●) enzymes were assayed in the presence of glutamine (A) or AMP (B). The effect of glutamine on the activity of wild-type GS in the presence of TnrA (□) is shown in A. Each data point is presented as a percentage of the activity present in the absence of inhibitors and is the average of at least two independent determinations that did vary by more than 10% (S.E.).

IC50 of 2.4 mM, the S186F enzyme is refractory to inhibition by glutamine (Fig. 2A). The AMP IC50 of wild-type GS (0.5 mM) is 12-fold lower than that of the S186F GS (6.0 mM) (Fig. 2B). The inhibition of wild-type GS by glutamine was also examined in the presence of TnrA. In these
experiments, the concentration of TnrA dimers was in 2-fold molar excess to GS subunits. Under these experimental conditions, GS activity is not inhibited by TnrA unless glutamine is present. Interestingly, the glutamine IC₅₀ was 6-fold lower in the presence of TnrA (0.4 mM) than when TnrA was absent (Fig. 2A). A previous mutational study, which suggested that TnrA binds to GS at the glutamate entrance of the active site, provide a possible explanation for this observation (24). Because glutamine is known to bind at the glutamate substrate site (23), the interaction between TnrA and feedback-inhibited GS may stabilize the binding of glutamine at the active site and thus inhibit GS enzymatic activity. It is also possible that TnrA bound to GS may block the glutamate entrance to the active site and thus reduce catalytic activity by preventing the release of glutamine and the entry of glutamate.

To confirm that glutamine mediates inhibition of wild-type GS by binding at the glutamate substrate site, the enzymatic activity of GS in response to varying concentrations of glutamate was examined in the presence and absence of glutamine. Although a hyperbolic saturation curve typical of Michaelis-Menten kinetics was seen in the absence of glutamine, a sigmoidal curve was obtained when glutamine was present (Fig. 3A). Sigmoidal kinetics in enzyme velocity curves can have a number of different explanations (32), and the cause of the sigmoidal curve with GS was not investigated. Nonetheless, these data reveal that high glutamate concentrations abolish the inhibition of GS activity by glutamine and that the apparent Kₘ values for glutamine increase in the presence of glutamate. These observations argue that glutamine is a competitive inhibitor with respect to glutamate. To determine the Kᵢ for glutamine, the apparent Kᵢ values obtained at various glutamine concentrations were plotted as a function of glutamine concentration, and the data points were used to construct a straight line by linear regression analysis (Fig. 3B). From the intercept with the x-axis, a glutamine Kᵢ of 0.6 mM was obtained. The resistance of S186F GS to inhibition by glutamine precluded the determination of a glutamine Kᵢ for this enzyme.

The AMP inhibition constants for wild-type and S186F GS were determined by the same approach that was used with glutamine. These experiments indicated that AMP was a competitive inhibitor with respect to ATP (data not shown). This result was not unexpected in that crystallographic studies have shown that AMP binds to the ATP substrate site of GS (22). In addition, previous kinetic analysis of S. typhimurium GS demonstrated that AMP is a competitive inhibitor of this enzyme (22). The S186F enzyme had an AMP Kᵢ (1.1 mM) that was 14-fold higher than the value obtained for wild-type GS (0.08 mM).

1-Methionine-S-sulfoximine is an analog of glutamate that inhibits the enzymatic activity of GS. 1-Methionine-S-sulfoximine binds to the glutamate substrate site and is phosphorylated in the presence of ATP (23, 33, 34). The phosphorylated 1-methionine-S-sulfoximine and ADP remain tightly bound to GS resulting in essentially irreversible inhibition of the enzyme (33, 34). In contrast to the results obtained with glutamine, the S186F enzyme has a 1-methionine-S-sulfoximine Kᵢ (13 μM) that is only 1.8-fold higher than wild-type GS (7.3 μM). This difference between the inhibitory effects of glutamine and 1-methionine-S-sulfoximine on the S186F enzyme most likely reflects the difference in their mechanism of inhibition.

The effect of the S186F mutation on the feedback inhibition of the Mn²⁺-dependent biosynthetic and transferase reactions was also examined (data not shown). Inhibition of the Mn²⁺-dependent biosynthetic activity by alanine and serine was relieved 33 and 13%, respectively, by the S186F mutation. Although the transferase reaction of the S186F enzyme is resistant to inhibition by AMP, no alteration in the sensitivity to inhibition by amino acids was observed.

Binding of Glutamine and AMP to GS—Equilibrium binding experiments were carried out to quantify the interactions of glutamine and AMP with wild-type and S186F GS. Interestingly, in vitro binding of glutamine to GS was only observed when the binding buffer contained phosphate. Structural analysis of glutamine-bound B. subtilis GS revealed that the amide nitrogen of glutamine is hydrogen-bonded to a phosphate anion located within the active site (Fig. 4) (23). A similar interaction between glutamine and phosphate was inferred from the crystal structures of S. typhimurium GS (23). These observations suggest that the binding of glutamine to GS is stabilized by its interaction with the phosphate anion.

The data for the binding of glutamine to wild-type GS fit well to a simple hyperbolic curve corresponding to a Kᵢ of 0.59 ± 0.07 mM (Fig. 5A). This analysis also indicated that 0.93 ± 0.04 mol of glutamine bound per mol of GS subunit at saturation. Binding of glutamine to the S186F protein was too weak to be detected, indicating that the Kᵢ is greater than 10 mM.
Additional binding experiments revealed that S186F GS had a 6-fold lower affinity for AMP than wild-type GS. AMP bound to wild-type enzyme with a $K_d$ of $19 \pm 2 \mu M$ (Fig. 5B), whereas the S186F GS had a binding constant of $120 \pm 10 \mu M$ (Fig. 5C). Binding of AMP at saturation was extrapolated to $0.65 \pm 0.03$ and $0.75 \pm 0.03$ mol of AMP/mol of GS subunit for the wild-type and S186F enzymes, respectively. The values obtained here for the binding of AMP to wild-type GS are in good agreement with measurements done by isothermal titration calorimetry in which a $K_d$ of $7.5 \mu M$ and a stoichiometry of $0.66$ mol of AMP/mol of GS subunit were obtained. Previous studies on the binding of AMP to E. coli GS found a binding constant of $125 \mu M$ and a stoichiometry of $1$ mol of AMP/mol of subunit (35). The reason for this difference in binding stoichiometries between the B. subtilis and E. coli enzymes is not known.

Inhibition of TnrA DNA Binding—The ability of S186F and wild-type GS to inhibit the binding of TnrA to amtB promoter DNA was examined in vitro with a DNA gel mobility shift assay. In the first set of experiments, fixed amounts of GS and TnrA were incubated with varying amounts of the feedback inhibitors (Fig. 6). Several different conclusions can be drawn from these results. As was reported previously (13), glutamine is more effective than AMP in promoting the ability of wild-type GS to inhibit TnrA DNA binding. In addition, S186F GS is much less effective than wild-type GS in suppressing DNA binding by TnrA in the presence of either glutamine or AMP. The difference in the abilities of the wild-type and mutant enzymes to inhibit TnrA DNA binding most likely reflects their affinities for these compounds. Lastly, with wild-type GS, a glutamine concentration of only $0.014 \text{ mM}$ is required to inhibit TnrA DNA-binding by $50\%$. This glutamine concentration is 42-fold lower than the $K_d$ for the binding of glutamine to wild-type GS ($0.59 \text{ mM}$) and most likely results from glutamine binding to GS with a higher affinity when TnrA is present.

In the second set of experiments, a fixed amount of TnrA was incubated with varying amounts of GS in either the presence or absence of $20 \text{ mM}$ glutamine. This concentration closely matches the intracellular
glutamine levels of \textit{B. subtilis} cells grown in the presence of excess nitrogen (18). When glutamine was not present, both the wild-type and mutant enzymes had similar abilities in blocking TnrA DNA binding (Fig. 7A). This indicates that in the absence of feedback inhibitors, the wild-type and S186F enzymes are equally capable of assuming the conformation required for interaction with TnrA. In contrast, even though glutamine stimulated the ability of both proteins to inhibit TnrA binding, 50\% inhibition of TnrA DNA binding by the wild-type enzyme occurred at 20-fold lower protein concentrations than with the S186F GS (Fig. 7B). These results are consistent with the model where the inability of the S186F enzyme to regulate TnrA activity in \textit{vivo} is due to defective binding of the feedback inhibitors glutamine and AMP rather than the inability to assume the protein conformation necessary for the interaction with TnrA.

**DISCUSSION**

Feedback inhibition of \textit{B. subtilis} GS is analogous to the adenyllylation of GS in enteric bacteria in that both processes posttranslationally regulate the activity of these enzymes. Indeed, similar alterations in nitrogen metabolism are observed in the \textit{B. subtilis} \textit{glnA}186 mutant and in strains of \textit{S. typhimurium} that contain mutations in \textit{glnE}, the gene encoding GS adenylyltransferase (36). Both the \textit{B. subtilis} \textit{glnA}186 and the \textit{S. typhimurium} \textit{glnE} mutant strains excrete glutamine. In addition, compared with wild-type cells, the growth rate of both mutants is significantly reduced following a shift from nitrogen-limited to ammonium excess medium.

Although the glutamine biosynthetic activity of \textit{B. subtilis} GS can be assayed \textit{in vitro} with either Mg\textsuperscript{2+} or Mn\textsuperscript{2+} (17), several observations argue that the Mg\textsuperscript{2+}-dependent reaction is the physiologically relevant enzymatic activity. First, in vegetatively growing \textit{B. subtilis}, the intracellular levels of Mg\textsuperscript{2+} exceed those of Mn\textsuperscript{2+} by a ratio of 170:1 (37). Second, several different \textit{B. subtilis} \textit{glnA} mutants with glutamine growth requirements have been shown to produce enzymes that lack Mg\textsuperscript{2+}-dependent activity but retain normal or enhanced levels of the Mn\textsuperscript{2+}-dependent activity (3, 24, 38, 39). The glutamine growth requirement of these bradytrophs argues that the Mn\textsuperscript{2+}-dependent enzymatic reaction does not contribute significantly to the \textit{in vivo} synthesis of glutamine. Finally, the most significant enzymatic alteration of S186F GS is the resistance of the Mg\textsuperscript{2+}-dependent reaction to feedback inhibition by glutamine and AMP. Because \textit{glnA}186 mutant cells excrete sufficient glutamine to cross-feed \textit{Gln}^{-} cells, unregulated glutamine synthesis catalyzed by the Mg\textsuperscript{2+}-dependent reaction must occur in \textit{glnA}186 mutant cells. Taken together, these observations argue that the Mg\textsuperscript{2+}-dependent reaction is the major route for glutamine synthesis in \textit{B. subtilis} and that GS activity is primarily regulated \textit{in vivo} by the feedback inhibitors glutamine and AMP.

Because glutamine is a heat-labile amino acid, little or no glutamine is present in plates containing ammonium, glutamate, and casamino acids as the nitrogen source. As a result, the screening conditions used to isolate the \textit{glnA}186 mutant selected for glutamine prototrophs and thus required that the mutant GS retain enzymatic activity. As a result of this constraint, any mutant enzyme identified using this screen would need to retain binding affinities for substrates that are similar to the wild-type enzyme. This expectation is supported by the observation that the substrate \textit{K}_{m} values for the wild-type and S186F enzymes are essentially identical (TABLE TWO). Nonetheless, S186F GS has reduced binding affinities for the feedback inhibitors glutamine and AMP. Because glutamine utilizes a different set of residues to interact with GS than does AMP, the \textit{glnA}186 mutation cannot alter a residue directly involved in the binding of both inhibitors.

The Ser-186 residue of \textit{B. subtilis} GS is located on a \textit{B}-strand that lines the active site (Fig. 5). The side chain of Ser-186 is oriented so that it points away from the cylindrical cavity of the active site. Although Ser-186 is located in close proximity to the binding sites for glutamine and AMP, this residue does not contact any substrates or inhibitors that bind within the active site. These observations suggest that the S186F substitution confers resistance to glutamine and AMP by an indirect mechanism. Interestingly, the most significant conformational difference between the \textit{S. typhimurium} and \textit{M. tuberculosis} GS models is the arrangement of the \textit{B}-strand corresponding to the region where the Ser-186 residue of \textit{B. subtilis} GS is located (21). This observation suggests that considerable conformational flexibility is present within this region of the GS proteins and raises the possibility that the feedback-resistant properties of \textit{B. subtilis} S186F GS results from an altered conformation in this region of the active site. Replacement of Ser-186 with a larger phenylalanine residue could perturb the conformation of the \textit{B}-strand and possibly alter the side chain positions of residues Glu-184 and Glu-189 (Fig. 5). Although Glu-184 and Glu-189 do not contact substrates or inhibitors bound at the active site, the altered position of these two residues in the S186F enzyme might destabilize the binding of AMP and glutamine without affecting substrate binding.

The \textit{B. subtilis} S186F enzyme is comparable with \textit{E. coli} GS in that both of these enzymes are resistant to inhibition by glutamine (4). Interestingly, \textit{E. coli} GS contains a histidine residue at the position that corresponds to Ser-186 of \textit{B. subtilis} GS. Thus the presence of a bulky amino acid side chain at this location may account, at least in part, for the resistance of \textit{E. coli} GS to inhibition by glutamine. Regardless, the observation that the \textit{glnA}186 mutation is located within the active site is consistent with glutamine inhibiting \textit{B. subtilis} GS enzymatic activity by binding at the active site and not at an allosteric regulatory site situated at some other location on the protein. Additional analysis would be required to identify the exact structural alternations responsible for the feedback-resistant phenotype of the \textit{B. subtilis} S186F GS.

Altered regulation of TnrA- and GlnR-dependent genes has been observed previously in \textit{B. subtilis} \textit{glnA} mutant strains identified by screening for glutamine auxotrophy or for mutants that expressed TnrA-regulated promoters constitutively (2, 24, 35, 38–44). The \textit{glnA}186 mutation is one of three \textit{glnA} mutations (S186F, G302D, E424K) that result in the synthesis of mutant GS enzymes unable to regulate TnrA activity \textit{in vivo} but retain levels of enzymatic activity that are essentially identical to the wild-type enzyme (24). No significant defects in feedback inhibition were observed with the G302D or E424K mutant enzymes. Because these two mutant enzymes were unable to inhibit TnrA DNA binding \textit{in vitro} in the presence or absence of feedback inhibitors, these proteins most likely contain structural alternations that prevent the interaction with TnrA. The S186F enzyme is a unique GS mutant that is defective in feedback inhibition. Binding of feedback inhibitors is required for GS to assume the conformation required for high affinity interaction with TnrA. The \textit{in vitro} characterization of S186F GS indicates that its mutant phenotype most likely results from the defective binding of the feedback inhibitors glutamine and AMP rather than the inability to assume the protein conformation necessary for the interaction with TnrA. However, because the Ser-186 residue is located in the vicinity of the proposed TnrA binding site on GS, we cannot rule out the possibility that the glutamine-bound form of the S186F enzyme assumes a conformation that binds TnrA with reduced affinity. We have previously provided biochemical and genetic data supporting a model in which the regulation of TnrA activity involves a direct interaction between feedback-inhibited GS and TnrA (13, 24). The demonstration that a mutant of \textit{B. subtilis} encoding GS resistant to feedback inhibition is also defective in regulating TnrA provides additional support for this model.
Feedback-resistant Glutamine Synthetase

Acknowledgments—We thank H. Kagan and R. Brennan for helpful discussions. Molecular graphics images were produced using the UCSF Chimera package from the Computer Graphics Laboratory, University of California, San Francisco (supported by National Institutes of Health Grant P41 RR-01081).

REFERENCES
1. Ikeda, T. P., Shauger, A. E., and Kustu, S. (1996) J. Mol. Biol. 259, 589–607
2. Hu, P., Leighton, T., Ishkhanova, G., and Kustu, S. (1999) J. Bacteriol. 181, 5042–5050
3. Reitzer, L. (2003) Annu. Rev. Microbiol. 57, 155–176
4. Woolfolk, C. A., and Stadtman, E. R. (1967) Arch. Biochem. Biophys. 118, 736–755
5. Fisher, S. H. (1999) Mol. Microbiol. 32, 223–232
6. Strauch, M. A., Aronson, A. I., Brown, S. W., Schreier, H. J., and Sonenshein, A. L. (1988) Gene 71, 257–265
7. Schreier, H. J., Brown, S. W., Hirschi, K. D., Nomellini, J. F., and Sonenshein, A. L. (1999) J. Mol. Biol. 280, 571–593
8. Dean, D. R., Hoch, J. A., and Aronson, A. I. (1977) J. Bacteriol. 131, 981–987
9. Nakano, Y., and Kimura, K. (1990) J. Mol. Biol. 210, 736–755
10. Wray, L. V., Jr., Ferson, A. E., and Fisher, S. F. (2000) Mol. Microbiol. 38, 175–184
11. Belitsky, B. R., Wray, L. V., Jr., Fisher, S. H., Bohannon, D. E., and Sonenshein, A. L. (2000) J. Bacteriol. 182, 5939–5947
12. Yamanaka, M. K., Ohki, Y., Naka, Y., and Fujita, Y. (2003) Mol. Microbiol. 49, 157–165
13. Wray, L. V., Jr., Zalieckas, J. M., and Fisher, S. F. (2001) Cell 107, 427–435
14. Deuel, T. F., and Stadtman, E. R. (1970) J. Biol. Chem. 245, 5206–5213
15. Deuel, T. F., and Prusiner, S. (1974) J. Biol. Chem. 249, 257–264
16. Deuel, T. F., and Turner, D. C. (1972) J. Biol. Chem. 247, 3039–3047
17. Deuel, T. F., Ginsburg, A., Yeh, J., Shelton, E., and Stadtman, E. R. (1970) J. Biol. Chem. 245, 5195–5205
18. Fisher, S. H., and Sonenshein, A. L. (1984) J. Bacteriol. 157, 612–621
19. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) J. Comput. Chem. 25, 1605–1612
20. Gill, H. S., and Eisenberg, D. (2001) Biochemistry 40, 1903–1912
21. Gill, H. S., Pihlgren, G. M. U., and Eisenberg, D. (2002) Biochemistry 41, 9863–9872
22. Liaw, S.-H., Yun, G., and Eisenberg, D. (1994) Biochemistry 33, 11184–11188
23. Liaw, S.-H., and Eisenberg, D. (1994) Biochemistry 33, 675–681
24. Fisher, S. H., Brandenburg, J. L., and Wray, L. V., Jr. (2002) Mol. Microbiol. 45, 627–635
25. Atkinson, M. R., Wray, L. V., Jr., and Fisher, S. F. (1999) J. Bacteriol. 171, 4758–4765
26. Neidhardt, F. C., Bloch, P. L., and Smith, D. F. (1974) J. Bacteriol. 119, 736–747
27. Chasin, L. A., and Magasanik, B. (1968) J. Biol. Chem. 243, 5165–5178
28. Wray, L. V., Jr., Zalieckas, J. M., and Fisher, S. F. (2000) J. Mol. Biol. 300, 29–40
29. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) Protein Sci. 4, 2411–2423
30. Cornish-Bowden, A. (1995) Fundamentals of Enzyme Kinetics, pp. 207–209, Portland Press, London
31. London, W. P., and Steck, T. L. (1969) Biochemistry 8, 1767–1779
32. Copeland, R. A. (2000) Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis, pp. 382–383, Wiley-VCH, New York
33. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) Protein Sci. 4, 2411–2423
34. Chasin, L. A., and Magasanik, B. (1968) J. Biol. Chem. 243, 5165–5178
35. Wray, L. V., Jr., Zalieckas, J. M., and Fisher, S. F. (2000) J. Mol. Biol. 300, 29–40
36. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) Protein Sci. 4, 2411–2423
37. Cornish-Bowden, A. (1995) Fundamentals of Enzyme Kinetics, pp. 207–209, Portland Press, London
38. London, W. P., and Steck, T. L. (1969) Biochemistry 8, 1767–1779
39. Copeland, R. A. (2000) Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis, pp. 382–383, Wiley-VCH, New York
40. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) Protein Sci. 4, 2411–2423
41. Cornish-Bowden, A. (1995) Fundamentals of Enzyme Kinetics, pp. 207–209, Portland Press, London
32. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) Protein Sci. 4, 2411–2423
43. Cornish-Bowden, A. (1995) Fundamentals of Enzyme Kinetics, pp. 207–209, Portland Press, London
34. Chasin, L. A., and Magasanik, B. (1968) J. Biol. Chem. 243, 5165–5178
44. Dean, D. R., Hoch, J. A., and Aronson, A. I. (1977) J. Bacteriol. 131, 981–987
45. Nakano, Y., and Kimura, K. (1990) J. Mol. Biol. 210, 736–755
46. Schreier, H. J., and Sonenshein, A. L. (1985) J. Mol. Biol. 245, 921–933
47. Deuel, T. F., and Stadtman, E. R. (1970) J. Biol. Chem. 245, 5039–5047
A Feedback-resistant Mutant of Bacillus subtilis Glutamine Synthetase with Pleiotropic Defects in Nitrogen-regulated Gene Expression
Lewis V. Wray, Jr. and Susan H. Fisher

J. Biol. Chem. 2005, 280:33298-33304.
doi: 10.1074/jbc.M504957200 originally published online July 29, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M504957200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 21 of which can be accessed free at http://www.jbc.org/content/280/39/33298.full.html#ref-list-1