Studies with GMP Synthetase from Ehrlich Ascites Cells

PURIFICATION, PROPERTIES, AND INTERACTIONS WITH NUCLEOTIDE ANALOGS

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GMP synthetase has been purified 57-fold from Ehrlich ascites cells. The enzyme was found to be stable and to have an approximate molecular weight of 85,000 (determined by gel filtration). Its activity was stimulated by dithiothreitol and inhibited by 2-mercaptoethanol, p-chloromercuribenzoate, and hydroxylamine. Both ammonia and glutamine could serve as amino group donors.

While none of the 10 triphosphate purine and pyrimidine nucleotides studied were able to substitute for ATP as the energy donor for the reaction, all of these compounds were able to bind to the ATP site. The $K_m$ values for CTP, $\beta$-d-arabinofuranosyl-ATP, and $I^-N^\bullet$-ethenoATP were slightly lower than the $K_m$ of ATP (0.28 mM).

Six monophosphate nucleotides were aminated by this enzyme. Listed in order of their substrate specificity towards a number of monophosphate and triphosphate nucleotides: XMP, 0.25 mM; ATP, 2.0 mM, glutamine, 2.0 mM; MgSO$_4$, 10 mM; Tris-HCl, pH 7.6, 75 mM. The reaction temperature was 37°C. At these conditions, 1 unit of enzyme is that quantity that will produce 1.0 nmol of GMP/min. Velocities for Assays I to III were monitored on the 0 to 0.1 absorbance scale of a Gilford recording spectrophotometer.

The biological conversion of xanthosine 5'-phosphate to GMP is catalyzed by GMP synthetase (XMP: L-glutamine amidoligase (AMP), EC 6.3.5.2). While GMP synthetase from certain bacterial sources can be readily isolated in large quantities (2, 3), the enzyme from many mammalian sources is detected only at very low concentration (4). In 1960, a report indicated that GMP synthetase is present at a relatively high specific activity in Ehrlich ascites cells (5). The study presented in the following manuscript describes the purification of the enzyme from this murine tumor to a stable state free from any contaminating enzymes that would interfere with kinetic assays. In addition to ascertaining some of the general and catalytic properties of this enzyme, its substrate and inhibitor specificities towards a number of monophosphate and triphosphate nucleotide analogs were investigated.

**EXPERIMENTAL PROCEDURES**

**GMP Synthetase Assays**

Four different product assays were developed (summarized in Table I) to meet the special requirements of individual experiments described below. Details of Assays I and IV were previously reported (6). The rates observed when either GMP synthetase or XMP was omitted ranged from nondetectable to less than 10% of the rates of the complete reactions. When present, these blank rates were subtracted from the rate observed. The rates observed when either GMP synthetase or XMP was omitted as follows: XMP, 0.25 mM; ATP, 2.0 mM, glutamine, 2.0 mM; MgSO$_4$, 10 mM; Tris-HCl, pH 7.6, 75 mM. The reaction temperature was 37°C. At these conditions, 1 unit of enzyme is that quantity that will produce 1.0 nmol of GMP/min. Velocities for Assays I to III were monitored on the 0 to 0.1 absorbance scale of a Gilford recording spectrophotometer.

**Standard Assay**

The concentrations of reagents for the standard reaction assay were as follows: XMP, 0.25 mM; ATP, 2.0 mM, glutamine, 2.0 mM; MgSO$_4$, 10 mM; Tris-HCl, pH 7.6, 75 mM. The reaction temperature was 37°C. At these conditions, 1 unit of enzyme is that quantity that will produce 1.0 nmol of GMP/min. Velocities for Assays I to III were monitored on the 0 to 0.1 absorbance scale of a Gilford recording spectrophotometer.


**Table I**

**Assays for GMP synthetase**

**Reaction catalyzed:**

<table>
<thead>
<tr>
<th>Assay</th>
<th>AMP kinase</th>
<th>GMP kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Pyruvate kinase</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>dehydrogenase</strong></td>
<td><strong>phosphoroenolpyruvate</strong></td>
</tr>
<tr>
<td></td>
<td><strong>5'-P</strong></td>
<td><strong>7-</strong></td>
</tr>
<tr>
<td></td>
<td><strong>X</strong></td>
<td><strong>X</strong></td>
</tr>
<tr>
<td></td>
<td><strong>max -1 cm -1</strong></td>
<td><strong>12.44</strong></td>
</tr>
</tbody>
</table>

\*Analysis of 3C-labeled bases following acid hydrolysis and paper chromatography (6).

**High Pressure Liquid Chromatography**

Nucleotides were analyzed with a Varian LCS-1000 liquid chromatograph. The details of this procedure are described elsewhere (7). References to the spectral extinction coefficients for the nucleotides and nucleotide analogs were previously reported (6).

**Protein Assays**

The method of Polin-Clower as modified by Lowry et al. (8) was used to determine the concentrations of protein.

**Biochemicals**

6-ThioXMP, 6-thioGMP, 6-NH2-1-Alox-5-P, 8-azaXMP, 1-[6-\*C]-Oxi-5-P, 8-\*C]-XMP, 7-Oxi-5-P, and 5-\*C]-XMP were synthesized; these laboratories as previously described (6, 9). AraXMP and araGMP were synthesized\* at these laboratories and provided by Dr. R. L. Miller.

\*dXMP was prepared by deaminating \*dGMP at 4° according to a published procedure (10). The product was collected by adsorption onto activated charcoal and removed by three washes of 5% NH4OH/20% ethanol solution. The solution was concentrated to 2 ml with a rotory flash evaporator at 30°, applied onto a 400 mesh NH4OH/25% ethanol solution. The solution was concentrated to 2 ml by evaporation and then diluted with water. Fractions containing the product (identified by thin layer chromatography on DEAE-cellulose plates in 0.2 N ammonium formate, pH 5.0, Rf = 0.98) were pooled and lyophilized. The isolated product appeared homogeneous as analyzed by high pressure liquid chromatography.

Cyclic XMP, \*dGMP, and TTP were purchased from Sigma; 8-azaGMP, XMP, and all triphosphate nucleotides except ITTP, from P-L Biochemicals. GMP Kinase was obtained as a byproduct (from the gel filtration column step) of the preparation of GMP synthetase. Sources for the other reagents used in this study have been reported (6).

**Purification of GMP Synthetase**

**Acetone Powder Preparation**—One hundred white male mice (18 to 20 g), inoculated 6½ days earlier with 0.1 ml of Ehrlich ascites fluid, were sacrificed by cervical dislocation. The ascitic fluid was collected in a flask containing 500 ml of 0.14 M heparin-treated NaCl at 4°. The cells were pelleted by centrifugation, resuspended in 600 ml of 0.07 M heparin-treated NaCl for 5 min (to hemolyze any contaminating erythrocytes), and collected again by centrifugation. The hemolytic fraction was then washed for 4½ hours with Buffer A at a flow rate of 1 ml/min. By this time, all of the visibly colored protein had eluted, and the A260 was <0.2. A linear gradient from 0 to 0.25 M KCl in Buffer A (600 ml total volume) was used for elution. The GMP synthetase eluted in a colorless peak at approximately 0.15 M KCl in the tail of a broad peak of GMP kinase. Since the latter enzyme was present, Assay III was necessarily used.

**Protein Concentration and Gel Filtration**—The peak fractions from the above column were pooled, and the enzyme activity was precipitated by the addition of solid ammonium sulfate to 80% saturation (51.6 g/100 ml). The precipitate was collected by centrifugation and resuspended in less than 2 ml of Buffer A. The suspension was dialyzed against 1 liter of Buffer A containing 0.05 M KCl for 1 hour, clarified by centrifugation, and applied onto a Sephadex G 100 column equilibrated with the same buffer. Elution with the same buffer was performed under a 25-cm pressure head and 3-ml fractions were collected. The GMP synthetase appeared in a sharp peak (Assay I), and the three tubes containing the highest activity were pooled. The GMP kinase eluted after the GMP synthetase. When necessary, the enzyme was concentrated as described above or by ultratiltrafiltration. Occasionally, a portion of the activity was lost following concentration, but was regained after storage for 1 day at -80°. The activity then remained constant for over 1 year at -80° and was not diminished by thawing and re-freezing.

**RESULTS**

**Enzyme Activity in Ehrlich Ascites Cells**—While the specific activity of GMP synthetase in freshly obtained cells decreased steadily after 4 days of growth, the tumor mass greatly increased through Day 7. For example, almost twice as much acetone powder could be obtained from cells harvested on Day 7 versus Day 6. Therefore, as a compromise, cells were routinely harvested after 6½ days.

**Enzyme Purification**—The results of a typical purification sequence are presented in Table II. The enzyme obtained had a specific activity comparable to the highest value (4) (or twice that value in the presence of 10 mM dithiothreitol) reported for this enzyme from nonbacterial sources. An important aspect of this procedure is that the lability of the enzyme in the acetone powder extract necessitates the immediate application of the extract onto the DEAE-Sephadex column. Attempts to partially purify the enzyme from the extract, or to stabilize it with substrate or a protease inhibitor prior to ion exchange chromatography resulted in large losses of activity. Conversely, after the ion exchange chromatographic step, the enzyme was quite stable during storage at -80°, or when catalyzing the amination of XMP at 37°.

**Contaminating Enzyme Activities**—When assayed at pH 7.6 in 75 mm Tris-HCl, the preparation obtained after the gel filtration step was found to be free from any detectable quantities of GMP kinase, glutaminase, nucleotide-5'-P phosphatase, adenosine kinase, IMP dehydrogenase, NADH oxidase, and NAD reductase. The activity of ATPase ranged from nondetectable to <10% of the GMP synthetase activity; AMP kinase and adenosine deaminase, from 10 to 20%; and inorganic pyrophosphatase, from 200 to 3000.

**pH Optimum**—The activity was evaluated over a pH range of 7.4 to 8.6 in 75 mm Tris-HCl. It was found to decrease by less than 20% at the extremes and to be maximal at pH 7.6. All subsequent assays were therefore performed at pH 7.6.

**Molecular Weight**—The molecular weight was estimated...
according to the method of Andrews (11). The G-100 column described under "Purification of GMP Synthetase" was calibrated with xanthine oxidase, aldolase, bovine serum albumin, GMP kinase, and cytochrome c. The elution pattern of GMP synthetase corresponded to a molecular weight of 85,000.

Sulfhydryl Involvement in Catalysis—Unlike the enzyme isolated from calf thymus (4), the activity of this enzyme did not have an absolute requirement for exogenous sulfhydryl-containing compounds. However, under standard assay conditions (Assay IV), the enzyme was inhibited 50% with 10 mM 2-mercaptoethanol and stimulated to 192% with 10 mM dithiothreitol. Furthermore, p-chloromercuribenzoate was a powerful inhibitor, producing 72% inhibition at 1 mM and complete inhibition above 5 mM. Conversely, 10 mM concentrations of reduced glutathione, ethanol, or methanol affected the reaction velocity by less than 10%. To avoid complicating the interpretation of results, all of the above reagents were omitted from the following studies.

Inhibition by Hydroxylamine—Hydroxylamine has been shown to be a strong inhibitor of GMP synthetase isolated from Aerobacter aerogenes (2) and Escherichia coli (12), and a weak inhibitor of the enzyme from calf thymus (4). Furthermore, when the hydroxylamine was preincubated with the enzyme, XMP, ATP, and Mg²⁺, and the reaction was initiated with the amino group donor, there was an apparent inactivation of the bacterial enzymes, but not the calf thymus enzyme. In the present studies (Assay IV), hydroxylamine was found to be a strong inhibitor of enzyme from Ehrlich ascites cells, producing 50% inhibition at a concentration of 2 mM, but the inhibition was relatively unaffected by preincubation in the absence of the amino group donor.

Kinetic Constants—Michaelis-Menten constants were determined from Lineweaver-Burk plots for each substrate, while the other substrates were fixed at their standard assay concentrations. Assay I was used for rate measurements with ATP, glutamine, and (NH₄)₂SO₄ as the variable substrates. The exogenous ammonium sulfate was removed from the coupling enzymes as described under "Experimental Procedure" for determinations with glutamine and (NH₄)₂SO₄. The Kₘ determination for XMP required the increased sensitivity of Assay III. The results are summarized in Table III. In agreement with the studies of GMP synthetase from other sources (1, 4, 13, 28), both glutamine and (NH₄)₂SO₄ can serve as the amino group donor. Glutamine, which was the more efficient donor, demonstrated very weak substrate inhibition at concentrations above 2 mM.

Interactions with Triphosphate Nucleotides—While none of the purine and pyrimidine triphosphate nucleotides in Table IV were able to substitute for ATP as the energy donor of the reaction, all of these compounds were able to inhibit the reaction. Three compounds were investigated in greater detail and were found to be competitive inhibitors with respect to ATP. Their Kₑ values are listed in Table IV. If it is assumed that the other compounds also compete with ATP, an inhibition value of 50% in Table IV would correspond to a Kₑ of 1.5 mM.

Interactions with Monophosphate Nucleotides—The interactions of eight XMP analogs with GMP synthetase were investigated. With the exception of the two analogs with binding constants greater than 15 mM, their substrate activities were assessed and are presented in Table V. It is clear that the modifications of the parent XMP structure resulted in either an increased Kₑ (Kₑ)⁴ or a decreased V_max.

Some of the aminated products were examined as inhibitors of the enzyme and were found to be competitive with respect to XMP. Their Kₑ values are listed in Table V. Among the monophosphate nucleotides studied, 6-thioXMP, with a Kₑ of 5 mM, (Fig. 1), was the outstanding inhibitor.

DISCUSSION

These studies performed with GMP synthetase isolated from Ehrlich ascites cells revealed that this enzyme has a number of properties that are common to the enzymes isolated from bacterial, bovine, and avian sources. One of these properties is the apparent importance of a sulfhydryl group to the catalytic activity. Although only the enzyme from calf thymus had an absolute requirement for an exogenous sulfhydryl-containing compound (4), the tumor enzyme and the enzymes from the other sources (13, 16) were highly sensitive to inhibition by the sulfhydryl reagent p-chloromercuribenzoate. Another aspect of similarity is that the bacterial (2, 12), bovine (4), and this tumor enzyme are inhibited by hydroxylamine. However, as described under "Results," it appears that the inhibition of the mammalian and bacterial enzymes occurs by different mechanisms.

Interspecies similarities are also noted with the Michaelis-Menten kinetic constants. The reported Kₑ values compared closely for each substrate (4, 6, 13, 17–19) except XMP. There seems to be a dichotomy between the mammalian and nonmammalian enzymes concerning the Kₑ values for XMP. The value reported here for the tumor enzyme is 3.6 mM, and that for the calf thymus enzyme is < 8 mM (4). In contrast, the values for the bacterial (6, 17, 18) and avian (13) enzymes average an order of magnitude higher.

In view of the enzyme’s strict substrate specificity at the ATP binding site, its broad specificity towards the triphosphate nucleotide inhibitors is of interest. The inhibition by the pyrimidine nucleotides is particularly intriguing. Although

*Since, with both this enzyme and the one from Escherichia coli B-96 (6), the Kₑ and Kₘ values of a given XMP analog approximate each other, the two constants are used interchangeably.

---

### Table II

<table>
<thead>
<tr>
<th>Step</th>
<th>Vol</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Fold purified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Acetone powder extract</td>
<td>nil</td>
<td>318</td>
<td>798</td>
<td>2.5</td>
<td>3.3*</td>
</tr>
<tr>
<td>2 &amp; 3. DEAE-sephadex and concentration</td>
<td>1.3</td>
<td>8.5</td>
<td>132</td>
<td>15.5</td>
<td>21</td>
</tr>
<tr>
<td>4. G-100 gel filtration</td>
<td>8.2</td>
<td>2.0</td>
<td>86</td>
<td>42.8</td>
<td>57</td>
</tr>
</tbody>
</table>

*a The specific activity of cells that were lysed and assayed prior to the preparation of the acetone powder was 0.75 unit/mg.

---

### Table III

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kₑ</th>
<th>V_max</th>
</tr>
</thead>
<tbody>
<tr>
<td>XMP</td>
<td>0.0036</td>
<td>1.0</td>
</tr>
<tr>
<td>ATP</td>
<td>0.28</td>
<td>1.1</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.68</td>
<td>1.37</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>36.0</td>
<td>0.56</td>
</tr>
</tbody>
</table>

* Determined with the nonvariable substrates at their 'standard' concentrations.
CTP, araATP and 1,N\(^6\)-ethenoATP have \(K_i\) values lower than the \(K_m\) for ATP. The high concentrations in vivo of ATP diminish the possibility that these compounds could control this enzyme under physiological conditions.

On the other hand, the inhibition by 6-thioXMP may be of pharmacological significance. Metabolic studies have demonstrated that following the administration of 6-mercaptopurine, 6-thioXMP can occur at concentrations as high as 50 \(\mu\)M in Ehrlich (20, 21) and L1210 (22) ascites cells and in adenocar-

**TABLE IV**

**Interactions of triphosphate nucleotides with GMP synthetase**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Substrate activity*</th>
<th>% Inhibition at 3 mM(^b)</th>
<th>(K_i)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2'-dATP</td>
<td>&lt;0.002</td>
<td>47</td>
<td>0.18</td>
</tr>
<tr>
<td>AraATP</td>
<td>&lt;0.002</td>
<td>99</td>
<td>0.26</td>
</tr>
<tr>
<td>1,N(^6)-EthenoATP</td>
<td>&lt;0.002</td>
<td>90</td>
<td>0.26</td>
</tr>
<tr>
<td>AMP-P(NH)P(^d)</td>
<td>&lt;0.002</td>
<td></td>
<td>96</td>
</tr>
<tr>
<td>XTP</td>
<td>&lt;0.002</td>
<td>56</td>
<td>44</td>
</tr>
<tr>
<td>GTP</td>
<td>&lt;0.002</td>
<td></td>
<td>46</td>
</tr>
<tr>
<td>ITP</td>
<td>&lt;0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrimidinos</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTP</td>
<td>&lt;0.002</td>
<td>91</td>
<td>0.23</td>
</tr>
<tr>
<td>AraCTP</td>
<td>&lt;0.002</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>UTP</td>
<td>&lt;0.002</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>2'-dITTP</td>
<td>&lt;0.002</td>
<td>39</td>
<td></td>
</tr>
</tbody>
</table>

* Analyzed with Assay IV after 10- and 60-min incubations with the "standard reaction mixture" and the analog substituted for ATP. The activity with ATP was 1.0. The lower limit for detecting activity was 0.002 nmol/min/unit.

* Assay IV was used under standard conditions with the exception that the concentration of ATP was 0.3 \(\mu\)M. Assays were done in duplicate.

* Determined from Dixon plots (14) using Assay IV. The two fixed concentrations of ATP were 0.30 and 0.48 \(\mu\)M. The inhibitors were competitive with respect to ATP.

* AMP-P(NH)P, 5'-adenylylimidodiphosphate.

**TABLE V**

**Interactions of monophosphate nucleotides with GMP synthetase**

<table>
<thead>
<tr>
<th>Compound</th>
<th>(V_{max})(^a)</th>
<th>(K_m) (\times 100)</th>
<th>(K_i)</th>
<th>(V_{max}/K_m)</th>
<th>Product(^c)</th>
<th>(K_i)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XMP</td>
<td>100(^a)</td>
<td>0.0006</td>
<td>28,000</td>
<td>GMP</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>2'-dXMP</td>
<td>149(^a)</td>
<td>0.125</td>
<td>1,300</td>
<td>2'- dGMP</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>8-AzaXMP(^e)</td>
<td>18</td>
<td>0.08</td>
<td>0.03</td>
<td>320</td>
<td>8-AzaGMP</td>
<td>0.04</td>
</tr>
<tr>
<td>6-ThioXMP</td>
<td>1(^f)</td>
<td>0.005</td>
<td>200</td>
<td>6-ThioGMP</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>AraXMP</td>
<td>6.5</td>
<td>0.09</td>
<td>72</td>
<td>AraGMP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-[(^14)C]Oxi-5'-P</td>
<td>0.65(^f)</td>
<td>1.3</td>
<td>1.2</td>
<td>0.5</td>
<td>6-NH(_2)-1-[(^14)C]Alo-5'-P</td>
<td></td>
</tr>
<tr>
<td>3-[(^14)C]XMP</td>
<td>&lt;0.2(^f)</td>
<td>0.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-Oxi-5'-P</td>
<td>Not tested</td>
<td>1(^f)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclic XMP</td>
<td>Not tested</td>
<td>&gt;20</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

* Determined by a Dixon plot (14). Competitive with respect to XMP.

* \(K_i\) values used where \(K_m\) values are not available.

* All nonradiolabeled products were analyzed by high pressure chromatography (see "Experimental Procedure"). The co-chromatography of the reaction product with authentic compound under conditions where individual peaks for all nucleotide substrates and products could be clearly distinguished served to verify the formation of the product. The radiolabeled products were verified by co-chromatographing with the authentic base after acid hydrolysis (see "Experimental Procedure").

* Determination by Assay III.

* Determination by Assay I.

* Approximated high pressure chromatographic analysis of the amount of product formed after a prolonged timed incubation (2 to 3 hours).

* Determined by Assay IV (6). The lowest detectable rate was 0.002 nmol/min/unit.
GMP analogs, that of 1-Oxi-5'-P is insufficient (25). The maximal S' for 1-Oxi-5'-P can be calculated from the K, determined in this study and the [1-Oxi-5'-P] determined earlier from metabolic data (25) obtained following the administration of large doses of its precursor, allopurinol. This value, 0.00056, supports the unlikelihood of the reaction occurring and explains the failure of investigators to detect any aminated products of 1-Oxi-5'-P in animal tissue following treatment with allopurinol (25–27).

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Studies with GMP synthetase from Ehrlich ascites cells. Purification, properties, and interactions with nucleotide analogs.

T Spector


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