The Glutamine Hydrolysis Function of Human GMP Synthetase

IDENTIFICATION OF AN ESSENTIAL ACTIVE SITE CYSTEINE*

(Received for publication, June 7, 1995, and in revised form, July 31, 1995)

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GMP synthetase (EC 6.3.5.2) is an amidotransferase that catalyzes the amination of xanthosine 5'-monophosphate to form GMP in the presence of glutamine and ATP. Glutamine hydrolysis produces the necessary amino group while ATP hydrolysis drives the reaction. Ammonia can also serve as an amino group donor. GMP synthetase contains two functional domains, which are well coordinated. The “glutamine amide transfer” or glutaminase domain is responsible for glutamine hydrolysis. The synthetase domain is responsible for ATP hydrolysis and GMP formation. Inorganic pyrophosphate inhibits the synthetase and uncouples the two domain functions by allowing glutamine hydrolysis to take place in the absence of ATP hydrolysis or GMP formation. Acivicin, a glutamine analog, selectively abolishes the glutaminase activity. It inhibits the synthetase activity only when glutamine is the amino donor. When ammonia is used in place of glutamine, acivicin has no effect on the synthetase activity. Acivicin inhibits GMP synthetase irreversibly by covalent modification. Enzyme inactivation is greatly facilitated by the presence of substrates. Acivicin labels GMP synthetase at a single site, and a tryptic peptide containing the modified residue was isolated. Mass spectrometry and Edman sequence analysis show that Cys104 is the site of modification. This residue is conserved among GMP synthetases and is located within a predicted glutamine amide transfer domain. These data suggest that Cys104 is an essential residue involved in the hydrolysis of glutamine to produce an amino group and is not needed for the hydrolysis of ATP or amination of xanthosine 5'-monophosphate to produce GMP.

The amidotransferases are a group of biosynthetic enzymes that incorporate nitrogen atoms into a variety of metabolites. These enzymes are constructed with two domains of distinct functions. The GAT function (or glutaminase) is responsible for utilizing glutamine to produce the nitrogen source. The synthetase function is responsible for using the nitrogen source produced from glutamine hydrolysis in the amination of the nitrogen-accepting metabolite (for a review of amidotransferases, see Ref. 1).

GMP synthetase is a member of the G-type amidotransferases that catalyzes the amination of XMP to produce GMP in the de novo synthesis of guanine nucleotides. The designation of “G-type” is based on sequence similarities with the trpG-encoded anthranilate synthase component I. Similar to other family members, GMP synthetase can utilize either ammonia or glutamine as a source of nitrogen (2-5). During catalysis, glutamine is hydrolyzed to generate the amino group needed for the amination reaction. In the presence of magnesium ion, ATP drives the reaction. GMP synthetase is unique among the G-type amidotransferases in that it hydrolyzes ATP to AMP and inorganic pyrophosphate. The other ATP-utilizing family members produce ADP and inorganic phosphate.

The GAT domains of different G-type amidotransferases share significant sequence similarities. There are a number of residues within this domain that are absolutely conserved in all family members and across species. Among these invariant residues is a cysteine in a consensus sequence: PXXG(I/V)/C/L/YG(H/M)/Q, where X represents variable hydrophobic residues. This cysteine residue is thought to be important in catalysis for two prokaryotic amidotransferases, carbamyl phosphate synthetase and anthranilate synthetase (Ref. 1 and references therein).

GMP synthetases from both prokaryotic and eukaryotic sources contain a conserved cysteine within the GAT domain. In human, this cysteine is at position 104 of the predicted sequence (6). The role for the conserved cysteine has not yet been clearly defined for GMP synthetase from any source. For the Escherichia coli enzyme, iodoacetamide inhibits the enzyme activity by cysteine modification (7). However, the bulk of the iodoacetamide alkylation was not at the conserved cysteine (Cys104) but rather a cysteine residue (Cys163) not known to be catalytically important (8). The sequence surrounding Cys163 has no similarity with other GMP synthetases or any amidotransferases. It is unknown whether the Cys163 is important for the function of the GAT domain.

GMP synthetase is particularly important as a potential target for anticancer and immunosuppressive therapies (6, 9, 10). An understanding of the active site topography of a target enzyme from human is crucial for designing potent and selective drug candidates. Recently, human GMP synthetase has been purified and characterized (5, 6). Here, we report on the glutamine binding and hydrolyzing functions of this enzyme. We show that the glutamine hydrolysis can be uncoupled from GMP synthesis, thus distinguishing the GAT and synthetase functions of the enzyme. An affinity probe specific for the glutamine site was used to label an active site residue. Biochemical and physical evidence are provided that identify the conserved cysteine (Cys104) as an active site residue essential for the GAT function of human GMP synthetase.

EXPERIMENTAL PROCEDURES

Materials—Human GMP synthetase was purified according to procedures previously described (6). The enzyme was obtained from either...
Covalent Modification of Cys\textsuperscript{104} by Acivicin

**RESULTS**

Glutamine Hydrolyzing Activity of GMP Synthetase—Glutamine hydrolysis was used as an index to monitor the GAT function of GMP synthetase. The glutaminase activity of GMP synthetase is not constitutively active. Glutamine hydrolysis requires the presence of all substrates: Mg\textsuperscript{2+}, ATP, and XMP. Omitting any one of the substrates results in a dramatic loss of the glutaminase activity (Table I). Substituting XMP with GMP, or ATP with AMP, or ATP with a non-hydrolyzable analog AMP-PNP, does not support glutamine hydrolysis. However, the ATP requirement can be fulfilled by inorganic pyrophosphate, a product of ATP hydrolysis. In fact, pyrophosphate is twice as active as ATP in stimulating the glutaminase activity. It appears that the requirement for ATP is not dependent on the hydrolytic event per se but rather on the presence of a product of hydrolysis. These results indicate that the occupation of the Mg\textsuperscript{2+}, pyrophosphate, and XMP-binding sites is necessary and sufficient to stimulate glutamine hydrolysis. Pyrophosphate, a competitive inhibitor toward ATP, can uncouple glutamine hydrolysis from GMP formation, since the latter absolutely requires ATP hydrolysis.

Inhibition of GMP Synthetase by Glutamine Analogs—To understand the GAT function of GMP synthetase and to identify key residue(s) involved in catalysis, we probed the GAT domain with a site-specific affinity reagent. Acivicin is a glutamine analog that inactivates several amidotransferases by covalent modification of Cys\textsuperscript{104} by acivicin.

**Table I**

| Sample | Glutamic acid formation (fractional activity) |
|--------|---------------------------------------------|
| Complete reaction | 1.00 |
| Omit XMP | 0.01 |
| Omit Mg\textsuperscript{2+} | 0.04 |
| Omit ATP | 0.00 |
| Omit XMP, add GMP | 0.00 |
| Omit ATP, add AMP and PP \textsuperscript{a} | 1.92 ± 0.23 |
| Omit ATP, add AMP | 0.00 |
| Omit ATP, add PP \textsuperscript{a} | 1.98 ± 0.17 |
| Omit ATP, add PP, and EDTA | 0.06 |
| Omit ATP, add AMP-PNP | 0.04 |

Glutaminease activity was measured using the standard concentrations of substrates as described under “Experimental Procedures.” Each added compound was present at 2 mM. The values are reported as the average of four experiments or the average ± S.E. AMP-PNP, β,γimidoadenosine 5\textsuperscript{′}-triphosphate.

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\( E_{\text{cat}} \) of \(-15 \text{ to } -30 \text{ eV} \). Edman microsequencing was carried out using standard methods on a ABI model 421 sequencer (Applied Biosystems). These data allowed the assignment of all of the major components observed in the tryptic digest of GMP synthetase and accounted for approximately 96% of the predicted sequence of the enzyme.

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**Generation of Tryptic Peptides and Sequence Analysis**—Samples of pure GMP synthetase (40 μg at 3.3 μM) were treated with acivicin (6.6 μM) in the presence of standard concentrations of Mg\textsuperscript{2+}, ATP, and XMP. After 30 min at 40 °C, the unbound small molecules were removed from the protein by gel filtration chromatography using NICK columns (pre-packed G-50 Sephadex, Pharmacia Biotech Inc.). GMP synthetase activity was assayed immediately to confirm the inactivation. Control samples were treated with the identical procedures except that acivicin was omitted in the initial incubation. Protein samples were reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin as described (12). Tryptic peptides were separated, and their molecular weights were determined by on-line HPLC-mass spectrometry as described below. The identities of peptides were verified by sequencing experiments using tandem mass spectrometry analysis and Edman microsequencing.

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On-line HPLC-mass spectrometry was carried out using a microspraying pump (ABI model 140B; Applied Biosystems) interfaced to a triple stage quadrupole mass spectrometer (TSQ700; Finnigan-MAT, San Jose, CA) equipped with an electrospray ionization source. Separations were carried out on a Vydac C4 column (2.1 × 150 mm) using a linear gradient consisting of solvents A (0.1% trifluoroacetic acid in water) and B (0.1% trifluoroacetic acid in 80:20 acetonitrile:water) as follows: 0–10 min, 2% B; 10–140 min, linear gradient to 100% B. The flow rate was 100 μl/min. The column effluent was split 50:1, and 2 μl/min was directed to the mass spectrometer. The major portion of the column effluent was directed to a fraction collector, and peptides in each fraction were subsequently analyzed by tandem mass spectrometry and by Edman microsequencing. Tandem mass spectrometry was performed by using collision-assisted dissociation of the multiply charged ions, using argon as a collision gas at pressures of 2–3 millitorr and energies \( E_{\text{col}} \) of \(-15 \text{ to } -30 \text{ eV} \). Edman microsequencing was carried out using standard methods on a ABI model 421 sequencer (Applied Biosystems). These data allowed the assignment of all of the major components observed in the tryptic digest of GMP synthetase and accounted for approximately 96% of the predicted sequence of the enzyme.

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**Determination of Inhibition Constants**—Inhibition constants were calculated by non-linear regression fit of the initial velocity data to appropriate equations using the Systat computer software.

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FIG. 1. Structure of acivicin.

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A native (A3.01 cells, T lymphoblastoma) or a recombinant source (baculovirus-infected insect cells). Since the native and recombinant enzymes were nearly identical in kinetic and biochemical properties (11), the two were used interchangeably. Acivicin (3-amino-3-deoxy-4,5-dihydro-5-isoxazoleacetic acid, NCS 163501), azaserine (O-diazooacetyl-l-serine, NSC 742), and DON (6-diazo-5-oxo-l-norleucine, NSC 7365) were purchased from Sigma (see Fig. 1 for structure of acivicin).

**Enzyme Activity Assay—**GMP synthetase activity was measured by \[^{14}C\]GMP formation from \[^{14}C\]XMP. When specified, enzyme activity was also measured by \[^{14}C\]glutamic acid formation from \[^{14}C\]glutamine, \[^{14}C\]AMP formation from \[^{14}C\]ATP, or AMP formation in a spectrophotometrically coupled assay. All of these procedures have been previously described, and the initial velocity determined by these procedures is virtually identical (5, 6). In general, initial velocity was measured with “standard” concentration of each substrate at pH 7.8, 40 °C for 4 min. “Standard” concentration is defined as 10 mM MgCl\textsubscript{2}, 2 mM ATP, 1 mM XMP, and 5 mM glutamine (10–20 times the \( K_{m} \) value of each). GMP synthetase was present at 40 nM unless specified.

**Preincubation with Acivicin—**To access the effect of acivicin treatment on enzyme activity, GMP synthetase was incubated with the inhibitor at 40 °C in a preincubation reaction prior to the enzyme activity assay. During the preincubation, GMP synthetase was present at 0.4 μM and the other components at concentrations and for time periods described in the legend of each figure. At the end of the preincubation periods, GMP synthetase activity was measured in a separate activity assay as described in the above paragraph. During the activity assay, all components in the preincubation mixture were 10 times less concentrated, unless specified.

**Glutaminase activity** was determined by the standard method on an ABI model 421 sequencer (Applied Biosystems). Determination of inhibition constants—Inhibition constants were calculated by non-linear regression fit of the initial velocity data to appropriate equations using the Systat computer software.
pounds are much less potent than acivicin, and acivicin was selected to be characterized further.

Acivicin Inhibition of GMP Synthetase—Fig. 3 shows that the inhibition of GMP synthetase by acivicin is a time-dependent reaction. The rate of inactivation is dramatically accelerated when all substrates are present. Omitting Mg\(^{2+}\), ATP, or XMP or replacing XMP with GMP eliminates this acceleration (data not shown). However, omitting glutamine further accelerates the rate of inactivation, suggesting that glutamine provides protection against acivicin. Similar to the glutaminase activity, pyrophosphate can replace ATP in accelerating the inactivation. The inactivation of GMP synthetase by acivicin is most efficient when Mg\(^{2+}\), pyrophosphate (or ATP), and XMP are present. This group of ligands also represents the requirement for the stimulation of the glutaminase activity. Thus, both glutamine and acivicin may interact with the same enzyme conformation that is conferred by the binding of XMP, Mg\(^{2+}\), and pyrophosphate (or ATP). Inhibition by acivicin is likely to involve the same elements as the glutaminase activity.

Acivicin inhibits the glutaminase activity and also inhibits the synthetase function when the only nitrogen source is glutamine. Interestingly, the effect of acivicin is dramatically different when glutamine is replaced by ammonium chloride. Whereas GMP formation in the presence of glutamine can be completely inhibited, the reaction in the presence of ammonia is not affected at all by acivicin (Fig. 4). Similar to GMP formation, ATP hydrolysis in the presence of ammonia is also not inhibited by acivicin (data not shown). These data show that acivicin inhibition interferes only with the glutaminase activity in releasing the amino group. As long as a source of the amino group is available, such as in the presence of ammonia, the synthetase activity of GMP synthetase is not affected by acivicin. But when the amino group release by glutamine hydrolysis is blocked by acivicin, the synthetase activity is also prevented. These results clearly distinguish the two domain functions. Furthermore, they also demonstrate that the two are well coordinated.

Acivicin inhibition of GMP synthetase is not readily reversible. Enzyme activity is not recovered by dilution or by gel filtration chromatography. These data are consistent with acivicin covalently modifying GMP synthetase at the glutamine binding site.

Kinetics of Acivicin Inhibition—Covalent modification of an enzyme often proceeds via a two-step process of initial formation of an enzyme-inhibitor complex (non-covalent) followed by inactivation (covalent). Equation 1 describes the relationship (17):

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K_i / [E] + K_{II} / [E] + K_{III} / [E] = K_i + K_{II} + K_{III}
\]
For the inhibition of GMP synthetase by acivicin, pseudo-first order kinetics is observed for the first 70–80% inactivation (Fig. 5a). From these data, the half-time of inactivation (t½) can be determined. Kinetically, t = ln 2/kobs, where kobs represents the first order rate constant. However, an examination of kobs as a function of [I] reveals that within the acivicin concentrations that were tested, saturation kinetics was not observed (Fig. 5b). Since kobs = kobs * [I]/(K_i + [I]), and kobs versus [I] shows a linear relationship, it is likely that K_i is much larger than 90 μM, the highest [I] tested. This estimate is somewhat consistent with the result described above that without preincubation, acivicin inhibits GMP synthetase with an IC_{50} of 178 μM. Based on this assumption, under the conditions tested, fractional activity = e^{-k_{inact} * [I] / K_i} * t (Eq. 1)

where the term k_{inact}/K_i represents a second order rate constant. For the inactivation of GMP synthetase by acivicin, this value is estimated to be 31.9 ± 1.3 min⁻¹ M⁻¹.

Modification of Cys^{104} by Acivicin—The stoichiometry of acivicin inactivation was examined by titrating GMP synthetase with limiting amounts of inhibitor. Complete inactivation can be extrapolated to approximately 0.8 acivicin per GMP synthetase monomer (Fig. 6).

A comparison of the tryptic digestion patterns reveals that the control and the inhibited samples were essentially identical except for one specific peptide, designated as T9 in the control (Fig. 7). Acivicin inhibition leads to the disappearance of the T9 peptide and the concomitant appearance of a T9a peptide. Based on molecular weight analysis and Edman microsequencing, both T9 and T9a correspond to residues 71 to 112, which reside within the predicted GAT domain of human GMP synthetase. This stretch of sequence contains a highly conserved region of the GAT domain including the invariant cysteine residue at position 104, which is the only cysteine residue in this sequence.

The observed molecular weight of T9 is 4574.1 ± 1.0, and that of T9a is 4659.0 ± 1.5. The former is in good agreement with the predicted mass (M_r = 4,575) of a peptide spanning residues 71–112 that contains a single cysteine that has undergone modification by iodoacetamide. The difference in the observed molecular weights between T9 and T9a corresponds exactly to the difference in mass between iodoacetamide and acivicin, indicating that in T9a, the iodoacetamide modification...
is replaced by acivicin. The observed molecular weights of T9a agrees with the predicted weight of residues 71–112 labeled with acivicin ($M_r$ 54,660).

Edman microsequencing of both T9 and T9a confirmed the identities of residues 71 through 90. Since this subsequence contains a single cysteine residue and the mass difference between the two peptides corresponds to substitution of an acetamide by an acivicin moiety, these data strongly suggest that the site of acivicin modification is Cys104. Fig. 8 shows the proposed structures of T9 and T9a, where Cys104 in T9 is modified by iodoacetamide and that in T9a is modified by acivicin.

The appearance of T9a is well correlated with the disappearance of T9. More importantly, it is also well correlated with the time dependence of GMP synthetase inactivation (Fig. 9). These results show that the modification of Cys104 is the cause of the inhibition of GMP synthetase by acivicin.

**DISCUSSION**

The GAT domain of human GMP synthetase has been characterized and probed with glutamine affinity analogs. Since acivicin is the most potent of the analogs tested, it was selected as a tool to dissect the enzymatic activity of GMP synthetase. Through the action of acivicin, the synthetase and glutaminase functions of GMP synthetase are clearly distinguished since ATP hydrolysis and GMP formation can take place in the absence of glutamine hydrolysis. Acivicin fits the classical definition of a syncatalytic modifier, where it is an affinity substrate analog whose action is facilitated by substrate turnover (18).

Enzyme inactivation by acivicin is complete and stoichiometric. Our data strongly suggest that the site of acivicin modification is Cys104, the conserved cysteine in GAT domain of the human sequence. This is the only modification detected, and it correlates well with the inactivation of enzyme activity (Fig. 9). These results demonstrate the catalytic importance of the conserved cysteine residue and are consistent with Cys104 being essential for the GAT function of human GMP synthetase. Modification of Cys104 impairs the catalytic ability to release the amino group from glutamine but without any interference with theamination of XMP. Acivicin inactivation appears to be highly selective toward the glutaminase function of GMP synthetase while leaving the synthetase function unaltered (Fig. 4). These results are evidence for not only a functional but also a physical distinction between the GAT and the synthetase domains of GMP synthetase, since the addition of a bulk selectively abolishes the former without affecting the latter.
An interesting feature of acivicin inhibition is its robust requirement for Mg\(^{2+}\), ATP (or pyrophosphate), and XMP. If any one of these ligands is missing, acivicin is rather inactive toward the enzyme. This could be an indication that Cys\(^{104}\) is dormant in a naïve enzyme not undergoing catalysis. However, in the presence of substrates, the sulfhydryl group of Cys\(^{104}\) becomes a highly reactive nucleophile. In the case of acivicin modification, the enzyme cysteine sulfhydryl group attacks carbon 3 in the isoxazole ring of acivicin and displaces chloride.

“Activation” of Cys\(^{104}\) is likely to involve a change in enzyme conformation. It is intriguing that the same substrate requirement for acivicin inactivation is also observed for glutamine hydrolysis. These results suggest that the reaction toward acivicin and glutamine may require the same enzyme conformation, which can be induced by the occupation of the Mg\(^{2+}\) and XMP binding sites and the pyrophosphate portion of the ATP binding site.

There are likely to be common elements in the catalytic site that are involved in both acivicin inhibition and glutamine hydrolysis. Cys\(^{104}\) could be such an element. Previous studies on other amidotransferases, CTP synthetase (19) and formylglycinamid ribonucleotide amidotransferase (20), have demonstrated the formation of a glutamyl-enzyme complex during catalysis where the glutamyl group is covalently bound via a thioester linkage. A catalytic cysteine is thought to serve as a docking site for the glutamyl group during the hydrolysis of glutamine to release the amino group. Although there is no direct evidence that the conserved cysteine in any amidotransferase is the attachment site for the glutamyl group, Cys\(^{104}\) in the human GMP synthetase is a reasonable candidate. Since it appears that Cys\(^{104}\) becomes highly reactive upon the binding of substrates, it is possible that the Cys\(^{104}\) sulfhydryl participates in the glutamine hydrolysis as a nucleophile releasing the amino group, resulting in the formation of a γ-glutamyl thioester-enzyme complex.

Two functional domains of GMP synthetase are responsible for catalyzing three reactions. The GAT domain is involved in hydrolyzing glutamine and releasing the amino group. The synthetase domain is involved in utilizing ATP hydrolysis to drive the amination of XMP. The involvement of ATP was convincingly shown to be linked to the formation of an adenyl-XMP intermediate during catalysis (21, 22). The displacement of the adenyl group at the C2 position of XMP by an amino group releases AMP and subsequently forms GMP. Presumably, the source of the amino group can be either glutamine or ammonia. Our data show that acivicin inhibits all three functions of GMP synthetase, namely glutamine hydrolysis, ATP hydrolysis, and GMP formation when glutamine is the only amino source (Fig. 4). However, when ammonia replaces glutamine as the amino source, neither ATP hydrolysis or GMP formation is affected by acivicin (Fig. 4). Since the formation of AMP is linked to the formation of the adenyl-XMP intermediate, these results imply that adenyl-XMP will form regardless of whether the glutaminase is functional. Conversely, glutamine hydrolysis will occur regardless of whether the adenyl-XMP intermediate can be formed, as long as certain pockets of the nucleotide binding sites are occupied. The binding of Mg\(^{2+}\), pyrophosphate, and XMP allows Cys\(^{104}\) to be involved in catalysis in the absence of adenyl-XMP formation. Since pyrophosphate is normally not present in cells but is produced from ATP hydrolysis during catalysis, our data suggest that the formation of adenyl-XMP may trigger glutamine hydrolysis.

In summary, our data show that although the two domains of GMP synthetase are distinct in functional characteristics, the activities of the two domains are well coordinated. Acivicin is a useful tool for identifying an essential active site residue and for elucidating the catalytic mechanism of GMP synthetase.

Acknowledgments—We thank Dr. Kurt Jarnigan and Natalie Saldou for peptide sequencing and Dr. Jim Barnett and assistants in the Protein Production Group for preparing the recombinant GMP synthetase.

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