Regulation of the Activity of the *Bacillus licheniformis* A5 Glutamine Synthetase

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The regulation of glutamine synthetase activity by positive and negative effectors of enzyme activity singularly and in combinations was studied by using a homogeneous enzyme preparation from *Bacillus licheniformis* A5. Phosphor-ylribosyl pyrophosphate at concentrations greater than 2 mM stimulated glutamine synthetase activity by approximately 70%. The concentration of phosphor-ylribosyl pyrophosphate required for half-maximal stimulation of enzyme activity was 0.4 mM. Results obtained from studies of fractional inhibition of glutamine synthetase activity were consistent with the presence of one allosteric site for glutamine binding (apparent $I_{0.5}$, 2.2 mM) per active enzyme unit at a glutamate concentration of 50 mM. At a glutamate concentration of 30 mM or less, the data were consistent with the enzyme containing two binding sites for glutamine (one of which was an allosteric site with an apparent $I_{0.5}$ of 0.4 mM). Based on an analysis of the response of glutamine synthetase activity to positive and negative effectors in vitro and to the intracellular concentration of these effectors in vivo, the primary modulators of glutamine synthetase activity in *B. licheniformis* A5 appear to be glutamine and alanine (apparent $I_{0.5}$, 5.1 mM).

The central position and the importance of the amino acid glutamine in cellular nitrogen metabolism are well documented (20, 29, 30). Since glutamine is used for such a variety of metabolic functions, it might be expected that the enzyme glutamine synthetase (GS) would be regulated by mechanisms which respond specifically to cellular need for glutamine. The relative levels of enzymes such as GS, glutamate synthase, glutamate dehydrogenase and glutaminase appear to be controlled differently (20, 29, 30), even within *Bacillus* spp. (5, 18, 19, 21, 22, 26). Thus, the nature of the regulatory mechanisms exerting their influence on GS might be expected to be different in some cells.

Studies by Hubbard and Stadtman (14) first suggested that differences may occur in the regulation of GS in various microorganisms. In particular, results obtained with a partially purified GS preparation from *B. licheniformis* ATCC 9945a (15, 16) indicated that the GS of *Bacillus* spp. was regulated differently from the *Escherichia coli* enzyme. Experiments with homogeneous preparations of GS from *B. licheniformis* A5 (9), *B. subtilis* (6), and *B. stearothermophilus* (33) and the two forms of GS isolated from *B. caldolyticus* (34) have failed to provide any evidence for regulation of GS activity by reversible adenylation. However, the GS isolated from these bacteria is more susceptible to direct regulation by feedback inhibition than is the *E. coli* GS.

An ample intracellular supply of glutamine is necessary for both growth and sporulation in *Bacillus* spp. The isolation and characterization of glutamine auxotrophs of *B. subtilis* (1, 4, 11) and *B. megaterium* (10, 24) have shown that both growth and sporulation of the mutants are glutamine dependent, with some auxotrophs requiring as much as 0.2 mg of glutamine per ml of culture to be able to utilize 1 mg of glucose per ml (11). Experiments performed in this laboratory have shown that intracellular glutamine pools in *B. licheniformis* A5 remain relatively constant during growth of this bacterium under a variety of cultural conditions, including those cultural conditions which lead to the release of nitrogen catabolite repression and the formation of significant numbers of spores during exponential growth (26).

The physical and kinetic properties of the GS purified from *B. licheniformis* A5 grown under different states of nitrogen limitation have been reported (9). From these studies, we have concluded that *B. licheniformis* A5 contains one GS whose activity is not regulated by a covalent
modification system. This paper further investigates the response of the purified *B. licheniformis* A5 GS to compounds previously shown to be regulators of enzyme activity (9). The intracellular concentrations of many metabolites linked with GS as either substrates, products, or regulators of enzyme activity have been measured during growth and sporulation of *B. licheniformis* A5 under a variety of cultural conditions (2, 7, 8, 26). The intracellular pools of some of these compounds change drastically during the transition between growth and sporulation in this bacterium. By comparing the response of the *B. licheniformis* A5 GS to positive and negative effectors in vitro with the measured intracellular pools of these effectors, we identified the most likely physiologically significant regulators of GS activity in vivo during growth and sporulation.

**MATERIALS AND METHODS**

**GS assays.** Conditions used for the growth of *B. licheniformis* A5, the purification of GS, and the determination of GS activity have been described previously (9). Biosynthetic GS activity was measured on homogeneous GS preparations by a modification of the radiochemical method of Prusiner and Milner (23). Except as noted below, standard GS assays contained the following components (micromoles) in a final volume of 0.1 ml: morpholinepropanesulfonic acid (pH 6.6), 5.0; NH₄Cl, 0.5; ATP, 0.5; MnCl₂, 0.5; glutamate, 5.0; and [U-¹⁴C]glutamate, 0.001 (approximately 0.25 μCi). These conditions, including metal ion specificity and concentration, have been previously shown to be optimal for the measurement of *B. licheniformis* GS activity (9). All incubations were at 37°C for 10 min. Enzyme assays were terminated by the addition of 2 ml of ice-cold distilled water; each solution was immediately passed over a column (0.7 by 3.0 cm) of Dowex 1-chloride. The glutamine-containing eluate was collected in a scintillation vial, and the radioactivity was determined after the addition of 10 ml of "tritosol" scintillation fluid (13). All enzyme assays were initiated by the addition of 0.9 μg (approximately 0.003 U) of GS purified from exponentially growing cells with ammonia as the sole nitrogen source. One unit of GS activity is defined as the amount of enzyme required to catalyze the conversion of 1 μmol of glutamate to glutamine per min under the above assay conditions.

In studies in which nucleotides were used as effectors of GS activity, the nucleotides were added to assay mixtures in equimolar amounts with MnCl₂ to prevent inhibition of enzyme activity by disturbing the 1:1 ratio of Mn²⁺ to ATP previously shown to be required for maximal *B. licheniformis* A5 GS activity (9). Because of their instability, carbamyl phosphate was crystallized (28), and the concentration of freshly prepared stock solutions of phosphorylribosyl PP, (PRPP) was determined immediately before use (12). All effectors of enzyme activity used were added to enzyme assays from stock solutions which had been previously adjusted to pH 6.6.

**Materials.** L-[U-¹⁴C]glutamic acid (296 mCi/mmole) was purchased from New England Nuclear Corp. (Boston, Mass) and was purified by passage through a column (0.9 by 9 cm) of AG 1 × 8-chloride (Bio-Rad Laboratories, Richmond, Calif.) by the method of Prusiner and Milner (23). [¹⁴C]Glutamic acid was eluted from these columns with 10 mM HCl. Plastic tips for a 2.5-ml Sarrette (Walter Sarstedt Inc., Princeton, N.J.) were plugged with glass wool and were used as disposable chromatographic columns for GS assays. All biochemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals used were of reagent grade. Double-distilled water was used to prepare all solutions.

**RESULTS**

Regulation of GS activity by negative effectors. The metabolites which inhibit GS activity could be divided into two groups: those which resulted in greater than 90% inhibition of enzyme activity (AMP and PP) and those which caused approximately 50% inhibition of activity when present at a concentration of 5 mM or less (Table 1, column 1). Of particular importance for later discussion was the finding that 5 mM glutamine inhibited GS activity by approximately 50%.

To determine whether the various inhibitors of enzyme activity acted independently, the capacity of each substance to inhibit enzyme activity was measured in the presence of a second inhibitor. The activity observed when two inhibitors were present was approximately that expected, assuming that the two inhibitors were acting independently (Table 1). There were no strong antagonistic or synergistic effects seen in

<table>
<thead>
<tr>
<th>Compound</th>
<th>None</th>
<th>Alanine</th>
<th>Glutamine</th>
<th>AMP (1 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>53</td>
<td>50</td>
<td>39</td>
</tr>
<tr>
<td>AMP (1 mM)</td>
<td>99</td>
<td>26 (21)*</td>
<td>25 (20)</td>
<td>25 (20)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>50</td>
<td>25 (27)</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Alanine</td>
<td>53</td>
<td>25 (27)</td>
<td>26 (21)</td>
<td>26 (21)</td>
</tr>
<tr>
<td>Serine</td>
<td>43</td>
<td>25 (21)</td>
<td>22 (22)</td>
<td>17 (17)</td>
</tr>
<tr>
<td>Glycine</td>
<td>60</td>
<td>27 (22)</td>
<td>28 (30)</td>
<td>25 (23)</td>
</tr>
<tr>
<td>Aspartate</td>
<td>53</td>
<td>25 (15)</td>
<td>33 (27)</td>
<td>26 (21)</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>41</td>
<td>24 (18)</td>
<td>26 (21)</td>
<td>20 (16)</td>
</tr>
<tr>
<td>Carbamyl phosphate</td>
<td>56</td>
<td>18 (19)</td>
<td>28 (28)</td>
<td>25 (22)</td>
</tr>
<tr>
<td>ADP</td>
<td>45</td>
<td>33 (20)</td>
<td>30 (23)</td>
<td>22 (18)</td>
</tr>
</tbody>
</table>

*All compounds were present at 5 mM, except where noted. Results are the average of at least nine determinations and have a variation of ±5% in the worst cases.

Numbers in parentheses are the activities expected assuming that both inhibitors act independently. In this case the percent enzyme activity remaining in the presence of two effectors of enzyme activity is the product of the percent enzyme activity measured in the presence of each inhibitor when tested alone.

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**Table 1. Percent enzyme activity remaining in the presence of two inhibitors of GS activity**

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the presence of the inhibitor combinations tested.

Stimulation of GS activity by PRPP. The B. licheniformis A5 GS is apparently unique with respect to its response to PRPP (9). Figure 1 shows the effect of increasing concentrations of PRPP on GS activity. There was a hyperbolic response of enzyme activity to increasing concentrations of PRPP, with a maximum stimulation of enzyme activity of approximately 70%. The concentration of PRPP required for half-maximal stimulation of enzyme activity was approximately 0.4 mM; this is the intracellular concentration of PRPP measured during growth of Salmonella sp. in minimal medium (25). Results of experiments in which PRPP was mixed with compounds previously shown to inhibit enzyme activity showed that the enzyme activity measured in the presence of any of the inhibitors tested was the same with and without the addition of 2 mM PRPP (Table 2). Thus, unless in vivo conditions prevent the complete reversal of PRPP stimulation of GS activity by the inhibitors observed in vitro, the in vivo physiological significance of stimulation of GS activity by PRPP seems to be minimal.

Kinetics of inhibition. Based solely on a comparison between the strength of inhibition of enzyme activity produced by the compounds listed in Table 1 and the approximate intracellular concentration of these effectors (2, 7, 8, 26), it appears that the most potent effectors of GS activity in vivo are alanine, glutamine, aspartate, serine, and AMP. To test this hypothesis, we studied the nature of inhibition exhibited by these compounds to determine the Kᵢ value of each of the effectors for GS.

Figure 2 shows the results obtained when the affinity of the B. licheniformis A5 GS for glutamate was tested at various concentrations of alanine. The results indicated that alanine exhibited mixed inhibition kinetics with respect to glutamate (3, 27). The slope and y-intercept of these double-reciprocal plots were not linearly related to the alanine concentration (Fig. 2, insert). Because of this nonlinear, mixed inhibition of enzyme activity by alanine with respect to glutamate, the apparent Kᵢ values calculated from the individual double-reciprocal plots shown in Fig. 2 were a function of the alanine concentration. The Kᵢ value, which can be calculated, is a measure of the affinity of GS for alanine and does not relate to the concentration of alanine required for 50% inhibition of enzyme activity (27). Thus, a quantitative analysis of alanine inhibition of GS activity by a calculation of a Kᵢ value proved to be relatively meaningless to regulation of enzyme activity.

The double-reciprocal plots obtained when the affinity of the B. licheniformis A5 GS for Mn-ATP was tested at various concentrations of Mn-AMP are shown in Fig. 3. Although some of the lines appear to converge around a single y-intercept, linear regression analysis of the lines generated at each concentration of Mn-AMP (correlation coefficient ≥ 0.98) indicated that the lines do not cross the y-axis at a single point. Thus, inhibition of the B. licheniformis A5 GS by Mn-AMP is not competitive, but mixed with respect to Mn-ATP (3, 27). The inhibition of enzyme activity produced by Mn-AMP was linearly related to the slopes of the individual double-reciprocal plots (Fig. 3, insert). The Kᵢ value

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**Table 2. Effect of PRPP on inhibition of GS activity**

<table>
<thead>
<tr>
<th>Addition*</th>
<th>Observed</th>
<th>Expected*</th>
<th>Observed without added PRPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRPP</td>
<td>173</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>PRPP + AMP (1 mM)</td>
<td>41</td>
<td>66</td>
<td>39</td>
</tr>
<tr>
<td>PRPP + glutamine</td>
<td>40</td>
<td>85</td>
<td>50</td>
</tr>
<tr>
<td>PRPP + alanine</td>
<td>55</td>
<td>90</td>
<td>53</td>
</tr>
<tr>
<td>PRPP + serine</td>
<td>48</td>
<td>73</td>
<td>43</td>
</tr>
<tr>
<td>PRPP + glycine</td>
<td>53</td>
<td>102</td>
<td>60</td>
</tr>
<tr>
<td>PRPP + carbamyl phosphate</td>
<td>64</td>
<td>95</td>
<td>56</td>
</tr>
<tr>
<td>PRPP + α-ketoglutarate</td>
<td>48</td>
<td>70</td>
<td>41</td>
</tr>
<tr>
<td>PRPP + aspartate</td>
<td>58</td>
<td>90</td>
<td>53</td>
</tr>
<tr>
<td>PRPP + ADP</td>
<td>34</td>
<td>77</td>
<td>45</td>
</tr>
</tbody>
</table>

* PRPP concentration was 2 mM in all cases and, except where noted, all GS inhibitors were present at 5 mM. Results are the average of at least nine enzyme assays and have a variation of ±5% in the worst cases.
* Activity calculated assuming that both compounds act independently. In this case the % enzyme activity remaining in the presence of two effectors of enzyme activity is the product of the % enzyme activity measured in the presence of each effector when tested alone.
* Taken from Table 1.

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![Fig. 1. Effect of increasing PRPP concentrations on GS activity. All assays were initiated and performed as described in the text.](http://jb.asm.org/ on May 13, 2021 by guest)
for Mn-AMP calculated either from the slopes of the individual double-reciprocal plots or from the replot shown in the insert to Fig. 3 was 0.1 mM. Essentially the same $K_i$ value for Mn-AMP was obtained when the apparent affinity of the enzyme for glutamate was tested at the same concentrations of Mn-AMP (data not shown).

The double-reciprocal plots obtained when the apparent affinity of the B. licheniformis A5 GS for glutamate was tested at various concentrations of glutamine are shown in Fig. 4. The results indicated that glutamine inhibition of GS activity was mixed with respect to glutamate, and the response of the slopes of the individual double-reciprocal plots to increasing glutamine concentrations (Fig. 4, insert) is consistent with the presence of two binding sites for glutamine per active enzyme unit (3, 27). The average slope of a Hill plot (27) in the region between 10 and...
90% inhibition of enzyme activity ($n_{\text{apparent}}$ number) when glutamine inhibition of GS activity was tested at a glutamate concentration of 30 mM was 1.7 (data not shown). Though the data are consistent with the presence of two binding sites for glutamine per unit of GS activity at a glutamate concentration of 30 mM, they do not preclude the possibility of a complex kinetic response of enzyme activity to glutamine which is mediated via a single glutamine-binding site.

Since glutamine produced parabolic mixed inhibition of GS activity with respect to glutamate, the apparent $K_i$ values which could be calculated are a measure of the affinity of GS for glutamine and thus cannot be directly related to the concentration of glutamine required to inhibit enzyme activity by 50% (27). However, Fig. 4 shows that glutamine was a very potent inhibitor of enzyme activity under these conditions. The apparent $K_{i,slope}$ values calculated from the slopes of individual double-reciprocal plots (Fig. 4) were in the range of 0.1 to 0.4 mM. This is in contrast with the data presented previously (Table 1) which showed that GS activity was inhibited by approximately 50% by 5 mM glutamine. A possible explanation for this apparent paradox will be presented below.

**Fractional inhibition studies.** The previously described experiments indicated that the kinetic response of the *B. licheniformis* A5 GS to compounds which inhibited enzyme activity was very complex and often of such a nature that an analysis of the inhibition of GS activity by calculating apparent inhibitor constants was of little quantitative meaning to regulation of enzyme activity. To circumvent this problem and attempt to quantitatively study regulation of enzyme activity by feedback inhibitors, we analyzed regulation of GS activity by negative effectors in terms of the fractional inhibition of enzyme activity produced as a function of inhibitor concentration (31, 32).

For these experiments, the fractional inhibition of enzyme activity, $i$, is defined as $(V_0 - V)/V_0$, where $V_0$ and $V$ represent the reaction velocities at zero and at a finite level of the inhibitor being tested, respectively. Equations relating $i$ to inhibitor concentration, $[I]$, have been extensively discussed elsewhere (31, 32). Plotting $1/i$ versus $1/[I]$, in analogy to a Lineweaver-Burk plot, yields values for $i_{\text{max}}$ and apparent $I_0.5$ in a manner similar to the determination of $V_{\text{max}}$ and apparent $K_m$ values. An effector which inhibits enzyme activity by 100% at saturating concentrations of effector produces an $i_{\text{max}}$ of 1.0. Incomplete (<100%) inhibition at an infinite effector concentration gives an $i_{\text{max}}$ less than 1.0 (1/$i_{\text{max}}$ greater than 1.0) and indicates that interaction of the effector with the enzyme does not occur at the active site (31, 32). As an indicator of inhibitor potency, one may compare the $i_{\text{max}}/I_{0.5}$ ratio for various effectors, in analogy to the specificity constant ($K_{\text{cat}}/K_m$) for substrates (31, 32).

With the exception of glutamine, all of the inhibitors of GS activity tested were capable of

Fig. 4. Double-reciprocal plots for glutamate saturation of the *B. licheniformis* A5 GS at various concentrations of glutamine. Assay conditions were as described in the text. (Insert) Replot of the slopes of the double-reciprocal plots obtained at the various glutamine concentrations tested.
inhibiting enzyme activity by 100% at saturating concentrations of the inhibitor (Table 3). Thus, for all of the inhibitors tested (except glutamine), the $i_{\text{max}}/I_{0.5}$ ratio is simply a measure of the relative apparent $I_{0.5}$ value of each inhibitor for GS.

### Table 3. Analysis of $i/i$ versus $1/i$ selected inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Apparent $I_{0.5}$ (mM)</th>
<th>$i_{\text{max}}$</th>
<th>$i_{\text{max}}/I_{0.5}$</th>
<th>Approx intracellular concn (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>5.1</td>
<td>1.0</td>
<td>0.20</td>
<td>6-18 (2)$^a$</td>
</tr>
<tr>
<td>Serine</td>
<td>6.6</td>
<td>1.0</td>
<td>0.15</td>
<td>4 (2)</td>
</tr>
<tr>
<td>Glycine</td>
<td>13.3</td>
<td>1.0</td>
<td>0.08</td>
<td>2 (2)</td>
</tr>
<tr>
<td>AMP</td>
<td>0.4</td>
<td>1.0</td>
<td>2.50</td>
<td>0.1-0.2 (7, 8)</td>
</tr>
<tr>
<td>Aspartate</td>
<td>5.5</td>
<td>1.0</td>
<td>0.18</td>
<td>5 (2)</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>3.7</td>
<td>1.0</td>
<td>0.27</td>
<td>≤1 (7, 8, 26)</td>
</tr>
<tr>
<td>Carbamyl phosphate</td>
<td>7.1</td>
<td>1.0</td>
<td>0.14</td>
<td>≤0.5 (7, 8)</td>
</tr>
<tr>
<td>ADP</td>
<td>3.9</td>
<td>1.0</td>
<td>0.26</td>
<td>2-6 (26)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>50 mM glutamate</td>
<td>2.2</td>
<td>0.81</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>20 mM glutamate</td>
<td>0.4</td>
<td>0.56</td>
<td>1.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.4</td>
<td>1.0</td>
<td>0.42</td>
</tr>
</tbody>
</table>

$^a$ Abbreviations are as defined in the text. Apparent $I_{0.5}$ and $i_{\text{max}}$ values were obtained by linear regression analysis of the response of enzyme activity to at least seven concentrations of each inhibitor tested. The correlation coefficients for the double-reciprocal plots generated were $>0.95$ in all cases. Inhibitor concentrations were varied over a 10-fold range and were set at levels such that appropriately distributed points were obtained on the double-reciprocal plots (as in Fig. 5). Inhibitor concentrations were varied such that the inhibitors were added at concentrations ranging between 0.25 and 4 times their respective apparent $I_{0.5}$ values in extreme cases.

$^b$ Numbers in parentheses are references.

The results obtained when glutamine was tested as an inhibitor of GS activity particularly warrant discussion. When tested under standard assay conditions (50 mM glutamate and saturating concentrations of all other substrates), the plot of $1/i$ versus $1/[I]$ obtained was linear (Fig. 5) and indicated an $i_{\text{max}}$ of 0.81 and an apparent $I_{0.5}$ of 2.2 mM for glutamine. The interaction of glutamine with the enzyme under these conditions was at a site other than the active site because the $i_{\text{max}}$ obtained was less than 1.0 (31, 32). The results obtained for glutamine inhibition of GS activity under these conditions were consistent with those previously discussed; the addition of 5 mM glutamine to the standard GS reaction mixture caused approximately 50% inhibition of enzyme activity (Table 1).

When the same experiment was repeated at a glutamate concentration of 20 mM, which is approximately five times the $K_m$ of the enzyme for glutamate (9), and at saturating concentrations of all other substrates, a different response was seen (Table 3 and Fig. 5). Under these conditions the plot of $1/i$ versus $1/[I]$ was curved. Extrapolation of the two portions of the curve obtained at 20 mM glutamate (Fig. 5) and calculation of apparent $I_{0.5}$ and $i_{\text{max}}$ values (Table 3) suggest the presence of a low (apparent $I_{0.5} = 2.4$ mM) and a high (apparent $I_{0.5} = 0.4$ mM) affinity site for glutamine binding per active enzyme unit (31, 32). Since the $i_{\text{max}}$ for the high-affinity glutamine-binding site is less than 1.0,
the data indicate that this glutamine-binding site is not at the active site.

The insert to Fig. 5 shows the Hill plots constructed from the data generated when glutamine inhibition of enzyme activity was tested in the presence of 20 or 50 mM glutamate. The Hill plot generated from GS assays conducted at a glutamate concentration of 20 mM was curved and indicated an $n_{\text{apparent}}$ for glutamine of 2.2. In contrast, the Hill plot obtained when glutamine inhibition of enzyme activity was measured in the presence of 50 mM glutamate was not curved, and the calculated $n_{\text{apparent}}$ for glutamine of 1.2 was consistent with the exposure of only one glutamine-binding site per unit of enzyme activity under these conditions (27). Although the data demonstrate a glutamate-dependent heterogeneity in the kinetic response of enzyme activity to glutamine, definitive proof of a glutamate-dependent change in the number of available glutamine-binding sites on the enzyme requires further documentation.

**DISCUSSION**

The in vivo flux through a given metabolic reaction can be regulated by enzyme quantity; by the relative concentrations of substrates, products, activators, and inhibitors; and by the availability of cofactors, metal ions, and any other factors which influence enzyme activity. The data compiled in both this and in a previous paper (9) support the conclusion that many, if not all, of these mechanisms could be operative in vivo to regulate glutamine synthase at the level of GS during growth and sporulation of *B. licheniformis* A5.

Compounds such as ADP and glycine can be eliminated as major physiological regulators of enzyme activity because the apparent $I_{0.5}$ values determined for these compounds are significantly higher than their intracellular pools in *B. licheniformis* A5 (2, 7, 8) (Table 3). Although there are no available data on intracellular pools of carbamyl phosphate, most carbamyl phosphate-requiring enzymes have $K_m$ values for carbamyl phosphate in the micromolar range (17). Thus, we would expect that the intracellular level of this metabolite is much lower than the apparent $I_{0.5}$ value determined for carbamyl phosphate. $\alpha$-Ketoglutamate pools are relatively low during growth and sporulation (7, 8, 26), so it is doubtful that this metabolite plays a major role in the regulation of the GS activity in vivo. Finally, aspartate and serine are two amino acids which have relatively invariant concentrations during growth and sporulation of *B. licheniformis* A5 in minimal medium supplemented with glucose and ammonia (2). Both of these amino acids are present intracellularly at concentrations approximately equal to their respective apparent $I_{0.5}$ values.

AMP was shown to have the greatest affinity for the enzyme (apparent $I_{0.5} = 0.4$ mM). Intracellular AMP concentrations are approximately 0.1 to 0.2 mM under most conditions tested in *B. licheniformis* A5 (7, 8). The intracellular concentration of AMP transiently rises to 0.9 mM immediately after the end of exponential growth in minimal medium supplemented with glucose and ammonia (8); thus, AMP inhibition of GS activity at this time could be very significant.

Previous work from this laboratory has shown that *B. licheniformis* A5 accumulates a relatively large pool of alanine (18 mM) during sporulation in minimal medium containing glucose and ammonia (2). The size of the alanine pool at this time is approximately three times the apparent $I_{0.5}$ value of alanine for GS. Since the size of the alanine pool during growth in the same medium is approximately 6 mM, it is expected that alanine would be a more potent regulator of GS activity in vivo during sporulation than during growth in this medium.

The last inhibitor of GS activity shown in Table 3 is glutamine, a reaction product whose inhibitory action is apparently modulated by the concentration of the substrate glutamate. The intracellular glutamate pool changes drastically during growth and sporulation of *B. licheniformis* A5 in minimal medium supplemented with glucose and ammonia (2). At midexponential growth, the glutamate pool is approximately 100 mM and comprises 85% of the total amino acid pool. By 2 h after the end of exponential growth in the same medium, the glutamate pool has dropped to approximately 15 mM, and this amino acid now accounts for only 28% of the total amino acid pool (2).

It is interesting that the intracellular glutamate pool undergoes such large fluctuations in view of the response of the GS to glutamine in vitro (Table 3 and Fig. 5). Both the single glutamine-binding site found at 50 mM glutamate and the high-affinity glutamine-binding site present at 20 mM glutamate have $i_{\text{max}}$ values of less than 1, indicating that both of these sites are not at the active site (31, 32). It is tempting to speculate that the allosteric glutamine-binding site present at 50 mM glutamate (apparent $I_{0.5} = 2.2$ mM; $i_{\text{max}} = 0.81$) is identical to the high-affinity allosteric glutamine-binding site (apparent $I_{0.5} = 0.4$ mM; $i_{\text{max}} = 0.56$) present at 20 mM glutamate and that the affinity of this site for glutamine is modulated by the glutamate concentration. Because the experiments performed do not allow such a precise identification
of the allosteric glutamine-binding site(s), alternative kinetic explanations for the glutamate concentration dependence of the inhibition of GS activity by glutamine are also feasible.

Intracellular glutamine pools have been measured during growth and sporulation of *B. licheniformis* A5 and have been found to be in the range of 2 to 6 mM under all physiological conditions tested (26; H. J. Schreier and R. W. Bernlohr, unpublished data). The mechanism responsible for maintaining the glutamine pool (transamination, protein turnover, or GS activity) under all physiological conditions is unknown, but the fact that the high-affinity glutamine-binding site present at 20 mM glutamate has an $K_m$ of 0.56 may prevent the total restriction of glutamine synthesis at the level of GS by glutamine when the glutamate pool drops and the glutamine pool remains relatively high.

The availability of substrates other than glutamate may also play a role in the regulation of GS activity, especially during sporulation. The ATP pool is approximately threefold higher than the apparent $K_m$ of Mn-ATP for GS (0.9 mM) during growth of *B. licheniformis* A5 in minimal medium containing glucose and ammonia (7, 8). The intracellular ATP pool measured during growth has been found to be relatively insensitive to both carbon and nitrogen supplies in this bacterium, but it does decrease to a level (1 mM) approximately equal to the apparent $K_m$ value of Mn-ATP for GS (9) during sporulation under some cultural conditions (7, 8). Growth of *B. licheniformis* A5 under a variety of physiological conditions, including those which lead to the release of nitrogen catabolite repression and the formation of significant numbers of spores during exponential growth, produces cells which have ammonia pools ranging between two and eight times the apparent $K_m$ (0.4 mM) of ammonia for GS (26).

The cumulative effect on enzyme activity observed with the combinations of inhibitors tested and the strength of inhibition of GS activity by some of these compounds at concentrations near or below their intracellular concentrations lead to the conclusion that GS activity is strongly inhibited (≥75%) in vivo under most physiological conditions. Given the number of potential regulators of flux through GS and the variations in the intracellular pools of some of these compounds, it is not possible to identify one factor which is most important to the regulation of GS activity in *B. licheniformis* A5 under all physiological conditions. However, when the effects on GS activity produced by individual negative effectors in vitro are compared with the intracellular pools of these compounds, the primary regulators of the *B. licheniformis* A5 GS in vivo appear to be glutamine and alanine. In addition, regulation of GS activity by AMP may be particularly important during the period in the developmental cycle when growth ceases and sporulation begins.

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LITERATURE CITED


