The Enzymology of Feedback Inhibition of Glutamine Phosphoribosylpyrophosphate Amidotransferase by Purine Ribonucleotides*

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(Received for publication, August 19, 1963)

An important biochemical regulatory mechanism is that of feedback control in which an end product adjusts the rate of its own production by inhibiting the activity of the first enzyme unique to its biochemical pathway (2). When first discovered, several inhibitions were interpreted in traditional terms of competition between inhibitor and substrate for a given binding site because the interactions were kinetically "strictly competitive." Further experience disclosed that other feedback inhibitions were noncompetitive or of mixed type. Additionally, it was observed that there was no necessary analogy between inhibitor and substrate size, charge, stereochemistry, or metabolism (3).

It is now known that some feedback-regulated enzymes possess special inhibitor binding sites that are distinct from those involved in binding of substrate. Existence of special binding groups has been demonstrated by desensitizing the enzyme to the effects of its inhibitor (4-7) and by mutant selection (8-10). In some cases, the evidence allows the possibility that substrate and inhibitors share portions of the same site. In others, the evidence is compelling that the inhibitor site is quite distinct from the substrate site. Several examples of inhibition suggest an effect upon the conformation of the enzyme, which results in a reduction of catalytic activity (4-6).

The biosynthetic sequence by which purine ribonucleotides are formed is under feedback control. In bacteria (11-13), HeLa and L cells (14, 15), and ascites cells (16, 17) both adenine and guanine nucleotides function as inhibitors of some early step in this sequence. The enzyme glutamine phosphoribosylpyrophosphate (glutamine-PP-ribose-P) amidotransferase (18) catalyzes the first reaction specifically concerned with purine biosynthesis.

Glutamine + PP-ribose-P + H2O → Glutamate + 5-phosphoribosylamine + PPi

In 1958 we showed that the amidotransferase from pigeon liver was inhibited by ATP and ADP and less strongly by GMP, GDP, and IMP (19). The inhibitions were competitive against PP-ribose-P. In view of the structural analogies between PP-ribose-P and the ribonucleotide inhibitors, we initially suggested that these inhibitors competed with PP-ribose-P for its binding site. We have now reinvestigated the control of glutamine-PP-ribose-P amidotransferase of pigeon liver by adeny ribonucleotides. We find that this enzyme too can be desensitized to the effects of ribonucleotide inhibitors. Studies with combinations of 6-aminopurine and 6-hydroxypurine ribonucleotides show inhibitions greater than can be attributed to interaction of both inhibitors at a single site; hence two distinct regulatory sites are postulated.

**EXPERIMENTAL PROCEDURE**

**Materials**—AMP, ADP, ATP, GMP, GDP, IMP, 5-phosphoribosyl 1-pyrophosphate, the 3-acetylpyridine analogue of DPN (Pabst Laboratories), L-glutamine (Mann Research Laboratories), milk xanthine oxidase and beef liver catalase (Worthington Biochemical Corp.) and horse hemoglobin (Sigma Chemical Co.), recrystallized three times, were commercial preparations. 6-Mercaptopurine ribonucleotide was prepared biosynthetically from 6-mercaptopurine and PP-ribose-P (20). Solutions of PP-ribose-P were prepared in water, 6.0 mg per ml, and assayed with orotidylic pyrophosphorylase and deoxyribonuclease (21). Average purity was 65%. Solutions were stored at −10° and were consumed in a few days. They were reassayed frequently, especially if stored longer. Glutamate dehydrogenase was purchased from Sigma Chemical Corp. as a crystalline suspension in sodium sulfate or as a solution in 50% glycerol, diluted 5-fold with 0.1 M phosphate buffer, pH 7.4, and assayed with glutamic acid and AcPyAD.

**Methods**—In the assays of glutamate dehydrogenase and of amidotransferase, kinetic measurements of rate of reduction of AcPyAD (19) were followed in a Beckman model DU ultraviolet spectrophotometer equipped with a Gilford automatic cuvette positioner, blank compensator, and optical density recorder. Protein was calculated from the absorbances at 260 and 280 μg according to Warburg and Christian (22). Sucrose density gradient centrifugation of pigeon liver amidotransferase was performed according to Martin and Ames (23), employing both 5- and 33-mi cells. Catalase standards were located by spectrophotometric assay of hydrogen peroxide disappearance (23). Hemoglobin standards were located by measurement of absorbance in the Soret region.

*These studies were supported by Research Grant A-1391 and CY-4142 from the United States Public Health Service. A preliminary report of this work has appeared (1).
†Student Research Trainee, Duke University Medical Center; supported by Training Grant 20-516 from the United States Public Health Service.

*1* In this paper, "binding site" will be used to designate a family of functional groups or "subsites" on the enzyme that are capable of interacting with groups on the substrate or inhibitor.
RESULTS

Pigeon Liver Glutamine Phosphoribosylpyrophosphate Amidotransferase Preparation—Our initial goal was to obtain a highly purified preparation of the enzyme. Accordingly, an additional step of chromatography on DEAE-cellulose was employed, with minor modifications of the previously published procedure (19) as follows. The 100,000 × g supernatant solution of 1:2 homogenate of pigeon liver in electrolyte buffer (24) was fractionated between 30 and 50% of saturation with neutralized ammonium sulfate solution a total of three times and dialyzed against continually changing distilled water for 14 hours. The resulting precipitate was collected and extracted with 0.05 M glycine, pH 8.0, made 0.1 M with phosphate buffer, pH 7.4, and heated at 60° for 4 minutes. (When a preparation was dialyzed only a few hours in order to avoid precipitation of the enzyme, as described below, the fraction was diluted with 0.4 volume of 0.25 M phosphate buffer, pH 7.4, and then heated.) The heat stable supernatant fraction was diluted to contain 10 mg of protein per ml and was treated two or three times with 0.3 volume of aged calcium phosphate gel (10 mg of solids per ml) to adsorb inactive protein. After the enzyme was equilibrated by dialysis for 2 hours with 0.01 M PO₄, pH 6.8, 25 to 30 mg of protein were placed on a DEAE-cellulose column 1 cm in diameter and 15 cm in height. Elution was effected by 0.2 M NaCl in 0.01 M phosphate buffer, pH 6.8 at 4°, as shown in Fig. 1. The pooled peak generally yielded an enzyme fraction having a specific activity of 2500 to 3200 units per mg of protein. The specific activity of the most active fraction was 800 to 1000 times greater than that of the soluble extract of liver (Table I). The enzyme was quite labile at this stage unless stored in 10−3 M mercaptoethanol.

Kinetics of Amidotransferase Reaction—The standard assay of amidotransferase employed in this study involves measurement of glutamate by coupling the reaction to the reduction of 3-AcPyAD in the presence of glutamate dehydrogenase. The assay is conducted in a 1-ml mixture of 0.25 mM PP-ribose-P, 1.0 mM glutamine, 3 mM MgCl₂, 0.6 mM 3-AcPyAD, 150 units of glutamate dehydrogenase (19), and the amidotransferase. As pointed out before (19), there is an initial lag period of some minutes before the rate of reduction of the DPN analogue becomes linear. Velocity measurements are made during the linear phase of the assay. The velocity was strictly proportional to the amount of amidotransferase up to rates of 0.100 absorbance unit per minute at 363 m. All rate studies reported herein were conducted at rates of less than 0.08 units per minute.

Plots of velocity against substrate concentrations showed a typical hyperbolic curve, with a linear initial phase arising from the origin, for both PP-ribose-P and glutamine. Kinetic data previously presented (19) indicate that glutamine and PP-ribose-P are bound in random sequence. Double reciprocal plots, 1/v against 1/S, showed the curvilinear shape expected of an enzyme reacting with two substrates (25, 26). The upward departure from linearity occurred at substrate concentrations below 10−4 M PP-ribose-P when glutamine was 1 mM, or below 4 × 10−4 M glutamine when PP-ribose-P was 0.95 mM. Hence all evaluations of mechanism of amidotransferase inhibition by ribonucleotides were conducted at concentrations above 10−4 M PP-ribose-P with glutamine constant at 1 mM, and above 4 × 10−4 M glutamine with PP-ribose-P constant at 0.25 mM.

The Km value for PP-ribose-P was 2.4 ± 0.2 × 10−4 M. This

![Fig. 1. Elution of glutamine PP-ribose-P amidotransferase from a DEAE-cellulose resin column. Protein, 31 mg, specific activity 285 units per mg, was applied to a column, 1 × 15 cm. Elution was effected by 0.01 M phosphate buffer, pH 6.8, with stepwise addition of NaCl of increasing molarity as indicated by arrows at top of diagram. Protein is indicated by black circle, and enzyme activity by O.](http://www.jbc.org/)

### Table I

Purification of glutamine PP-ribose-P amidotransferase of pigeon liver

<table>
<thead>
<tr>
<th>Step and procedure</th>
<th>Volume</th>
<th>Protein</th>
<th>Activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Soluble extract</td>
<td>67.0</td>
<td>72.0</td>
<td>5</td>
<td>20,630</td>
</tr>
<tr>
<td>II. Ammonium sulfate, 30 to 50% fraction</td>
<td>25.2</td>
<td>54.3</td>
<td>18</td>
<td>24,624</td>
</tr>
<tr>
<td>III. Enzyme dialyzed 4 hours, diluted with buffer</td>
<td>34.8</td>
<td>20.3</td>
<td>44</td>
<td>40,200t</td>
</tr>
<tr>
<td>IV. Heat-stable fraction</td>
<td>16.5</td>
<td>20.3</td>
<td>119</td>
<td>20,746</td>
</tr>
<tr>
<td>V. Calcium phosphate gel supernatant</td>
<td>23.0</td>
<td>5.2</td>
<td>283</td>
<td>35,100</td>
</tr>
<tr>
<td>VI. DEAE-cellulose eluate</td>
<td>6.0‡</td>
<td>0.18</td>
<td>4,000</td>
<td>3,680</td>
</tr>
</tbody>
</table>

* A small amount of inactive precipitate was removed.
† Activation of this magnitude was frequently though not invariably encountered after a short dialysis. Among the explanations are (a) removal of ammonium ion, which interferes with the assay; (b) removal of endogenous inhibitor nucleotides; (c) change in properties of enzyme, with reduction in sensitivity to inhibitors; and (d) conversion to a more active conformational form.
‡ A 6-ml aliquot of Fraction V was submitted to chromatography. The corrected recovery in the two best eluate fractions was 14,210 units, or 35% of Fraction III.
value was constant throughout purification, and from one preparation to the next, irrespective of the sensitivity of the preparation to nucleotide inhibition. The $K_m$ for glutamine showed a small variation from one preparation to another, and averaged $1.0 \times 10^{-4}\text{ M}$ for purified preparations devoid of glutaminase activity.

**Inhibition by Purine Ribonucleotides**—We had previously found that glutamine PP-ribose-P amidotransferase was strongly inhibited by purine 5'-ribonucleotides. The enzyme was not inhibited by ribose 5'-phosphate, purine ribonucleotides or bases, by 2'- or 3'-phosphate or deoxyribose phosphate analogues, or by pyrimidine ribonucleotides (19). The inhibitions by purine 5'-ribonucleotides were competitive against PP-ribose-P. $K_i$ values ranged from $3.7 \times 10^{-5}\text{ M}$ for ATP to $3.8 \times 10^{-4}\text{ M}$ for GDP. Data obtained on two consecutive large scale enzyme preparations had been in good agreement. Hartman (27) found that a highly purified amidotransferase from chicken liver was insensitive to purine ribonucleotides. In the present study, the same purine ribonucleotides were again found to inhibit the amidotransferase, although sensitivity was lost on extensive purification (see below). In double reciprocal plots the inhibitions appeared competitive against PP-ribose-P as before (19). At high levels of AMP, GMP, 6-mercaptopurine ribonucleotide (28), or ATP, 100% inhibition was demonstrated. Thus, the inhibitions were also reexamined with respect to glutamine. In double reciprocal plots the apparent $K_m$ value was increased but maximal velocities were decreased (Fig. 2). Thus, the inhibitions were of the mixed competitive-noncompetitive type against glutamine.

In a series of experiments with different preparations of purified amidotransferase a remarkable variation in sensitivity to nucleotide inhibitors was encountered. This variation involved all ribonucleotide inhibitors. Enzyme fractions with specific activities in the range between 2500 and 3500 units per mg generally showed inhibition by AMP, with $K_i$ values of the order of $10^{-4}\text{ M}$, but some preparations showed no inhibition even at $2 \times 10^{-2}\text{ M}$. About one-half of preparations were inhibited by ADP, and showed $K_i$ values of about $4 \times 10^{-4}\text{ M}$. When an enzyme was ADP-sensitive, the $K_i$ value was invariably lower than that of AMP, signifying that inhibition did not merely reflect conversion of ADP to AMP. Almost all purified preparations were resistant to ATP inhibition at a concentration of nucleotide of $2 \times 10^{-3}\text{ M}$, and some were resistant even at $2 \times 10^{-2}\text{ M}$. Table II shows the range of $K_i$ values obtained with various partially purified preparations of amidotransferase which showed significant nucleotide sensitivity. The ratios of $K_i$ values for various nucleotide pairs differed widely from one enzyme preparation to the next. The variations in $K_i$ values of different preparations which showed constancy of $K_m$ values, and in particular the total insensitivity of certain preparations to nucleotide inhibitors, indicate that the binding of inhibitors involves some functional groups of the enzyme that are not concerned with, and do not influence, binding of substrates.

**Preparation of ATP-sensitive Enzyme**—The results cited above, showing a difference between earlier and more recent preparations of amidotransferase with respect to ATP sensitivity, suggested that alterations of the enzyme were occurring during purification. Systematic study of responsiveness of less highly purified enzyme showed that the heat stable fraction (Stage IV) and all later fractions were affected little by ATP, if at all. Attention was therefore directed to earlier stages of purification. Although glutaminase activity of the redissolved ammonium sulfate fraction was considerable, it was possible to demonstrate that the enzyme was regularly ATP-sensitive at this stage. During prolonged dialysis against distilled water the enzyme precipitated, and the extracted enzyme was generally ATP-resistant even before being heated. The clue that a fundamental change in the amidotransferase may have occurred when it precipitated during dialysis prompted a review of records of earlier preparations. The initial preparations that were highly sensitive to purine ribonucleotides were in each instance preparations that had behaved atypically, inasmuch as the bulk of the enzyme had remained in solution during dialysis. Hence, in several preparations, dialysis of the ammonium sulfate fraction was interrupted after 1 hour, before precipitation had begun.

![Fig. 2. Double reciprocal plot of velocity and glutamine concentration data in presence and absence of 2 mM AMP, GMP, and IMP. PP-ribose-P was constant at 0.25 mM. The inhibitions are of the mixed competitive-noncompetitive type with respect to glutamine.](http://www.jbc.org/)

**FIG. 2.** Double reciprocal plot of velocity and glutamine concentration data in presence and absence of 2 mM AMP, GMP, and IMP. PP-ribose-P was constant at $0.25\text{ mM}$. The inhibitions are of the mixed competitive-noncompetitive type with respect to glutamine. $v = \text{change in optical density at 363 nm per 10 minutes.}$

### Table II

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>No. of determinations</th>
<th>$K_i$ (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>26</td>
<td>$9.2 \times 10^{-6}$ to $2.5 \times 10^{-3}$</td>
</tr>
<tr>
<td>ADP</td>
<td>10</td>
<td>$3.8 \times 10^{-6}$ to $4.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>ATP</td>
<td>10</td>
<td>$3.1 \times 10^{-6}$ to $1.1 \times 10^{-3}$</td>
</tr>
<tr>
<td>GMP</td>
<td>12</td>
<td>$8.6 \times 10^{-5}$ to $3.5 \times 10^{-4}$</td>
</tr>
<tr>
<td>GDP</td>
<td>6</td>
<td>$3.8 \times 10^{-5}$ to $5.4 \times 10^{-4}$</td>
</tr>
<tr>
<td>IMP</td>
<td>6</td>
<td>$1.8 \times 10^{-3}$ to $3.5 \times 10^{-2}$</td>
</tr>
</tbody>
</table>
These preparations were sensitive to AMP and ATP, but their sensitivity to ATP inhibition was variable and frequently disappeared over a period of a few hours at 4°. During this period the specific activity increased spontaneously, at times by 3-fold or even more. Several different preparations of enzyme which had lost ATP sensitivity in this manner were observed to regain ATP sensitivity when frozen overnight at -10°, only to lose it once more during a few hours at 0°. This was not solely a matter of concentration of the protein as water was extruded during freezing, since removal of the upper dilute fraction after thawing did not stabilize ATP inhibition of residual enzyme. Fig. 3 shows a particularly instructive experiment in which a dramatic increase in enzyme activity, fluctuating ATP responsiveness, and a gradual decline of sensitivity to AMP were observed during ageing alternately at 4° and -10°. These surprising variations in three properties of the enzyme each occurred independently of the others.

The highest sensitivity to ATP observed in preparations dialyzed for short periods of time approached that previously reported (19). Inhibition by ATP was normally competitive against PP-ribosyl-P and $K_I$ values of 1.8 to 3.0 x $10^{-4}$ M were observed at that stage. When a preparation having a sensitivity to ATP was heated at 60° for 3 to 6 minutes, ATP sensitivity was reduced or abolished, often with little or no loss of enzyme activity (Table III). Similarly, passage of an ATP-sensitive enzyme through a small column of Sephadex G-25 resulted in immediate total loss of ATP sensitivity. Many attempts were made to standardize the conditions responsible for ATP inhibition, without notable success. These included use of neutralized EDTA, 0.0001 to 0.001 M, for ATP inhibition, without notable success. These included use of neutralized EDTA, 0.0001 to 0.001 M, and mercaptoethanol, 0.001 M, in all solutions in contact with the enzyme after the experience of Gerhart and Pardee (5), as well as storage in ATP, 0.001 M, long or short chain polyphosphate polymers, 4 x $10^{-5}$ M, 0.1 M phosphate buffer, pH 7.4, 0.25 M NaCl, or 0.1 M KCl.

In other experiments, enzyme preparations showed instability of response to AMP and GMP comparable to that shown for ATP. Examples are shown in Fig. 4A and B, in which sensitivities of enzyme to these two nucleotides was initially great, diminished markedly during 1 to 2 days at -10°, and was followed by significant recovery of sensitivity on further ageing at 4° and overnight freezing. Although the trends of sensitivities to AMP and GMP showed a general parallelism, several departures from a strictly coordinate variation are to be noted. Compare, for example, the sensitivities to AMP and GMP at 0.5 to 1.0 mM concentrations on Day 5, a.m., in Fig. 4.

Cooperative Inhibition—The studies described above suggested that the enzyme might possess more than one inhibitor binding site. A series of studies was conducted with different pairs of ribonucleotide inhibitors, in which inhibitions caused by each ribonucleotide alone were compared with inhibitions caused by two ribonucleotides acting together. It was reasoned that two inhibitors bound by identical subgroups of a single site should

\[ v = \frac{1}{K + A^2 + B^2} \]

where $A$ and $B$ are the concentrations of the two nucleotides.

1 Because of loss of ATP sensitivity on dialysis and on passage through Sephadex, the possibility that a low molecular weight substance was involved in ATP binding or in stabilization of enzyme in a particular conformation was considered. The lyophilized dialysate (distilled water) was added back to the enzyme, without restoring ATP responsiveness.

4 We are indebted to Dr. William S. Lynn for the polyphosphate polymers.

**Fig. 3. Variable sensitivity of amidotransferase to AMP and ATP.** The initial assays were conducted immediately after the ammonium sulfate fraction had been dialyzed 1 hour against Tris, 0.05 M, pH 8.0. Assays were repeated 1 hour later. The enzyme was then frozen at -10° for 12 hours, thawed quickly by hand, and kept at 4° for 2 hours, during which time three assays were performed. The enzyme was refrozen for 16 hours, thawed, kept at 4° for 6 hours, and finally frozen for 18 hours before it was reassayed. Note the remarkable gain in specific activity, the loss of ATP sensitivity at 4° and recovery at -10°, and independent decline in AMP responsiveness.

**Table III**

<table>
<thead>
<tr>
<th>Time of assay</th>
<th>Control</th>
<th>ATP</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before heating</td>
<td>0.059</td>
<td>0.017</td>
<td>71</td>
</tr>
<tr>
<td>After 0 minutes at 60°</td>
<td>0.044</td>
<td>0.038</td>
<td>18</td>
</tr>
</tbody>
</table>

**Fig. 4. Loss and recovery of sensitivity of amidotransferase to AMP and GMP.** The preparation was an ammonium sulfate fraction dialyzed against water 1 hour. Storage between assays was at -10°, except that the interval between a.m. and p.m. assays represented storage in an ice bath at 4°. The specific activity increased by 50% between day 1 and day 3.
compete with each other. Thus the effects of equimolar concentrations of two such inhibitors should be intermediate between those of the same total concentrations of either one alone. If, however, two inhibitors were bound at a single site in such a way that certain subgroups were shared, e.g., those concerned with binding of the phosphoribosyl moiety, and others were unshared, e.g., auxiliary subgroups concerned with binding of different purine substituents, the effects of equimolar concentrations of two such inhibitors might be greater than that of the same total concentration of either inhibitor alone, but should not exceed that of the sum of the fractional inhibitions of the individual ribonucleotides.

Cooperative inhibitions equal to such summed effects would also be consistent with two separate inhibitor binding sites but would not be sufficient evidence to indicate their existence. However, cooperative inhibitions by two nucleotides significantly exceeding the sums of their fractional inhibitions would indicate the existence of separate binding sites and a sensitization of the enzyme by one inhibitor to the effects of the subsequent binding of a second. Such effects would be incompatible with a single site model, even one with auxiliary subgroups which might allow differential binding affinities of different inhibitors.

In the studies to be described the enzyme fractions employed had been dialyzed overnight and then carried through the step of heating at 60° (Stage IV, Table I). These fractions were less sensitive to purine ribonucleotides than those employed in the studies recorded above, but had the advantage that the inhibitions were quite reproducible.

Representative results are shown in Fig. 5. In the cases of AMP + ADP, GMP + IMP (Fig. 5, upper frames), GMP + GDP, and AMP + ATP, the inhibitory effects of equal concentrations of the two nucleotides acting in concert were in no case greater than the inhibition caused by the same total concentrations of the more potent of the two inhibitors. Nor are they greater than the sum of the fractional inhibitions caused by each inhibitor acting alone. Thus, in the case of each of these pairs, the data are compatible with binding at a single site. However, in the cases of AMP + GMP, AMP + IMP (Fig. 5, lower frames), AMP + GDP, and AMP + 6-mercaptopurine ribonucleotide (28), the effects of the two inhibitors acting together were not only greater than those of the same concentration of either nucleotide acting alone, but were also greater than the sums of the fractional inhibitions of the individual nucleotides. Thus these results cannot be explained by interaction of these nucleotide pairs with the enzyme at a single binding site. This study indicates that purine ribonucleotides with a 6-amino group are bound at a site different from that at which purine ribonucleotides with a 6-hydroxy (or 6-sulphydryl) group are bound. They further indicate that both types of ribonucleotide can be bound simultaneously by the enzyme, and that binding of one inhibitor increases the effect of binding of the second.

Studies were also conducted with noninhibitory analogues. Adenosine, adenine, or ribose 5-phosphate (each 2 mM) or inorganic phosphate (0.1M) had no effect upon the inhibition of amidotransferase by AMP (2 mM).

Stoichiometry—Studies were conducted to evaluate the number of moles of substrate (PP-ribose-P) or of inhibitor bound per mole of active site. Data were obtained on the activity of amidotransferase as functions of substrate and inhibitor concentrations, and the relationships were represented by the expressions (10, 29):

\[
\log \frac{v}{v_{max}} = n \log S - \log K
\]

and

\[
\log \left( \frac{V_2}{V_1} - 1 \right) = n \log I - \log K
\]

For PP-ribose-P, \(n = 1\), regardless of the sensitivity of the enzyme to AMP or ATP (Fig. 6), indicating that both nucleotide-sensitive and -resistant forms of the enzyme bind 1 mole of PP-ribose-P. For the ribonucleotide inhibitors, however, the plots were not linear (Fig. 7) but showed an increasing slope with increasing concentrations of inhibitor. In several cases the slopes decreased again at high levels of inhibitor. 7 A sigmoidal relationship with slopes ranging from 1 to >2 may also be discerned in the plot of the interaction of L-isoleucine and L-threonine deaminase published by Monod, Changeux, and Jacob (10).
pears that interactions of inhibitors with the amidotransferase alter the properties of the enzyme, and that critical concentrations exist at which small increments in inhibitor concentration cause profound reductions in catalytic activity of the enzyme. Thus this kinetic approach is not suitable for exploring further the question of number of molecules of inhibitors bound in experiments of cooperative inhibition.

**Heat Inactivation of Amidotransferase**—When enzyme (Stage VI) was heated at 56° in 0.05 M Tris, pH 8.0, it was stable for about 10 minutes, but thereafter a progressive loss of activity occurred. The inactivation curve then showed first order kinetics (Fig. 8). When enzyme was heated in the presence of glutamine and PP-ribose-P it was initially more stable, but after 25 to 30 minutes, heat inactivation occurred at the same rate as in the unprotected enzyme. This loss of stabilization possibly reflects the heat lability of the substrates themselves, particularly of PP-ribose-P (21). In contrast, when the enzyme was heated in the presence of AMP it was immediately heat labile, and was inactivated at the same rate attained with untreated enzyme after 13 minutes and with substrate-protected enzyme after 30 minutes.

**Studies on Desensitization of Amidotransferase to Nucleotide Inhibition**—In studies of other end product-inhibited enzymes, it has sometimes been possible to effect a desensitization of the enzyme to its inhibitors by careful treatment with heat, metabolic inhibitors, or urea (4-6). These procedures were investigated in the case of the amidotransferase also.

Heating of enzyme at 52, 56, 62, or 70° in the absence or presence of substrates or inhibitors, never led to any major reduction in sensitivity of the enzyme to AMP, although as pointed out above, sensitivity to ATP was reduced by heat. A typical experiment with AMP is shown in Fig. 9.

The enzyme (Fraction V; specific activity, 450 units per mg of protein) was incubated with p-hydroxymercuribenzoate or N-ethylmaleimide in concentrations of 10^-5 to 10^-3 M for 5 minutes at 50°C. Mercaptoethanol could be added in the assay of N-ethylmaleimide-treated amidotransferase, since the inhibition of the amidotransferase by N-ethylmaleimide is irreversible. Under these circumstances N-ethylmaleimide did not inhibit GDH. The higher concentrations of p-hydroxymercuribenzoate and of urea employed partially inhibited glutamate dehydrogenase (10 to 50%). In all such cases, additional glutamate dehydrogenase was added to keep the activity of this enzyme constant in all cuvettes.
minutes at 25° in the presence or absence of substrates, after which activity and inhibition by AMP was determined. Typical results are shown in Fig. 10. In no case was there a reduction in sensitivity of enzyme to AMP following treatment with these agents (see inset, Fig. 10).

The enzyme (Fraction V, the same preparation as used above) was incubated in urea over a range of 0.05 to 0.8 M for 10 minutes at 25°. Inhibition of activity was 17% in 0.6 M urea (Fig. 11) and 50% in 0.8 M urea. These concentrations of urea potentiated inhibition by adenine nucleotides. For example (Fig. 11), in the presence of 0.6 M urea, 2 mM ATP was progressively inhibitory, whereas in the absence of urea the enzyme was unaffected by this level of ATP. Similarly, 0.6 M urea sensitized the enzyme to inhibition by ADP (Fig. 11), and 0.8 M urea markedly sensitized the amidotransferase to levels of AMP which were only minimally inhibitory in the absence of urea. The increases in sensitivity were progressive, and inhibition frequently became complete after about 15 minutes.

Since none of these procedures dissociated inhibition from catalysis, it appears that the active site itself is at least as labile as any portion of the enzyme molecule uniquely involved in
of insensitive and sensitive enzymes were compared. Amido-

Sucrose Density Gradient Studies—In view of the effects of the
dialysis procedure and of freezing on the response of the enzyme
to purine ribonucleotide inhibition, the question of conformational
tional changes of the enzyme became of great interest. A series
of studies was undertaken in which the sedimentation velocities
of insensitive and sensitive enzymes were compared. Amido-
transerase purified through Stage VI (elution from DEAE-
cellulose), which was totally insensitive to AMP or ATP at
concentrations of $2 \times 10^{-3}$ M, was distributed in a 6 to 20%
linear sucrose gradient in a single sharply defined activity peak
with $S$ values of 8.8 to 9.3 (mean, 9.0 S). These values are
based on an $S$ value of 11.3 for the catalas activity peak which
constituted an internal standard (Fig. 12A). The enzyme remained
insensitive to the same concentrations of AMP and ATP
after centrifugation.

About 20 samples of amidotransferase purified only through
Stage II (the 25 to 45% saturated ammonium sulfate fraction,
dialyzed against water only 1 hour) were also examined in the
sucrose gradient. These preparations were nucleotide-sensitive
and showed $K_i$ values for AMP of about $10^{-4}$ M and for ATP of
about $3 \times 10^{-4}$ M. Samples were examined immediately after
dialysis as well as after storage at $-10^\circ$C for periods of 1 to 18
days. Quantities of protein ranging from 15 to 36 mg were
applied in 1 to 32 ml of sucrose. The usual finding was a single
broad symmetrical peak of enzyme activity in the region between
9 and 11 $S$ (mean, 10.0 S; Fig. 12B). The sensitivity of the
enzyme to AMP and ATP was systematically examined im-
mediately after collection of drop fractions, and samples from
the leading and trailing slopes were examined as well as from
the peak tubes. The results may be summarized by stating
that sensitivity to both nucleotides has sometimes been identical
to that of the sample before centrifugation, and at other times
it has been drastically reduced during centrifugation. A given
fraction might be 60 to 70% inhibited by $2 \times 10^{-3}$ M concen-
trations of both AMP and ATP, by one only, or by neither. No
significant intra-peak variations were found. When desensitiza-
tion occurred, it was accompanied by a 20 to 50% gain in re-
covery of enzyme activity.

These latter studies were conducted with impure fractions,
and permit us to state only that desensitization does not appear
to be associated with a gross change of sedimentation velocity
of the enzyme. In this respect, pigeon liver amidotransferase
resembles phosphoribosyl-ATP-pyrophosphorylase, in which
both the sensitive and insensitive enzymes show sedimentation
constants of 9.6 S (6), and differ from aspartate transcarbamylase
in which desensitization is accompanied by a change in sedi-
mentation constant from 11.6 S to 5.9 S (5).

Glutamine Phosphoribosylpyrophosphate Amidotransferase of
Rat Liver—The existence of a control mechanism in avian liver
to potentiaaly capable of regulating purine synthesis is clearly
impied by the results reported above. Since a major function
of avian purine biosynthesis de novo is the disposition of excess
nitrogen as uric acid, the functional role of such a control in
avian liver is uncertain. Other evidence suggests that feedback
regulation is a general property of purine biosynthesis in many
species (11-17). In addition, Nierlich and Magasanik (31)
demonstrated that an amidotransferase from bacterial origin is
subject to control by repression and derepression. We con-
sidered it of interest to determine whether an amidotransferase
of mammalian origin possessed control properties similar to those
of avian sources.

Glutamine phosphoribosylpyrophosphate amidotransferase
was purified approximately 500-fold from rat liver by minor
adaptations of the procedure described above. Because of the
high ratio of glutaminase to amidotransferase activity in rat
liver the standard assay based upon measurement of production
of glutamate could not be employed with crude extracts. A
two stage assay of yield of 5 phosphoribosylamine was conducted,
resulted as described by Nierlich and Magasanik (32). When
the enzyme had been purified some 50-fold, the standard assay
was again employed. The activity of the enzyme after elution
from a DEAE-cellulose resin column was 250 units per mg of
protein. $K_m$ values for rat liver amidotransferase were $8.6 \times
10^{-4}$ M for PP-ribose-P and 5.3 $\times 10^{-4}$ M for glutamine. GMP,
AMP, and ATP were found to be competitive inhibitors against
PP-ribose-P and mixed competitive-noncompetitive inhibitors
against glutamine. Once again, $K_i$ values were quite variable
from one preparation to the next. No studies were conducted to
assess the responsiveness of undialyzed preparations of the en-
zeyme. The limited studies performed indicate a general simi-
larity of the amidotransferase of rat liver and that of pigeon or
chicken liver, and suggest that feedback regulation of purine
biosynthesis in mammalian systems (14-17) operates at this
enzyme locus.

DISCUSSION

Study of the enzymology of feedback inhibition of glutamine
phosphoribosylpyrophosphate amidotransferase has been ham-
pered by difficulties in stabilizing the nucleotide sensitivity of
the purified enzyme. The studies reported in this paper have
shown that amidotransferase purified 4- to 10-fold from the
soluble supernatant fraction of pigeon liver is highly sensitive to
ribonucleotides such as AMP and ATP, but that ageing, heating,
and further purification of the enzyme are associated with a
marked decrease, and sometimes a total loss, of feedback inhibi-
tion. Despite the great variations of sensitivity of different
preparations of amidotransferase to ribonucleotide inhibitors,
reflected in marked variations of $K_i$ values, the $K_m$ values of
PP-ribose-P and of glutamine were constant. These observa-

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tions of themselves establish that the inhibitors are attached to the enzyme by functional groups which are at least partially distinct from those required for binding of either PP-ribose-P or glutamine.

Two major models have been considered as explanations of the interactions in which desensitization to inhibitors may be shown (4, 5, 10). In the first model, it is postulated that the substrate site and the inhibitor site share certain functional groups. The inhibitor site contains in addition one or more unshared groups which may be selectively inactivated, or rendered unavailable by conformational changes of the enzyme. In the second model, a control site distinct from the substrate site is postulated. Inhibition involves interaction between sites, reducing enzymatic activity or stabilization of the enzyme in a conformation in which catalytic activity is reduced or absent. Desensitization involves selective inactivation of the inhibitor site, or an effect upon conformation that reduces the affinity of the enzyme for the inhibitor or renders inhibitor binding of no consequence if it occurs.12

The results of the present study may be considered in terms of these models. With reference to the first model, one might postulate, as we did initially (19) that the phosphoribosyl portion of the inhibitors bind to the PP-ribose-P site. Auxiliary sub sites may exist which contribute to the over-all binding energy of a particular inhibitor, and determine formation of a catalytically active conformation when inhibitor is bound. The conformation of the enzyme molecule determines the spatial relationships of the subsites and therefore the ability to bind inhibitors. Differences in binding energy of various inhibitors will exist among the types of enzyme molecules present in a mixed population. Thus in one conformation the enzyme may bind adenine ribonucleotides better than guanine ribonucleotides. In another conformation the reverse may obtain. In yet another conformation no inhibitor subsites would be suitably placed for binding, or the conformation would permit binding of AMP but not of ATP. At least qualitatively, this hypothesis would seem to allow many of the observations described in this paper: (a) strictly competitive inhibition toward PP-ribose-P, (b) complex inhibition toward glutamine, (c) variability in sensitivity toward individual inhibitors, including near-total desensitization of certain preparations toward AMP, ADP, ATP, and GMP, (d) noncoordinate variations in sensitivities of AMP, GMP, and ATP, (e) nonlinear relationship of log (Vo/Vi - 1) and log I for all inhibitors, (f) differences in effects of substrates and inhibitor (AMP) upon heat sensitivity of the enzyme, (g) sensitization of the enzyme to adenine ribonucleotide inhibitors by urea, and (h) approximately equal sedimentation constants for sensitive and insensitive enzymes.

There is one observation, however, which cannot be explained by a model which postulates that all ribonucleotide inhibitors bind to the phosphoribosyl binding points of the PP-ribose-P site. This is the finding that the simultaneous presence of equal concentrations of one 6-aminopurine and one 6-hydroxypurine ribonucleotide results in an inhibition greater than that caused by the same total concentration of either inhibitor alone, and greater than the sum of the fractional inhibitions of either inhibitor alone. These observations indicate that both types of inhibitor can act simultaneously upon the same enzyme molecule, and further imply that the binding of the first nucleotide may increase the inhibitory effect resulting from binding of the second nucleotide. These results require a model which postulates at least two inhibitor binding sites, one for adenine and one for guanine ribonucleotides. One site could share binding groups with the PP-ribose-P site, provided that auxiliary subgroups also exist, for the reasons discussed above. Both groups must depend upon conformational factors for competence as inhibitor sites. It is possible that both inhibitor sites are distinct from the substrate sites, that binding of either type of inhibitor excludes PP-ribose-P from its site by effects upon the conformation of the enzyme which cause distortion of the substrate site, and that PP-ribose-P stabilizes the enzyme in a conformation in which inhibitors cannot bind or do not affect catalytic activity if they do bind.12 These hypotheses will be recognized as variations of the induced-fit theory (39) with respect to both substrate and inhibitor sites.

In studies with other enzymes, three observations have been difficult to accommodate within the model of a substrate and inhibitor site with groups in common, and have led to postulates of separate inhibitor sites. These are (a) the inhibition does not reach 100% even at saturating levels of inhibitor (4, 5); (b) inhibition may be blocked by a structural analogue of the inhibitor (5); and (c) the inhibitor is bound equally well by sensitive and insensitive enzyme (6). In the present study, inhibition has repeatedly been found to reach 100% for AMP, ADP, ATP, GMP, and 6-mercaptopurine ribonucleotide. In instances in which it did not, the presence of a desensitized fraction of enzyme is likely. It was not possible to block the effect of one inhibitor by simultaneous addition of another inhibitor or of a noninhibitory analogue. No studies of binding of inhibitors by desensitized enzyme were attempted, since these would be meaningless without concomitant studies of binding of a highly purified, nucleotide-sensitive enzyme.

The results described in this paper indicate that feedback inhibition of glutamine phosphoribosylpyrophosphate amidotransferase by purine ribonucleotides is a potential control mechanism in vivo. Crude enzyme preparations regularly exhibited ATP sensitivity. The K, values observed with ADP and ATP in enzyme preparations showing stable inhibitions (19) were about 3.8 X 10^{-4} m. It would appear that feedback regulation of amidotransferase in vivo may be a complex process in which a number of members of the purine ribonucleotide family participate. The limited studies conducted with amidotransferases from rat and chicken liver indicate that their responses to purine ribonucleotide inhibitors are similar to those of the pigeon liver enzyme. A number of similarities exist between the amidotransferase and other regulated systems (2, 4-10), although certain differences emerge in a comparison of any two feedback-inhibited enzymes which indicate a multiplicity of variations in the details of individual control mechanisms.

**SUMMARY**

1. Glutamine phosphoribosylpyrophosphate amidotransferase has been purified 1000-fold from pigeon liver. Crude fractions of the enzyme are inhibited by adenosine monophosphate, ADP, ATP, GMP, GDP, and IMP, but desensitization to inhibitors occurs to variable extents during purification. Several of the most highly purified fractions were totally insensitive to ribo-

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12 Martin (5) has shown that desensitized phosphoribosyl-ATPpyrophosphorylase binds histidine just as well as does the sensitive enzyme.
nucleotides. The enzyme-inhibitor interactions of sensitive preparations are kinetically strictly competitive against phosphoribosylpyrophosphate and mixed competitive-noncompetitive against glutamine. A wide range of $K_i$ values was observed for all ribonucleotides, but $K_s$ values for phosphoribosylpyrophosphate and glutamine were identical for sensitive and insensitive enzymes.

2. Kinetic evidence suggests that the enzyme has separate binding sites for 6-aminopurine and 6-hydroxypurine ribonucleotide inhibitors, and that both types of inhibitors may act simultaneously upon the enzyme molecule. A number of studies are best explained by postulating that the enzyme is susceptible to inhibitors only when in a specific conformational state, and that inhibitors act by distorting the enzyme and reducing its activity.

3. The purified enzyme has a sedimentation coefficient of 9.0 S. The crude enzyme has a sedimentation coefficient of 9 to 11 S. No evidence was found that desensitization was associated with a major change in S value.

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The Enzymology of Feedback Inhibition of Glutamine Phosphoribosylpyrophosphate Amidotransferase by Purine Ribonucleotides
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