Regulation of *Bacillus subtilis* Glutamine Phosphoribosylpyrophosphate Amidotransferase Activity by End Products*

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General kinetic properties and inhibition by purine end products of highly purified glutamine phosphoribosylpyrophosphate amidotransferase from *Bacillus subtilis* have been studied. The enzyme was subject to specific inhibition by the purine nucleotides AMP, ADP, GMP, and GDP. AMP was by far the most effective inhibitor. The action of the inhibitors displayed positive cooperativity and was antagonized by increasing phosphoribosylpyrophosphate concentration. Phosphoribosylpyrophosphate saturation was hyperbolic in the absence of inhibitors ($K_m = 72 \pm 8 \mu M$), but became positively cooperative in the presence of inhibitors. The concentration of glutamine ($K_m = 4.3 \pm 0.4 \mu M$) did not have a large effect on the sensitivity of the enzyme to end product inhibition. Divalent cations were required for both activity and sensitivity to allosteric inhibition. The concentration dependence of these effects and the differential effects of various cations demonstrated that two different cation sites are involved. Three pairs of nucleotide inhibitors, which were weak inhibitors when tested singly, exhibited very pronounced synergistic inhibition; these were: ADP and GMP, ADP and GDP, and GMP and GDP. AMP did not show synergistic inhibition when tested in combination with other nucleotides. The results require the existence of at least one, and possibly two, allosteric nucleotide-binding sites per amidotransferase subunit. The findings in this paper provide a more complete picture of the regulation of *de novo* purine nucleotide biosynthesis in *B. subtilis* and a necessary background for the study of the regulation of the oxidative inactivation which this enzyme undergoes *in vitro* and *in vivo*.

Glutamine phosphoribosylpyrophosphate amidotransferase (EC 2.4.2.14, 5-phosphoribosylamine:pyrophosphate phosphoribosyltransferase (glutamate-amidating)), hereafter called "amidotransferase," catalyzes the first step of *de novo* purine nucleotide biosynthesis and is therefore an appropriate target for regulation of this pathway. This enzyme has been an object of study in our laboratory because it is rapidly inactivated in *Bacillus subtilis* cells when they enter stationary phase prior to endospore formation (1). The inactivation has been shown to result from reaction of oxygen with an iron-sulfur center in the enzyme (2, 3) and appears to be followed by proteolysis of the inactivated enzyme (3, 4). The rate of reaction of amidotransferase with oxygen *in vitro* is strongly affected by substrates and end product inhibitors of the enzyme (4, 5), and it has been proposed that these ligands could regulate the inactivation of the enzyme *in vivo* (5). An essential step in the further characterization of the regulation of the inactivation of amidotransferase, therefore, is a kinetic study of the specificity of end product inhibition and of the dependence of inhibition on substrate concentration and other experimental variables. In addition to inactivation, *B. subtilis* amidotransferase is subject to metabolic regulation by repression (6, 7) and end product inhibition (5, 8). A careful study of the interaction of amidotransferase with end product nucleotides would also clarify regulation of this enzyme by feedback inhibition. This paper reports such a study using kinetic methods and nearly homogeneous preparations of *B. subtilis* amidotransferase.

**Experimental Procedures**

Amidotransferase Assays—Except where indicated, all assays were performed with the two-step spectrophotometric glutamate production assay (1, 9). Standard reaction conditions for the first step were: 30 mM Tris/Cl, pH 8.2, 2 mM EGTA, 5 mM MgCl₂, 2.5 mM P-Rib-PP, 20 mM glutamine, 0.2 to 0.5 mg of bovine serum albumin, and 0.5 to 2 μg of amidotransferase added last in a final volume of 1.00 ml. Amidotransferase solutions were diluted with 50 mM Tris/Cl, pH 7.9, containing 5 to 10 mg of bovine serum albumin/ml prior to assay. For each reaction, an identical control containing no P-Rib-PP was incubated at the same time. All of the data shown represent the average of duplicate determinations, corrected for the control without P-Rib-PP. After reaction for a fixed time, usually 20 min at 37°C, the reaction was stopped by heating at 100°C for 2 min. The tubes were cooled and clarified by centrifugation for 5 min in a clinical centrifuge. The glutamate content of each tube was determined by transfer of a sample, usually 50 or 100 μl, from the first step into a second step reaction mixture, which contained in a final volume of 1.00 ml: 100 mM potassium phosphate, pH 8.0, 0.2 mM acetylpyridine adenine dinucleotide and 15 IU of glutamate dehydrogenase. The second step reaction mixtures were incubated at 37°C for 45 min, and the absorbance at 353 nm was determined. A net absorbance change of 8.9 corresponds to formation of 1 μmol of glutamate in the aliquot of the Step 1 reaction mixture. The amidotransferase assay was shown to be linear with time for at least 30 min and linear with enzyme up to 10 μg of enzyme/assay. The second step was linear in response up to detection of 100 nmol of glutamate. In all cases where nucleotide inhibitors were used with the second step of the assay, lack of interference by the highest level of inhibitor tested was demonstrated. Interference with the activity of amidotransferase by 1 mM Rib-5-P, PP₈, and P₆, which are potential impurities in commercial P-Rib-PP,

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† The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)N,N,N',N'-tetraacetate; P-Rib-PP, 5-phospho-D-ribofuranosyl-1-pyrophosphate.
was shown to be negligible. Determination of the amount of glutamate formed in blanks lacking P-Rib-PP established that the glutaminase activity of purified amidotransferase, if any exists, was less than 0.5% of the amidotransferase activity.

When NH₄⁺ was used as a substrate or when phosphoribosylamine formation was to be assayed directly, the assay procedure described by King and Holmes (10) was used. The assay conditions were as described (10) except that MgCl₂, P-Rib-PP, and glutamine or NH₄Cl concentrations were as given under "Results."

Substrate saturation curves giving linear double reciprocal plots were analyzed by the computer-fitting technique of Cleland (11). Hill coefficients were determined graphically.

Materials—Amidotransferase was purified to near homogeneity from B. subtilis 60164 as described by Wong (4). The concentration of the enzyme was determined from absorbance measurements at 278 nm, and the extinction coefficient was based on dry weight (2). Acetylpyridine adenosine diphosphate, recrystallized glutamate dehydrogenase, EGTA, P-Rib-PP, and all nucleotides were purchased from Sigma Chemical Co. Glutamine was purchased from Calbiochem or Sigma. The concentration and purity of P-Rib-PP solutions were routinely tested by enzymatic assay (12); the preparations used were about 85% pure by weight, which is corrected for in the concentrations given. The purity of nucleotides was tested by thin layer chromatography on polyethyleneimine plates using 0.3 m potassium phosphate, pH 7, as the solvent. No impurities were detected in the nucleoside monophosphates; the nucleotide diphosphates and triphosphates were contaminated with traces (<5%) of their corresponding tri-, di-, and monophosphate derivatives. [²⁵³C]Cystine was purchased from Ameraham/Searle. All metal ions were used as the chloride salts.

RESULTS

General Kinetic Characteristics of Amidotransferase

pH Activity Profile—Amidotransferase displayed a broad, flat pH-activity optimum from pH 6 to 8.5. The activity varied by less than 10% in this range (Fig. 1), but showed some dependence on the buffer used.

Activation by Divalent Cations—Amidotransferase showed an absolute requirement for divalent cation for activity. Activation by Mg²⁺ followed a hyperbolic concentration dependence with half-maximal activation at about 0.2 mM Mg²⁺. This value was essentially independent of the P-Rib-PP concentration, which suggests that Mg²⁺ binds to the enzyme more tightly than to P-Rib-PP (K⁺[Mg²⁺-P-Rib-PP] = 0.62 mM, Ref. 13). Little or no inhibition was seen with concentrations of Mg²⁺ up to 10 mM. Several divalent cations were tested for their ability to replace Mg²⁺, using Tris/maleate buffer, pH 6.5, to avoid the precipitation seen at higher pH. Mn²⁺, Fe²⁺, and Co²⁺ were nearly as effective as Mg²⁺ at 5 mM concentrations; Ca²⁺ gave 20 to 30% of the activity of Mg²⁺ and was inhibitory in excess. K⁺, Li⁺, Na⁺, Zn²⁺, Cu²⁺, and Fe³⁺ did not replace Mg²⁺. Because small amounts of Ca²⁺ ions were inhibitory, even in the presence of 5 mM Mg²⁺, the Ca²⁺-specific chelator EGTA was added in all assays unless otherwise indicated.

Substrate Saturation—Substrate saturation curves for both P-Rib-PP and glutamine were hyperbolic when determined in the absence of inhibitors and at saturating concentrations of Mg²⁺. A Kₘ value of 72 ± 8 μM was obtained for P-Rib-PP in the two-step glutamate formation assay used in most of the studies reported here (20 mM glutamine, 3 mM MgCl₂). The Kₘ for glutamine under standard conditions was 4.3 ± 0.4 mM. The sensitivity of the two-step assay was too low for precise steady state kinetic measurements at P-Rib-PP concentrations below the Kₘ. For this reason, a detailed analysis of steady state kinetics was not attempted.

The assay of King and Holmes (10) for phosphoribosylamine formation permitted determination of a Kₘ value for NH₄⁺ utilization of 34.8 ± 1.3 mM at 10 mM P-Rib-PP and 12 mM Mg²⁺. Under the same conditions, a Kₘ for glutamine of 9.0 ± 1.0 mM was observed; at 5 mM Mg²⁺, the Kₘ for glutamine was 6.3 ± 1.2 mM. The activity with saturating concentrations of NH₄⁺ was about 1.4 times as great as that at saturating levels of glutamine. We did not find the phosphoribosylamine assay procedure to be sufficiently sensitive or precise for determination of the Kₘ for P-Rib-PP or detailed kinetic studies.

Inhibition of Amidotransferase by End Products

Survey of Inhibitory Metabolites—Table I lists a series of purine derivatives that were examined for their ability to inhibit amidotransferase under standard assay conditions. By far the most effective inhibitor was 5'-AMP, which was completely inhibitory at 3 mM. Inhibition by AMP was very specific. The only other nucleotides capable of inhibiting amidotransferase significantly at 5 mM were ADP, GMP, and GDP, all of which were much less effective than AMP. The following pyrimidine nucleotides were also tested at 3 mM and

<table>
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<tr>
<th>Inhibitor tested</th>
<th>Percentage of control activity of amidotransferase inhibition by purine derivatives at 3 mM inhibitor</th>
<th>Percentage of control activity of amidotransferase inhibition by purine derivatives at 5 mM inhibitor</th>
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<tr>
<td>5'-AMP</td>
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</tr>
<tr>
<td>3'-AMP</td>
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<td>94</td>
</tr>
<tr>
<td>2'-dAMP</td>
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<td>94</td>
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<td>Cyclic 2',3'-AMP</td>
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<tr>
<td>XMP</td>
<td>89</td>
<td>99</td>
</tr>
</tbody>
</table>

| " Assayed under standard conditions. |
Regulation of B. subtilis P-Rib-PP Amidotransferase 5399

Inhibition of amidotransferase by purine nucleotides. Standard assay conditions were used. Panel A, inhibition by adenine nucleotides. □ AMP; ○ ADP; ● ATP. Panel B, inhibition by guanine nucleotides. △ GMP; ▲ GDP; ○ GTP.

Inhibition by adenine and guanine ribonucleotides was examined further (Fig. 2). Only AMP and ADP showed substantial inhibition at concentrations less than 5 mM under standard assay conditions. At higher concentrations, ATP, GMP, GDP, and GTP became inhibitory. The fact that the effectiveness of these nucleotides as inhibitors was not correlated with their ability to chelate Mg\(^{2+}\) excluded simple chelation as the mechanism of inhibition. Inhibition by AMP and ADP was strongly cooperative. The Hill coefficients for AMP and ADP inhibition for the data shown in Fig. 2 were 3.3 and 3.8, respectively. In other experiments, the Hill coefficient for AMP inhibition ranged between 3.3 and 3.8. GDP inhibition, while weak, had a Hill coefficient of 3.3. The other weak inhibitors displayed lower cooperativity of inhibition, having Hill coefficients of 1.2, 1.8, and 1.7 for GMP, ATP, and GTP, respectively.

Effect of pH on Sensitivity to AMP Inhibition—Amidotransferase has about the same activity at pH 6 as at pH 8.2 in the absence of inhibitors, but the enzyme is completely insensitive to 1.5 mM AMP at pH 6 (Fig. 1). This selective desensitization indicates that AMP acts at an allosteric site, rather than the active site, as does the specificity of AMP inhibition (Table I).

Relationships between Nucleotide Inhibition and Substrate Concentration—A decrease in the glutamine concentration from the standard value of 20 to 5 mM, which is near the \(K_m\) for glutamine, had only a small effect on the sensitivity of amidotransferase to inhibition by AMP (Fig. 3, Curve C versus Curve B). On the other hand, decreasing the P-Rib-PP concentration from 2.5 to 1 mM sharply increased the sensitivity of amidotransferase to AMP (Fig. 3, Curve C versus Curve A). Correspondingly, a 4-fold increase in P-Rib-PP concentration greatly decreased AMP inhibition (Fig. 3, Curve C versus Curve E). It should be noted that all of the concentrations of
Fig. 6. Divalent cation dependence of AMP inhibition of amidotransferase. Panel A, standard assay conditions were used except that EGTA was omitted and 5 mM CoCl₂ (○) or 5 mM MnCl₂ (●) was used in place of MgCl₂; and the buffer was 50 mM Tris/maleate, pH 6.5. Activities without AMP were 93 and 90% of the activity with 5 mM MgCl₂ when Co²⁺ and Mn²⁺, respectively, were used. Panel B, standard assay conditions were used, except that the following cations were used in place of MgCl₂: ○ 3.25 mM CaCl₂; □ 3.75 mM MgCl₂; △ 3.25 mM CaCl₂ plus 3.75 mM MgCl₂. Note that since 2 mM EGTA was present, the concentration of free Ca²⁺ ions was 1.25 mM. Activities without AMP were 38 and 77% of the activity with 3.75 mM MgCl₂ when Ca²⁺ and Ca²⁺ plus Mg²⁺, respectively, were used.

Fig. 7. Inhibition of amidotransferase by pairs of nucleotides. Standard assay conditions were used. Panel A, synergistic inhibition by ADP and GMP; ●, ADP; □, GMP; ○, equimolar mixtures of AMP and GMP. Panel B, nonsynergistic inhibition by AMP and GMP; ■, AMP; △, GMP; □, equimolar mixtures of AMP and GMP. The dashed lines represent calculated inhibition curves assuming independent (nonsynergistic) inhibition by the nucleotide pairs.

TABLE II

<table>
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<tr>
<th>Inhibitor(s)</th>
<th>Observed* __I₀₅</th>
<th>Calculated* __I₀₅</th>
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<td>ATP</td>
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<td></td>
<td></td>
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<td>GMP</td>
<td>12</td>
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</tr>
<tr>
<td>GDP</td>
<td>9</td>
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<td>GTP</td>
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<td></td>
</tr>
<tr>
<td>IDP</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMP + ADP</td>
<td>2.0</td>
<td>3.5</td>
<td>Weak</td>
</tr>
<tr>
<td>AMP + GMP</td>
<td>3.5</td>
<td>3.5</td>
<td>No</td>
</tr>
<tr>
<td>AMP + GDP</td>
<td>3.0</td>
<td>3.5</td>
<td>No</td>
</tr>
<tr>
<td>ADP + GMP</td>
<td>1.1</td>
<td>11</td>
<td>Strong</td>
</tr>
<tr>
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<td>3.2</td>
<td>11</td>
<td>Strong</td>
</tr>
<tr>
<td>ADP + ATP</td>
<td>10</td>
<td>11</td>
<td>No</td>
</tr>
<tr>
<td>GDP + GMP</td>
<td>5</td>
<td>15</td>
<td>Strong</td>
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<tr>
<td>GDP + GTP</td>
<td>13</td>
<td>15</td>
<td>No (?)</td>
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<tr>
<td>ATP + GTP</td>
<td>14</td>
<td>19</td>
<td>No (?)</td>
</tr>
<tr>
<td>IDP + GMP</td>
<td>7</td>
<td>11</td>
<td>Weak</td>
</tr>
</tbody>
</table>

* Determined by interpolation from concentration dependence of inhibition under standard assay conditions.

P-Rib-PP used in these experiments were at least 10 times the Kₘ for P-Rib-PP in the absence of AMP. The results indicated a strong antagonism between P-Rib-PP saturation and AMP inhibition. This antagonism was shown very clearly by examination of P-Rib-PP saturation curves in the presence of various concentrations of AMP (Fig. 4). The concentration of P-Rib-PP at half-saturation was 0.07, 0.5, and about 5 mM at 0, 0.5, and 1.5 mM AMP, respectively. P-Rib PP saturation curves were hyperbolic in the absence of AMP, but became strongly cooperative in the presence of the inhibitor. The Hill coefficients for P-Rib-PP were 1.0, 1.6, and 2.0 in the presence of 0, 0.5, and 1.5 mM AMP, respectively. It appeared that at 3.0 mM AMP the maximal velocity of amidotransferase was also reduced. Much higher concentrations of ADP (4 and 8 mM) and GDP (5 and 10 mM) had qualitatively similar effects on the shapes, half-saturation values, and maximal velocities of P-Rib-PP saturation curves (data not shown).

Requirement of Divalent Cations for Inhibition

The experiments shown in Fig. 5 document that the effectiveness of AMP as an inhibitor is strongly dependent on Mg²⁺. At 1 mM AMP, for example, amidotransferase was only inhibited by 4% at 1.25 mM Mg²⁺, but the enzyme was 47% inhibited at 5 mM Mg²⁺ and 85% inhibited at 8.5 mM Mg²⁺. It should be noted that the activity in the absence of AMP was the same at all of the concentrations of Mg²⁺ used. Thus, concentrations of Mg²⁺ sufficient to activate amidotransferase fully did not give full sensitivity to nucleotide inhibition. Experiments designed to maintain constant free Mg²⁺ concentration indicated that chelation of Mg²⁺ by AMP did not play a significant role in the shape of AMP inhibition curves (Fig. 5, Curve C versus Curve B). Mg²⁺-dependent enhancement of nucleotide inhibition was also demonstrated with ADP and GMP. Increasing the total Mg²⁺ concentration from 5 to 12.5 mM decreased the Iₜₕ value for ADP from 5.8 to 3.6 mM and decreased the Iₜₕ for GMP from 12 to 5.9 mM.

The idea that divalent cations may bind at different sites to play their roles in activating amidotransferase and permitting nucleotide inhibition, which was suggested from the different concentration dependencies demonstrated above, received further support from studies of the ability of various divalent cations to support activity and AMP inhibition. Co²⁺ and Mn²⁺ both activated amidotransferase nearly as well as Mg²⁺, but differed dramatically in the sensitivity to AMP inhibition they conferred on amidotransferase (Fig. 6A). Inhibition by AMP in the presence of Mn²⁺ was similar to that seen in the presence of Mg²⁺, but amidotransferase was much less sensitive to AMP in the presence of Co²⁺. A particularly complex situation was observed in studies with Ca²⁺ ions (Fig. 6B). Ca²⁺, which supported amidotransferase activity at about 30% of the rate of an equivalent concentration of Mg²⁺, resembled Co²⁺ in conferring insensitivity to AMP inhibition. Yet, addition of 1.25 mM Ca²⁺ plus 3.75 mM Mg²⁺ to assay mixtures rendered amidotransferase twice as sensitive (Iₜₕ decreased by a factor of 2) to AMP inhibition as in the presence of Mg²⁺ alone. The results with the various divalent cations indicated that amidotransferase possesses two different divalent cation sites, one that is essential for activity and a second that is involved in nucleotide inhibition.

Synergistic Inhibition by Nucleotide Pairs—Inhibition by single nucleotides was compared to the inhibition observed in the presence of equimolar concentrations of pairs of nucleotides. In each case, the expected inhibition by the pair was single nucleotides was compared to the inhibition observed in the presence of various concentrations of AMP and GMP. The shape of AMP inhibition curves (Fig. 5, Curve B). The idea that divalent cations may bind at different sites to play their roles in activating amidotransferase and permitting nucleotide inhibition, which was suggested from the different concentration dependencies demonstrated above, received further support from studies of the ability of various divalent cations to support activity and AMP inhibition. Co²⁺ and Mn²⁺ both activated amidotransferase nearly as well as Mg²⁺, but differed dramatically in the sensitivity to AMP inhibition they conferred on amidotransferase (Fig. 6A). Inhibition by AMP in the presence of Mn²⁺ was similar to that seen in the presence of Mg²⁺, but amidotransferase was much less sensitive to AMP in the presence of Co²⁺. A particularly complex situation was observed in studies with Ca²⁺ ions (Fig. 6B). Ca²⁺, which supported amidotransferase activity at about 30% of the rate of an equivalent concentration of Mg²⁺, resembled Co²⁺ in conferring insensitivity to AMP inhibition. Yet, addition of 1.25 mM Ca²⁺ plus 3.75 mM Mg²⁺ to assay mixtures rendered amidotransferase twice as sensitive (Iₜₕ decreased by a factor of 2) to AMP inhibition as in the presence of Mg²⁺ alone. The results with the various divalent cations indicated that amidotransferase possesses two different divalent cation sites, one that is essential for activity and a second that is involved in nucleotide inhibition.

Synergistic Inhibition by Nucleotide Pairs—Inhibition by single nucleotides was compared to the inhibition observed in the presence of equimolar concentrations of pairs of nucleotides. In each case, the expected inhibition by the pair was calculated assuming independent inhibition, as was done in studies of avian liver amidotransferase by Caskey et al. (14). Some pairs of weak inhibitors, e.g. ADP and GMP (Fig. 7,
Panel A), displayed strongly synergistic inhibition. This inhibition was cooperative; the Hill coefficient as a function of total nucleotide concentration was 3.3. Other pairs, particularly those involving AMP or very weak inhibitors, did not display significant synergism of inhibition (e.g. AMP and GMP, Fig. 7, Panel B). Table II lists all of the pairs examined and shows that three pairs of inhibitors—ADP and GMP, ADP and GDP, and GMP and GDP—displayed strongly synergistic inhibition. Other pairs were weakly or not at all synergistic. A series of pyrimidine nucleotides was also examined for synergistic inhibition in combination with GMP, but none was observed.

**DISCUSSION**

Kinetic studies of the regulation of glutamine P-Rib-PP amidotransferase by nucleotides have been conducted with enzymes from many sources (for reviews, see Refs. 15 and 16), but the only studies with highly purified enzymes prior to this work were performed with amidotransferases from avian liver (14, 17, 18) and from *Escherichia coli.* The studies with the avian enzymes were complicated by the instability of sensitivity of these enzymes to nucleotide inhibition (14, 17, 18). The purified chicken liver amidotransferase was completely insensitive to nucleotides (17), while the nucleotide sensitivity and other kinetic properties of the pigeon liver enzyme depended on the purification procedure and prior treatment (18) of the enzyme. For the avian liver enzymes, therefore, it is difficult to be confident that the kinetic studies provided an accurate description of the properties of the native enzyme. In contrast, the kinetic properties of *Bacillus* amidotransferase are quite stable. Most of the properties of the purified enzyme are very similar to those reported by Shiio and Ishii (8) for a preparation purified only 5-fold from crude extracts and to our own observations with crude extracts (1). There are only a few instances in which our results differ significantly from those of Shiio and Ishii (8). (a) We find AMP to be a much more effective inhibitor than ADP, while they found the two nucleotides to be similarly effective. It is possible that AMP was formed from ADP in the crude extract. (b) Shiio and Ishii reported that GTP was an effective, partial inhibitor, while we found it to be a poor inhibitor. This contradiction is difficult to explain, but it should be noted that GTP inhibition is the one property reported by Shiio and Ishii to change upon aging of the enzyme. (c) Shiio and Ishii did not observe synergism in nucleotide inhibition, but they did not test any of the pairs which we found to display strong synergism. Altogether, we believe that the kinetic properties of the purified enzyme reported here accurately reflect the properties of the enzyme in a fully native state.

While it is inappropriate in this discussion to relate the results reported here to all of the studies of amidotransferases from various sources, a few generalizations can be made. *Bacillus* amidotransferase shares several properties with most other amidotransferases: (a) positive cooperativity of nucleotide inhibition and of P-Rib-PP saturation in the presence of inhibitors, (b) stronger inhibition by nucleotide monophosphates than by di- and triphosphates, (c) a competitive relation between P-Rib PP and nucleotide inhibition, and (d) many general kinetic properties, such as *Km* values, divalent cation requirements, and ability to use NH₄⁺ as a substrate. With respect to end product inhibition, the *Bacillus* enzyme differs most dramatically from the other enzymes that have been examined in two respects: (a) by far the most effective inhibitors are AMP and ADP, and (b) some of the nucleotide pairs examined exhibit much stronger synergistic inhibition than has been previously reported for any other amidotransferase.

A few conclusions concerning the physical mechanism of inhibition of amidotransferase by nucleotides can be drawn. The specificity, kinetic properties, and selective desensitization of AMP inhibition at pH 6 or with Co²⁺ ions establish quite clearly that this nucleotide binds at an allosteric site. The native amidotransferase exists as a tetramer (2), so that the Hill coefficient of appreciably greater than 3 observed for AMP inhibition suggests the existence of one AMP site per subunit and very strong interaction between sites. We have shown that saturating concentrations of AMP convert *Bacillus* amidotransferase from a tetramer to a dimer (2, 19). *P-Rib-PP* appears to stabilize the tetrameric form (19). It is possible that this dimer-tetramer equilibrium plays a central role in activity modulation by nucleotides, but the results can also be explained by postulating multiple conformations of tetramer only. A complete study of the relationship between quaternary structure and allosteric inhibition is in progress. The observation of synergistic inhibition by specific nucleotide pairs suggests the existence of at least two different nucleotide binding sites per subunit. The experiments with divalent cations also clearly indicate two classes of divalent cation sites at different specificities and affinities for the enzyme. A high affinity site must be occupied for activation of the enzyme, and a lower affinity site is required to confer sensitivity to inhibitors. This dual role for cations does not appear to have been recognized in previous studies with this enzyme. It is not clear whether activation by Mg²⁺ involves formation of Mg-P-P-PP, but the enzyme is certainly capable of binding Mg²⁺ in the absence of P-Rib-PP. Dialyzed, purified enzyme has, in the one case tested, been shown by neutron activation analysis to contain Mg²⁺.

Finally, we wish to consider the implications of our results for the probable regulation of purine nucleotide biosynthesis *de novo* in *B. subtilis.* Amidotransferase is subject to three modes of regulation: repression, feedback inhibition, and inactivation. Complete repression of amidotransferase by guanosine plus adenosine has been reported previously (6, 7), and we have confirmed this observation. The feedback inhibition of amidotransferase is dominated by AMP. One can speculate that the unusual effectiveness of AMP serves to coordinate purine biosynthesis to the energy availability of the cell, even in the absence of high end product pools. The powerful synergism between ADP and GDP, ADP and GDP, and GDP and GDP can clearly serve to ensure that both guanine and adenosine nucleotide pools play a role in regulation of purine synthesis. The third mode of regulation of amidotransferase in *B. subtilis* is oxidative inactivation of the enzyme in the stationary phase of growth (1, 3–5). The rate of the inactivation is strongly affected by nucleotide inhibitors of the enzyme: AMP stabilizes, GMP destabilizes (4, 5). The studies in this paper provide a framework for a complete examination of the potential regulators of the oxidative inactivation of amidotransferase *in vitro* and, eventually, *in vivo*.

**Acknowledgments**—We are grateful to Katharine Gibson, Thomas Paulus, and Joseph Wong for many helpful discussions of this work and to Simon Rosenzweig for mass culturing of bacteria.

**REFERENCES**

Regulation of Bacillus subtilis glutamine phosphoribosylpyrophosphate amidotransferase activity by end products.
E Meyer and R L Switzer