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Biochemical Characterization of Human GMP Synthetase*

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GMP synthetase (EC 6.3.5.2) plays a key role in the *de novo* synthesis of guanine nucleotides. It is a potential target for immunosuppressive therapy. Recently, the human enzyme was purified to homogeneity (Hirst, M., Haliday, E., Nakamura, J., and Lou, L. (1994) *J. Biol. Chem.* 269, 23830–23837). We now report the characterization of this enzyme in terms of its biochemical and kinetic properties. We found that there are distinct features of the human enzyme that has not been reported for GMP synthetase from other sources. There are two variant forms of human GMP synthetase. Their catalytic properties are very similar, although their isoelectric points are different. They most likely represent post-translational modification variants. Magnesium ion is required for enzyme activity, and the requirement is beyond levels needed for ATP chelation. Magnesium appears to be an essential activator and there may be more than one binding site. Interaction of GMP synthetase with xanthosine 5′-monophosphate (XMP), a substrate, exhibits sigmoidal kinetics with a Hill coefficient of 1.48 ± 0.07. This positive cooperativity is not due to ligand-induced oligomerization, since GMP synthetase remains a monomer in the presence of XMP and other substrates. Decoyinine, a selective inhibitor of GMP synthetase, inhibits the human enzyme reversibly with uncompetitive inhibition kinetics toward glutamine and XMP and non-competitive kinetics toward ATP.

Proper nucleotide metabolism is an important factor for immune cell maturation and function. Inherited defects in adenosine deaminase and purine nucleotide phosphorylase result in immunodeficiency in patients (reviewed in Refs. 1 and 2). Selective inhibition of IMP dehydrogenase (EC 1.1.1.205) and dihydroorotate dehydrogenase (EC 1.3.99.11), which are two essential enzymes in *de novo* nucleotide biosynthesis, result in immunosuppression (3, 4). These findings suggest that lymphocytes are very sensitive to abnormal levels of nucleosides and nucleotides. Accumulation of some metabolites and depletion of others disable proper lymphocyte functions. To fully understand the importance of these metabolic pathways in immune cell functions, it is necessary to closely examine the link between the immunobiology and the cell biology of nucleotide biosynthesis. Furthermore, an understanding of the enzymatic activities in the pathways is required in order to design therapies for patients when immunomodulation is desired.

GMP synthetase is a key enzyme in the *de novo* synthesis of guanine nucleotides. It is a G-type amidotransferase catalyzing the final step of GMP synthesis in the amination of XMP[1] to GMP (Equation 1). The designation of "G-type" is based on sequence similarities with the *trpG*-encoded anthranilate synthase component II (for a review of amidotransferases, see Ref. 5).

$$\text{XMP} + \text{ATP} + \text{Gln (or NH}_3) \xrightarrow{\text{Mg}^{2+}} \text{GMP} + \text{AMP} + \text{PP}_i + \text{Glu} \quad \text{(Eq. 1)}$$

Because of the importance of guanine nucleotide synthesis in immune cell functions, GMP synthetase is a potential target for immunosuppressive therapy.

Previously, the biochemical properties and reaction mechanism of the *Escherichia coli* GMP synthetase were characterized (6–8). Strong evidence was provided for the formation of an adenyl-XMP intermediate during catalysis (9, 10). Through active site labeling and sequence comparison with other G-type amidotransferases, certain key conserved residues were identified and predicted to be essential for enzyme activity (11, 12). Molecular cloning and genetics studies provided a better understanding of the gene organization and regulation of guanine nucleotide synthesis in bacteria (for a review, see Ref. 13). The *gua* operon in *E. coli* is comprised of the structural genes for the two enzymes, IMP dehydrogenase and GMP synthetase, required for the biosynthesis of GMP from IMP (14). The *gua* operon is controlled by the *purR* repressor which uses hypoxanthine and guanine as corepressors (15–17). Unfortunately, similar knowledge of the catalytic and regulatory features of mammalian GMP synthetases is extremely limited. Information concerning the human enzyme is not available. In order to understand the critical relationship between nucleotide synthesis and immune cell functions in humans, it is important to fully examine the functional properties of this key enzyme in nucleotide metabolism.

The human GMP synthetase has been cloned recently (18). The enzyme is encoded by a single 2.4-kilobase message and one gene. RNA hybridization experiments reveal that the level of expression is substantially higher in proliferating, transformed cells than nontransformed cells. These expression levels are consistent with previous reports that GMP synthetase activity is noticeably higher in neoplastic and regenerating tissues than in the normal counterparts (19, 20). Furthermore, arresting the proliferation results in the dramatic down-regulation of the expression of both message and protein (18). These findings provide a foundation toward the understanding of the expression of an enzyme important for cell growth and development as related to

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[1] The abbreviations and trivial names used are: XMP, xanthosine 5′-monophosphate; 2′,3′-ddATP, 2′,3′-dideoxy-ATP; AMP-CPP, α,β-methyleneadenosine 5′-triphosphate; AMP-PCP, β,γ-methyleneadenosine 5′-triphosphate; AMP-PNP, β,γ-imidoadenosine 5′-triphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate) and ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); decoyinine, 9-β-D-(5,6-psicofuranoseenyl)-adenine; psicofuranine, 9-β-D-psicofuranosyl-adenine; vidarabine, 9-β-D-arabinofuranosyl-adenine; formycin A, 7-amino-3-[β-D-ribofuranosyl]-1*H*-pyrazolo[4,3-d]pyrimidine.

the cellular regulation of purine metabolism.

Recently, we reported the purification of the human GMP synthetase (18). The present paper is the first report of the characterization of the biochemical and kinetic properties of the human enzyme. These data are fundamental for understanding the mechanisms of enzyme catalysis and inhibitor action and hence for the discovery of potent and selective inhibitors as immunosuppressive agents.

EXPERIMENTAL PROCEDURES

Materials—Native GMP synthetase was purified from A3.01 cells (human T-lymphoblastoma) as described (18). Decoyinine and psicofuranine were synthesized at Syntex Discovery Research. Radiochemicals were purchased from either Moravek (Brea, CA) or DuPont NEN.

Determination of Enzyme Activity—Initial rates were measured by [8-^{14}C]GMP formation from [8-^{14}C]XMP at 40 °C, pH 7.8, for 10 min using saturating concentrations of substrates unless specified. "Saturating concentrations" of substrates is defined as 10 mM magnesium chloride, 2 mM ATP, 1 mM XMP, and 5 mM L-glutamine. The spectrophotometric coupled assay measuring AMP formation was also performed. Both of these assay procedures were described previously, (18, 21) and they were followed exactly, with the exception that purified preparations of GMP synthetase were used (30–45 nM or 2.3–3.5 μg/ml final concentration). Purified enzyme (1 mg) has a maximum activity of producing 3–4 μmol of product/min at 40 °C and one unit is defined as the amount of enzyme producing 1 μmol of product/min at 40 °C. For measuring radioactive AMP or glutamic acid formation, the assay conditions for [^{14}C]GMP formation were modified by omitting [8-^{14}C]XMP but including [2,8-^{3}H]ATP (10 mCi/mmol) or L-[U-^{14}C]glutamine (2 mCi/mmol), respectively. In experiments measuring [2,8-^{3}H]AMP formation, each reaction was stopped by the addition of 6 μl of quench solution, which contained 250 mM EDTA and 125 mM each of ATP and AMP as carriers. ATP and AMP were separated by thin layer chromatography and the amounts of [^{3}H]AMP produced were determined as described (18). The solvent used for the separation was 0.5 M lithium chloride. AMP formation as determined by the radioactive assay provides the same result as the spectrophotometric coupled assay (data not shown). In experiments measuring L-[U-^{14}C]glutamic acid formation, the reaction was quenched by the addition of 6 μl of 250 mM EDTA and 33 mM glutamic acid. Glutamine and glutamic acid were separated by thin layer chromatography on DC-plastikfolien cellulose plates (Merck) in a mixture of ethanol: *t*-butanol:formic acid:water (v/v, 12: 4: 1: 3). Plates were then sprayed with a ninhydrin solution which consisted of 1% ninhydrin (w/v), 3% glacial acetic acid (v/v) in acetone. The position of glutamic acid was visualized after heating at 95 °C for 1 min. The amounts of [^{14}C]glutamic acid produced were determined as described (18). For the determination of IC_{50} values, enzyme activity was measured in the presence of 10% dimethyl sulfoxide with 20 μM XMP, 40 μM ATP, and 200 μM glutamine.

Determination of Kinetic and Inhibition Constants—All kinetic and inhibition constants were determined by fitting the initial velocity data to appropriate equations using the computer program Systat. All determinations are reported as "value ± asymptotic standard error." To determine K_i values and pattern of inhibition, the data were fitted to the equations of Cleland (22), using Systat and nonlinear regression analysis by the computer program KinetAsyst. To calculate Hill coefficients, the data were fitted to the Hill equation (23), using the Systat program as above. For the determination of inhibition constants when varying XMP, the data were fitted to Equation 2 for competitive inhibition or Equation 3 for uncompetitive inhibition, assuming that each inhibitor only binds to one site.

$$v = V_m/\{(K_m/S)^n \times [1 + (I/K_{is})] + 1\} \quad \text{(Eq. 2)}$$

$$v = V_m/[(K_m/S)^n + 1 + (I/K_{ii})] \quad \text{(Eq. 3)}$$

In Equations 2 and 3, v is velocity, V_m is maximum velocity, K_m is Michaelis constant, S is substrate concentration, n is Hill coefficient, I is inhibitor concentration, K_{is} is inhibition constant for a competitive inhibitor, and K_{ii} is inhibition constant for an uncompetitive inhibitor.

Determination of Subunit Composition by Density Gradient Centrifugation—Samples of purified enzyme (3 μg each) were layered on top of 5-ml 10–30% glycerol gradients containing 20 mM Tris-HCl, pH 7.4, and 50 mM sodium chloride in polyallomer 13 × 51-mm tubes. Molecular weight markers were treated in parallel with identical procedures. The markers used were catalase (M_r = 245,000), aldolase (M_r = 155,000), bovine serum albumin (M_r = 66,000), ovalbumin (M_r = 43,000), and cytochrome *c* (M_r = 12, 400). Samples (100 μl) containing GMP synthetase, alone or together with the markers, were centrifuged at 250,000 × *g* in a Beckman SW 55 Ti rotor for 17 h at 4 °C. Fractions (100 μl) were collected. The sedimentation profiles were determined by enzyme activity for GMP synthetase, optical density at 414 nm for cytochrome *c*, and protein bands on SDS gels for the other markers.

Isoelectric Focusing Electrophoresis—Pre-cast isoelectric focusing gels were purchased from Novex (Encinitas, CA). Samples of purified GMP synthetase were prepared and electrophoresed according to a standard procedure provided by the manufacturer. The gels were calibrated by protein standards with known isoelectric points (Bio-Rad). The standards are: phycocyanin (pI = 4.65), β-lactoglobulin B (pI = 5.10), bovine carbonic anhydrase (pI = 6.00), human carbonic anhydrase (pI = 6.50), equine myoglobin (pI = 7.00), human hemoglobin A (pI = 7.10), human hemoglobin C (pI = 7.50), lentil lectin (three bands, pI = 7.80, 8.00, 8.20), and cytochrome *c* (pI = 9.60).

Determination of Metal-Nucleotide Concentrations—$MgATP^{2-}$ concentrations were calculated as described (24, 25). Equations 4 and 5 were solved simultaneously.

$$[M] = [M]_t - \{[MATP][1 + (K_1/K_2)([H]/K_H)]\} \quad \text{(Eq. 4)}$$

$$[MATP] = [ATP]_t/\{1 + (K_1/[M]) + (K_1/[M])([H]/K_H) + (K_1/K_2)([H]/K_H)]\} \quad \text{(Eq. 5)}$$

$[M]_t$ is the total Mg^{2+} concentration, $[ATP]_t$ is the total ATP concentration, [MATP] is the $MgATP^{2-}$ concentration, [M] is the free Mg^{2+} concentration, [H] is the hydrogen ion concentration, K_1 is the dissociation constant for $MgATP^{2-}$, K_2 for $MgHATP^-$, and K_H for $HATP^{3-}$ (24). The values for K_1, K_2, *and* K_H are 1.37×10^{-5}, 2×10^{-3}, and 1.12×10^{-7} M. These values were calculated as the reciprocal of the recommended stability constants for $MgATP^{2-}$, $MgHATP^-$ and $HATP^{3-}$, respectively (26).

To investigate the interaction of free ATP with the enzyme, initial velocity data were analyzed by the following equations (27, 28). These equations describe a situation when ATP is a competitive inhibitor toward $MgATP^{2-}$. Equations 6 is expressed in terms of free ATP and Equation 7 in free Mg^{2+}.

$$v = \frac{V_m}{1 + \frac{K_{MgATP}}{[MgATP]}\left\{1 + \frac{[ATP_f]}{K_{i(ATPf)}}\right\}} \quad \text{(Eq. 6)}$$

$$v = \frac{V_m}{1 + \frac{K_{MgATP}}{[MgATP]} + \frac{K_d K_{MgATP}}{K_{i(ATPf)}[Mg_f]}}, \text{ where } K_d = \frac{[ATP_f][Mg_f]}{[MgATP]} \quad \text{(Eq. 7)}$$

[MgATP] is $MgATP^{2-}$ concentration, $[Mg_f]$ is free Mg^{2+} concentration, $[ATP_f]$ is free ATP concentration, and K_S is K_m of each substrate.

RESULTS

Two Forms of GMP Synthetase—GMP synthetase can be separated into two distinct peaks of activity by anion exchange chromatography (Fig. 1). Four to five times more total activity elutes in the earlier peak (peak I) than in the later peak (peak II). Based on the Coomassie Blue staining of SDS gels, peak I appears to be homogeneous while peak II does not. Although there are other protein bands present, both peaks of enzyme activity correlate only to the relative intensity of the 75-kDa GMP synthetase band. In SDS gels, the GMP synthetase band in peak I and peak II have the same electrophoretic mobility. In isoelectric focusing gels, the two forms of enzyme have distinct profiles with peak I being more basic than peak II (Fig. 2). This difference in isoelectric points is consistent with the order of elution from an anion exchange column.

We considered the possibility that these two forms of enzyme may represent different populations in equilibrium. For instance, they may be at different stages of aggregation. Rechromatography of each separately or treating each individually with 0.75 M ammonium sulfate to dissemble any aggregates prior to rechromatography does not alter the elution position (not shown). Alternatively, perhaps one form represents a reaction intermediate. To test this possibility, each form was

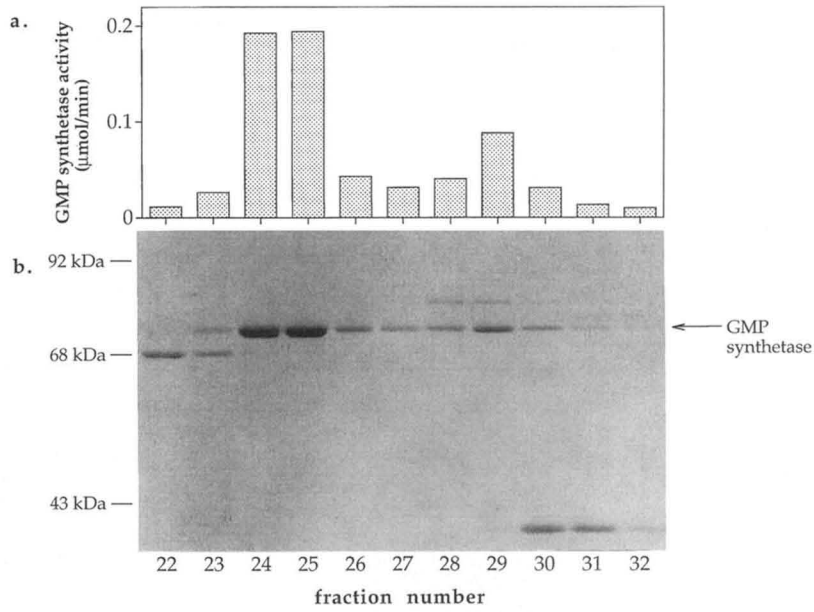

FIG. 1. **Separation of GMP synthetase into two distinct forms by anion exchange chromatography.** GMP synthetase was prepared according to the purification scheme described previously (18). The data in this figure represent the final step of the purification which is anion exchange chromatography on a Mono Q HR5/5 column. A total of 0.7 unit of enzyme was applied. The elution was accomplished using a 30-ml linear gradient of sodium chloride (0–0.25 M). Fraction 1 represents the start of the gradient. *a* shows the GMP synthetase activity in each fraction, and *b* is a Coomassie Blue-stained SDS-polyacrylamide gel. Enzyme activity was determined by the spectrophotometric coupled assay as described and the total amounts of eluted activity were reported. The materials eluted in fractions 24 and 25 (0.1 M salt, 12–12.5 ml elution volume) were designated as peak I and fraction 29 (0.12 M salt, 14.5 ml elution volume) as peak II.

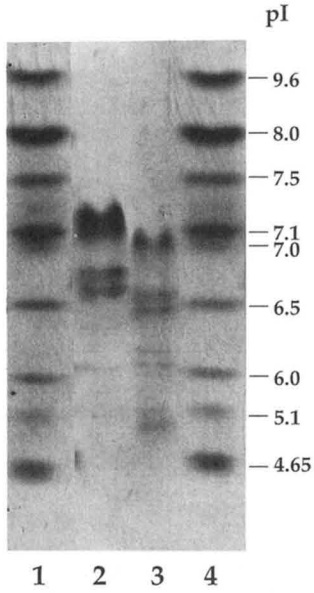

FIG. 2. **Isoelectric focusing of GMP synthetase.** Peak I and peak II GMP synthetase were prepared as described in the legend of Fig. 1. The samples (5 μg each) were electrophoresed on an isoelectric focusing gel and stained with Coomassie Blue as described under "Experimental Procedures." The samples were: calibration markers in *lanes 1* and *4*, peak I in *lane 2*, and peak II in *lane 3*. Samples that appear homogeneous in SDS gel routinely display multiple bands in isoelectric focusing gel. These bands are likely to represent protein aggregates, since the samples were electrophoresed under nondenaturing conditions. Alternatively, they may be isoelectric variants.

incubated with saturating amounts of substrates to allow single or multiple turnovers. Subsequent rechromatography showed that the elution profiles did not change. These results indicate that the two forms of GMP synthetase are not in equilibrium, and they are not readily interconvertible.

Both forms of GMP synthetase were characterized, and they were found to have very similar kinetics properties. The catalytic turnover numbers (k_{cat}) are 5.4 s^{-1} and 5.6 s^{-1} for peaks I and II, respectively. The K_m values toward ATP and glutamine are 132 ± 7 μM and 406 ± 49 μM for peak I and 180 ± 12 μM and 358 ± 34 μM for peak II, respectively. The interaction of GMP synthetase with ATP and glutamine obeys Michaelis-Menton equations. However, GMP synthetase displays sigmoidal kinetics when interacting with XMP (Fig. 3). This phenomenon is observed reproducibly for both forms of GMP synthetase. The half-saturation values toward XMP are 35.6 ± 1.8 μM and 45.4 ± 5.3 μM for peak I and II with the Hill coefficients of 1.48 ± 0.07 and 1.54 ± 0.16, respectively.

Both forms of the enzyme are inhibited by a selective GMP synthetase inhibitor decoyinine, and the modes of inhibition are identical. Decoyinine is an uncompetitive inhibitor with respect to both XMP and glutamine. The K_{ii} values toward XMP and glutamine are 50.4 ± 4.1 and 46.7 ± 4.6 μM for peak I, and 41.7 ± 5.3 and 43.1 ± 3.8 μM for peak II, respectively. The inhibition toward ATP is noncompetitive. The K_{ii} and K_{is} values are 30.4 ± 1.3 μM and 46.4 ± 6.1 μM for peak I, and 23.6 ± 3.0 μM and 29.0 ± 6.9 μM for peak II, respectively (see Fig. 4 for peak I; data not shown for peak II).

Since the two forms of GMP synthetase are not distinguishable catalytically, and peak I is higher than peak II in both purity and availability, peak I is selected for further characterization. The remaining results represent those of peak I, which will be referred to simply as "GMP synthetase."

Biochemical Characterization—To examine the subunit composition of human GMP synthetase, the enzyme was centrifuged together with molecular weight markers in a density gradient. The GMP synthetase activity sedimented as one peak and its profile is close to bovine serum albumin (M_r = 66,000). This result suggests that the human enzyme is a monomer (data not shown). Protein sequencing by Edman degradation shows that the enzyme is blocked at the N termini.

GMP synthetase converts XMP to GMP with stoichiometric hydrolysis of ATP to AMP and inorganic pyrophosphate, and L-glutamine to L-glutamic acid. The catalytic rate of GMP formation is identical to those of AMP and glutamic acid (data not shown). Ammonia can also serve as the amino group donor but the affinity is much lower. The K_m of ammonia is 5.1 ± 0.6 mM.[2] The ammonia-dependent k_{cat} is 50–60% of the glutamine-dependent activity.[3]

Dependence on Metal Ions—The presence of Mg^{2+} is required for the GMP synthetase reaction and the half-saturation value for the cofactor is 1.78 ± 0.07 mM. The specificity of the metal ion requirement was investigated. The results in Fig. 5*a*

[2] This value was calculated based on the K_m of ammonium chloride of 174 ± 21 mM and the pK_b of ammonia at 40 °C of 4.730.

[3] J. Nakamura and L. Lou, unpublished data.

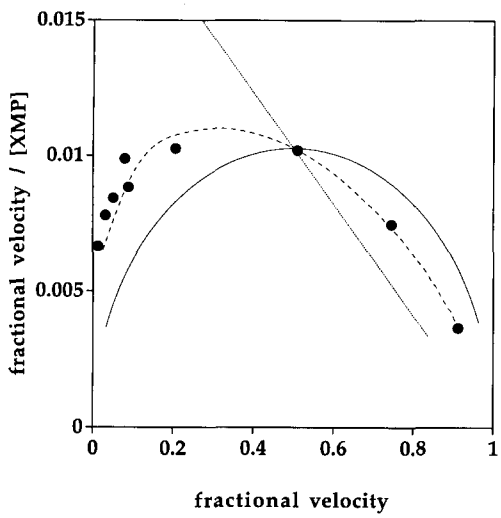

FIG. 3. **Binding of XMP to GMP synthetase.** The initial velocity of peak I GMP synthetase was determined at various concentrations of XMP. The other substrates were at saturating concentrations as described under "Experimental Procedures." Enzyme activity was determined by [^{14}C]GMP formation from [^{14}C]XMP, where the specific radioactivity was constant at 10 mCi/mmol in all samples. The data were normalized to maximum velocity (V_m) and shown as fractional velocity (●) in an Eadie-Hofstee plot. The lines represent theoretical data where the Hill coefficient is 1 (· · · ·), 1.48 (– – – –), or 2 (——).

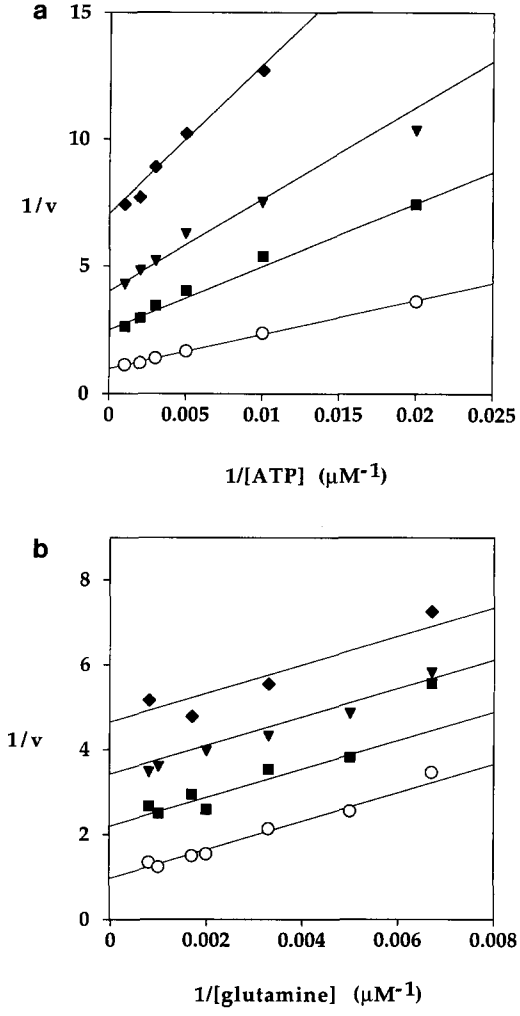

FIG. 4. **Inhibition of GMP synthetase by decoyinine.** Inhibition of peak I GMP synthetase by decoyinine is presented in double-reciprocal plots. Enzyme activity were measured as described under "Experimental Procedures," and all substrates except for the one that was varied were at saturating concentrations. *a*, ATP was the varying substrate and decoyinine was present at 0 μM (○), 50 μM (■), 100 μM (▼), and 200 μM (◆). *b*, glutamine was the varying substrate, and decoyinine was present at 0 μM (○), 50 μM (■), 100 μM (▼), and 150 μM (◆). The initial velocity was normalized to V_m. The fractional velocity relative to V_{m4} was fitted to the equations of Cleland for noncompetitive inhibition (varying ATP in a) and uncompetitive inhibition (varying glutamine in *b*). The *lines* represent the theoretical data based on the fit.

showed that Mn^{2+} is an effective cofactor at low concentrations, and Zn^{2+} or Ca^{2+} are not effective. The Mg^{2+} dependence displays sigmoidal kinetics and the Hill coefficient is 4.14.± 0.77. This apparent positive cooperativity can be an indication that there are multiple Mg^{2+} binding sites. For the experiment presented in Fig. 5*a*, when Mg^{2+} and ATP are each present at 2 mM total concentrations, $MgATP^{2-}$ is 1.7 mM (see *arrow* in Fig. 5*a* and see "Experimental Procedures" for calculation). The data show that although the complexation between metal ion and nucleotide is 85% complete, the reaction is only at 61% of maximum. The stoichiometry of Mg^{2+} requirement is higher than ATP, suggesting that additional Mg^{2+} must bind to the enzyme for maximum activity. This phenomenon was further illustrated in Fig. 5*b*. In this experiment, total ATP was lowered to 0.1 mM to exaggerate the ratio of free Mg^{2+} to free ATP. At this ATP concentration, the half-saturation value for Mg^{2+} is 1.93 ± 0.08 mM which is similar to the value when ATP is 2 mM (see above). When $MgATP^{2-}$ complexation is essentially complete (see *arrow* in Fig. 5*b*), enzyme activity is only less than 10% of maximum. As free Mg^{2+} continues to increase, the enzyme activity is also increased, although $MgATP^{2-}$ remains essentially constant throughout. These results clearly suggest that the GMP synthetase reaction not only depends on $MgATP^{2-}$ but requires free Mg^{2+}.

The data in Fig. 5*c* suggest that Mg^{2+} is an essential activator of GMP synthetase. In this experiment, Mg^{2+} was lowered to 1 mM in order to decrease the ratio of free Mg^{2+} to free ATP. Under these conditions, ATP initially causes an increase in enzyme activity, since $MgATP^{2-}$ is formed. As ATP continues to increase, it causes a depletion of free Mg^{2+} and a parallel decrease in enzyme activity. In spite of the increase in $MgATP^{2-}$, the enzyme activity continues to decrease until it is completely abolished. Note that the maximum activity does not occur at the point of maximum concentration of $MgATP^{2-}$, but at a concentration lower than that, while there is still free Mg^{2+}. These data suggest that $MgATP^{2-}$ alone is not sufficient for catalysis and the presence of free Mg^{2+} is essential.

Another possible interpretation of the data in Fig. 5*c* is that ATP may be an inhibitor competing with $MgATP^{2-}$, the true substrate of the reaction. Thus removal of the free ATP through Mg^{2+} chelation results in the apparent activation by Mg^{2+}. However the present data suggest that this is unlikely since the observed Mg^{2+} requirement is such that enzyme activation is not correlated with the decrease of free ATP. At low Mg^{2+} concentrations, GMP synthetase is rather inactive even when the ratio of free ATP to $MgATP^{2-}$ is low. For example, at 0.4 mM free Mg^{2+} when free ATP is 5 μM and $MgATP^{2-}$ is 95 μM, the enzyme activity is only 10% of maximum (see *arrow* in Fig. 5*b*).

Previously, a mathematical expression has been derived for a situation when free ATP binds to the catalytic site where $MgATP^{2-}$ binds and forms an abortive complex (Ref. 27; see Equations 6 and 7 under "Experimental Procedures"). Equation 7 predicts that if ATP is a competitive inhibitor toward $MgATP^{2-}$, a double-reciprocal plot of initial velocity against free Mg^{2+} concentration, as a function of $MgATP^{2-}$ concentration, would result in a parallel pattern (28). To investigate the effect of free ATP on GMP synthetase, initial velocity was measured at several $MgATP^{2-}$ concentrations while varying

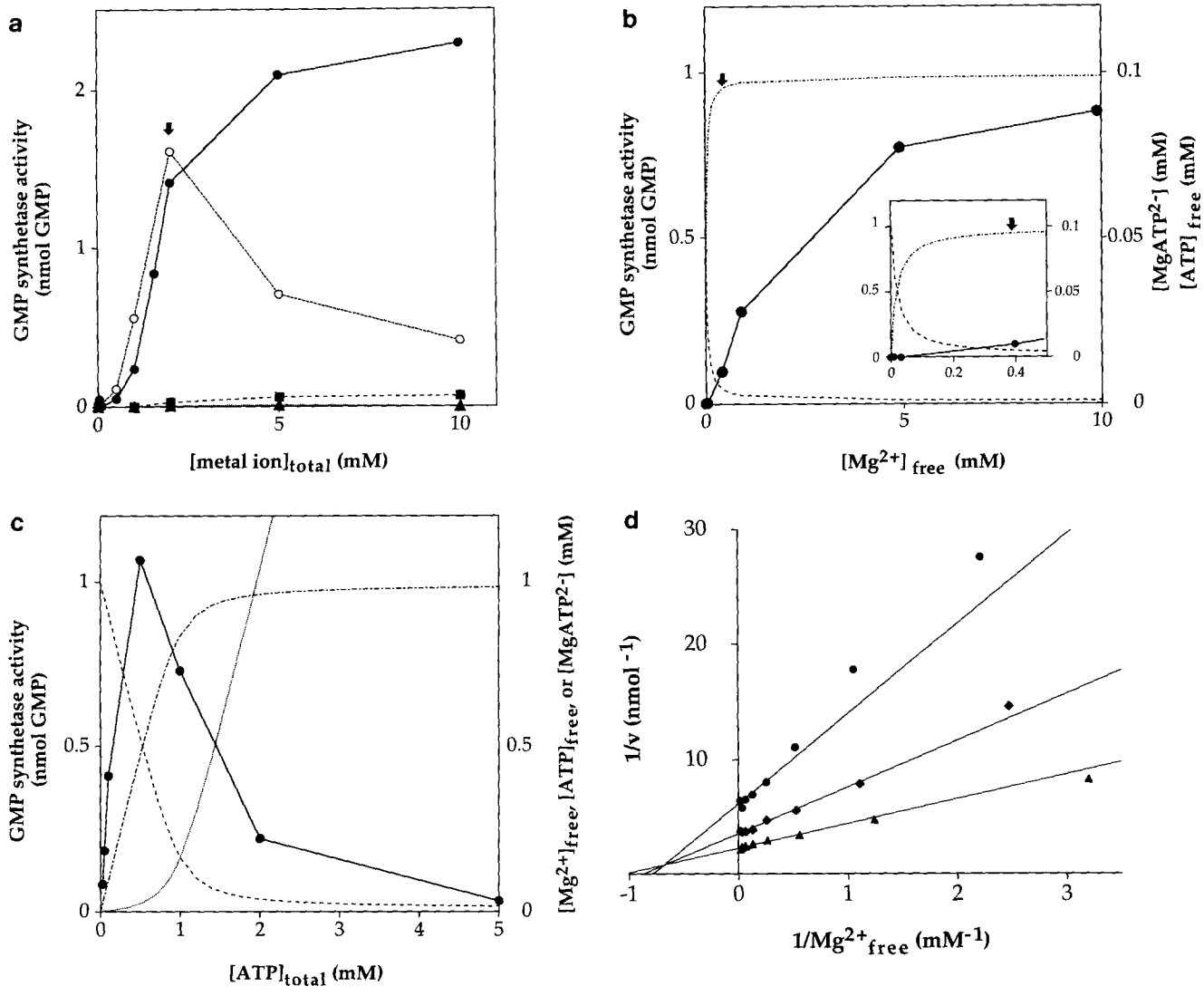

FIG. 5. **Dependence on metal ions.** GMP synthetase activity was assayed as described under "Experimental Procedures" except that concentrations of ATP and magnesium chloride were modified as indicated. The enzyme activity is represented by the amount of GMP formed in 10 min by 70 ng of GMP synthetase at 40 °C and pH 7.8. *a*, ATP was present at 2 mM (total) with the following metal ions: Mg^{2+} (●), Mn^{2+} (○), Zn^{2+} (▲), and Ca^{2+} (■). *b*, total ATP concentration was 0.1 mM. The maximum rate of product formation under these conditions is 1 nmol/10 min. Enzyme activity is represented by ——●——; the concentrations of $MgATP^{2-}$ (–·–·–) and free ATP (– – –) were calculated as described under "Experimental Procedures." The *arrow* points to 95 μM $MgATP^{2-}$ and 5 μM free ATP. The *inset* represents an expansion of the lower Mg^{2+} concentration data. *c*, total Mg^{2+} concentration was 1 mM. Enzyme activity is represented by ——●——. The concentrations of free Mg^{2+} (– – –), free ATP (· · ·), and $MgATP^{2-}$ (·–·–·) were calculated as described under "Experimental Procedures." *d*, initial velocity was measured in the presence of 0.049 mM (●), 0.099 mM (♦), and 0.198 mM (▲) $MgATP^{2-}$.

Mg^{2+} concentrations; Fig. 5*d* clearly shows that the initial velocity data of GMP synthetase exhibits not a parallel, but an intersecting, pattern. These results are inconsistent with the competitive inhibition by free ATP as the major reason for the activation by Mg^{2+}. So the observed inhibition by ATP is likely to be caused by ATP diminishing the free Mg^{2+}, which is essential for GMP synthetase activity.

Substrate Recognition of GMP Synthetase—The selectivity toward substrate was examined by substituting each substrate with corresponding analogs. We found that GMP synthetase was highly specific in terms of substrate recognition. ATP analogs such as GTP, 2′-dATP, 3′-dATP, 2′,3′-ddATP, AMP-CPP, AMP-PCP, AMP-PNP, ATPγS and ATPαS were tested for their ability to replace ATP in the formation of GMP. None of the above are active as substrate except ATPγS. The rate of GMP formation with ATPγS is 10% that of ATP (2 mM each). Three of these analogs, 3′-dATP, AMP-PNP, and ATPγS are somewhat active as inhibitors, whereas the remaining ones show less than 30% inhibition at 2 mM (Table I). Several glutamine analogs were also examined. D-Glutamine and the L-isomers of asparagine, homoglutamine, citrulline, ornithine, and methionine sulfoximine cannot provide an amino group for the formation of GMP and none inhibit the enzyme up to 5 mM. Another glutamine analog, glutamic acid-γ-methyl ester, is a competitive inhibitor toward glutamine (Table I). IMP is a poor inhibitor, causing less than 15% inhibition at 2 mM. These results show that GMP synthetase has very stringent requirements for substrate recognition and binding of small molecule substrate analogs.

Next the inhibition by products was examined (Table I). Inorganic pyrophosphate is the most effective product inhibitor and it is purely competitive toward ATP. The inhibition of inorganic pyrophosphate is not due to the depletion of $MgATP^{2-}$ caused by the chelation of Mg^{2+}. On the contrary, AMP is a poor product inhibitor, and the inhibition is mixed type toward ATP. Glutamic acid is virtually inactive as an

TABLE I
Inhibition of GMP synthetase by products and substrate analogs

GMP synthetase activity was determined by measuring [^{14}C]GMP formation as described under "Experimental Procedures." For the data presented in the column "% inhibition," initial velocity was measured with 0.2 mM XMP, 0.3 mM ATP, and 0.8 mM glutamine. The initial velocity under these conditions was approximately 90% of maximum velocity. Each compound was present at 2 mM except for glutamic acid γ-methyl ester (Glu-OCH_3) which was present at 5 mM. For the determination of inhibition constants (K_i), initial velocity was measured at saturating substrate concentrations except for the one that was varying (see legend of Fig. 3). Each inhibitor was present at four concentrations ranging from 0 to 5 times the K_i value.

Compound	Inhibition	Varying substrate	K_i	Mode
	%			
None	0			
3′-dATP	59.1	ATP	213 ± 112 μM	Competitive
AMP-PNP	73.2	ATP	299 ± 68 μM	Competitive
ATPγS	43.3		Not determined	
AMP	26.1	ATP	19.8 ± 4.4 mM (K_{is}) 131 ± 43 mM (K_{ii})	Mixed type
PP_i	Not determined	ATP	53.0 ± 7.8 μM	Competitive
Glu-OCH_3	41.8	Glutamine	3.7 ± 0.4 mM	Competitive
GMP	59.7	XMP	170 ± 38 μM	Competitive

inhibitor, whereas GMP inhibits relatively effectively.

Inhibition by Nucleoside Analogs—Decoyinine and psicofuranine are two adenosine antibiotics that selectively inhibit GMP synthetase (29). To investigate the structural requirements for selective inhibition, a panel of nucleoside analogs were tested. Several purine nucleosides were examined and these included adenosine, guanosine, xanthosine, inosine, purine riboside, 6-chloropurine riboside, and 6-mercaptopurine riboside. Several pyrimidine nucleosides, cytidine, uridine, thymidine, and orotidine, were also tested. Among these nucleosides, adenosine is the only active inhibitor, whereas all the others inhibit less than 5% up to 1 mM (Table II). The dramatic selectivity toward adenosine lead us to examine additional adenosine analogs. We tested vidarabine, 2′-deoxyadenosine, 3′-deoxyadenosine, and 5′-deoxyadenosine, analogs modified at the ribose moiety. We also tested formycin A and 2-chloroadenosine, analogs modified at the adenine ring, as well as adenine. Adenine is not active; only 5′-deoxyadenosine and 2-chloro-adenosine are active (Table II). These results suggest that the interaction at the decoyinine binding site of GMP synthetase requires a core adenosine-like structure, whereas modifications at the 2-position of the purine ring and the 1′- and 5′-positions of the ribose can be tolerated.

Inhibition of *E. coli* GMP synthetase by psicofuranine appears to be dependent on XMP, Mg^{2+}, and pyrophosphate, and it has been described as essentially irreversible (30). We found the inhibition of human GMP synthetase by decoyinine and the other adenosine analogs to be fully reversible and not dependent on any substrate or products.

TABLE II
Inhibition of GMP synthetase by nucleoside analogs

IC_{50} values (concentration of inhibitor at 50% inhibition) were determined as described under "Experimental Procedures." Initial velocity was measured in the presence of 10% dimethyl sulfoxide with 20 μM XMP, 40 μM ATP, and 200 μM glutamine.

Compound	IC_{50}
	μM
Decoyinine	17.3 ± 1.5
Psicofuranine	46.5 ± 3.2
Adenosine	408 ± 99
5′-Deoxyadenosine	268 ± 22
2-Chloro-adenosine	191 ± 34

DISCUSSION

GMP synthetase is a glutamine-dependent amidotransferase that uses ATP as the driving force generating AMP and inorganic pyrophosphate. The affinity of the human enzyme toward each substrate is such that the intracellular rate of GMP formation should not be limited by ATP or glutamine, but rather be dependent on the level of XMP, since XMP is generally present at extremely low levels in cells. The human enzyme can also use ammonia in place of glutamine, although the reaction is insignificant at physiological concentrations of ammonia. As expected for a key enzyme in a metobolic pathway, GMP synthetase has very stringent requirements for substrate recognition.

Only one form of GMP synthetase in *E. coli* (7) or Yoshida sarcoma ascites cells (19) has been described. However, human GMP synthetase exists as two distinct forms that have different isoelectric points. Since the human GMP synthetase is encoded by one gene and one message (18), it is unlikely that these forms represent isozymes of distinct gene products or splice variants. More importantly, the two forms of GMP synthetase were also observed when the cDNA of GMP synthetase was expressed in a baculovirus system.[4] The two forms do not represent different oligomeric states of the enzyme, since they are both monomers. There is also no evidence for the two to be reaction intermediates since they appear to be stable and not in equilibrium. It is likely that the two forms of GMP synthetase represent posttranslational modification variants. In spite of their difference in net charge, the two forms of enzymes are catalytically similar. Nothing is known about their regulatory properties and this topic is currently being investigated.

Examination of the metal ion dependence reveals that Mg^{2+} is an activator of GMP synthetase. The half-saturation value for Mg^{2+} is close to 2 mM, regardless of whether ATP is at 0.1 or 2 mM. The Mg^{2+} activation appears to be essential for enzyme activity, since the complete chelation of free Mg^{2+} by ATP results in the complete inactivation of the enzyme (Fig. 5c). If Mg^{2+} is not essential, the activity is expected to decrease to a certain plateau but not diminished completely. The decrease in enzyme activity parallels the depletion of free Mg^{2+}, not the increase of free ATP. It is unlikely that the observed requirement for free Mg^{2+} is due to inhibition by free ATP, since the activation of enzyme activity is not correlated with the decrease of free ATP (Fig. 5*b*). Furthermore, the initial velocity pattern in Fig. 5*d* suggests that any contribution of ATP inhibition to the observed Mg^{2+} activation, if exists at all, would be minor. Our results show that there may be more than one Mg^{2+} binding site, although it is not clear whether binding at one site augments the binding at subsequent site(s). Multiple Mg^{2+} sites has not been described for GMP synthetases from other sources.

[4] L. Lou, J. Nakamura, S. Tsing, B. Nguyen, J. Chow, K. Straub, H. Chan, and J. Barnett, unpublished data.

Interaction of the enzyme with XMP exhibits sigmoidal kinetics (Fig. 3). This phenomenon has not been reported for any bacterial or mammalian GMP synthetase. The apparent positive cooperativity is observed, and the Hill coefficient remains the same when activity is measured by [^{3}H]AMP formation, [^{14}C]glutamine formation, or when ATP or glutamine is not saturating (twice the respective K_m value). Previously, it was shown that CTP synthetase, a G-type amidotransferase involved in pyrimidine nucleotide biosynthesis, undergoes nucleotide-induced tetramerization that leads to cooperativity in substrate binding (31). Unlike CTP synthetase, the sigmoidal kinetics of GMP synthetase with XMP cannot be explained by substrate-induced oligomerization. The presence of saturating levels of XMP, either alone or in combination with ATP or glutamine, did not affect the monomeric composition of GMP synthetase.[4] The possible existence of multiple XMP binding sites in a monomeric GMP synthetase is currently under investigation.

Inhibition by decoyinine exhibits uncompetitive kinetics toward XMP and glutamine and noncompetitive toward ATP (Fig. 4). These results suggest that decoyinine binding to GMP synthetase occurs after the binding of XMP and glutamine. Decoyinine may bind to the enzyme either before or after ATP binds. Previously, it was proposed for the *E. coli* GMP synthetase that ATP was the first substrate to bind, followed by XMP and then glutamine (10). The current decoyinine inhibition data with the human enzyme are not entirely consistent with this proposal. According to the above order of substrate binding, if decoyinine binds after XMP and glutamine, then one would predict the inhibition to be uncompetitive toward ATP as well. We are currently pursuing the order of substrate binding for the human enzyme.

Our studies of GMP synthetase reveal some similarities between the enzyme from human and from other sources. More interestingly, we have identified some features that have not been described for any bacteria or mammalian GMP synthetases. These findings serve as a foundation to further investigate the mechanisms of enzyme catalysis and regulation. The knowledge concerning the human enzyme provides crucial information toward the search for immunosuppressive drugs.

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