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Ammonia Channeling in *Plasmodium falciparum* GMP Synthetase: Investigation by NMR Spectroscopy and Biochemical Assays

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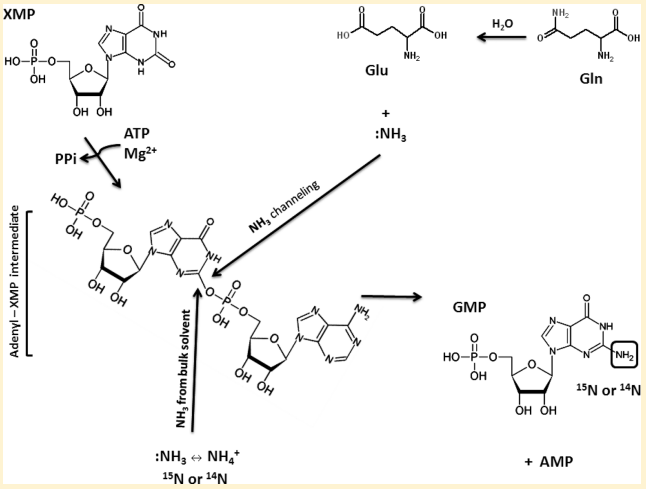
Supporting Information

ABSTRACT: GMP synthetase, a class I amidotransferase, catalyzes the last step of the purine biosynthetic pathway, where ammonia from glutamine is incorporated into xanthosine 5′-monophospate to yield guanosine 5′-monnophosphate as the main product. Combined biochemical, structural, and computational studies of glutamine amidotransferases have revealed the existence of physically separate active sites connected by molecular tunnels that efficiently transfer ammonia from the glutaminase site to the synthetase site. Here, we have investigated aspects of ammonia channeling in *P. falciparum* GMP synthetase using biochemical assays in conjunction with ^{15}N-edited proton NMR spectroscopy. Our results suggest that (1) ammonia released from glutamine is not equilibrated with the external medium, (2) saturating concentrations of glutamine do not obliterate the incorporation of external ammonia into GMP, and (3) ammonia in the external medium can access the thioester intermediate when the ATPPase domain is bound to substrates. Further, mutation of Cys-102 to alanine confirmed its identity as the catalytic residue in the glutaminase domain, and ammonia-dependent assays on the mutant indicated glutamine to be a partial uncompetitive inhibitor of the enzyme.



Glutamine-dependent amidotransferases (GATs) are a family of enzymes that generate ammonia by the hydrolysis of glutamine and subsequently transfer it to a diverse set of acceptor molecules. These enzymes are modular in nature, with each domain associated with a specific catalytic function.[1−6] Crystal structures of many of the GATs have shown that the glutaminase and the acceptor domains are spatially separated, with the tunnel connecting the two active sites for ammonia transfer being structurally diverse in nature across the different members.[6,7] Significant understanding of ammonia channeling has come from structural, kinetic, and computational studies done on carbamoyl phosphate synthetase (CPS)[8−11] and imidazole glycerol phosphate synthase (IGP synthetase),[12−15] the two class I amidotransferases. Use of ^{15}N NMR spectroscopy and MD simulations followed by site-directed mutational analysis in CPS has provided insights into mechanistic details of ammonia transfer in this enzyme.[9−11] The crystal structure of asparagine synthetase B (AS-B), a class II amidotransferase, highlighted a solvent inaccessible tunnel operating between the two domains of the enzyme.[16] However, a recent kinetic study carried out on this enzyme using isotope-edited ^{1}H NMR spectroscopy has revealed that $^{15}NH_4^+$ provided in the external medium can enter the enzyme in the presence of glutamine.[17] In *Aquifex aeolicus* GatCAB, a Glu-tRNA^{Gln}/Asp-tRNA^{Asn} glutamine-dependent amidotransferase, it has been shown that a water-filled ammonia channel is open throughout the length between the glutaminase and the synthetase active sites.[18] A structurally unique intersubunit ammonia tunnel has recently been shown in *Mycobacterium tuberculosis* NAD^+ synthetase.[19]

Guanosine monophosphate synthetase (GMPS), a class I glutamine-dependent amidotransferase (E.C. 6.3.5.2), catalyzes the conversion of xanthosine 5′-monophosphate (XMP) to guanosine 5′-monophosphate (GMP) in the purine biosynthetic pathway. In this reaction a molecule of adenosine 5′-triphosphate (ATP) is utilized (Scheme 1). Glutamine or external ammonia serves as a nitrogen source in eukaryotic, prokaryotic, and archaeal GMP synthetases.[20−23] This enzyme, like other amidotransferases, consists of a glutaminase domain for the hydrolysis of glutamine and a synthetase or ATP pyrophosphatase (ATPPase) domain where GMP is produced from ATP, XMP,

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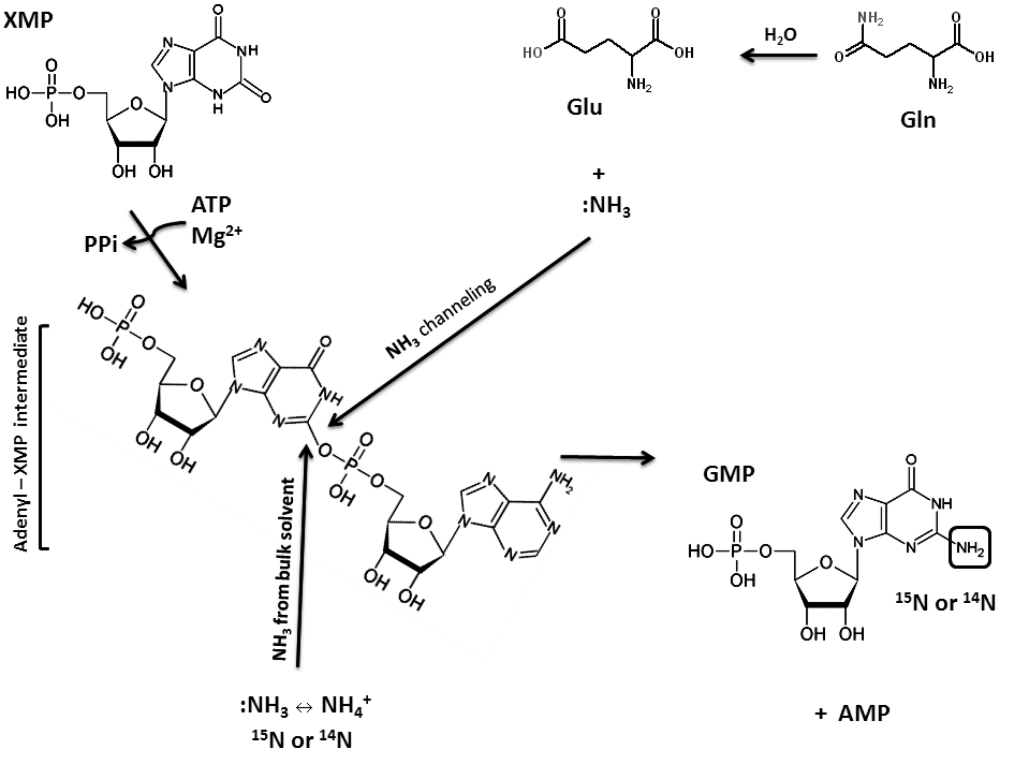
Scheme 1. Conversion of XMP to GMP by GMP Synthetase via the Formation of Adenyl-XMP Intermediate



and ammonia. In our previous report on *Plasmodium falciparum* GMP synthetase (PfGMPS), an essential enzyme for the parasite, it was shown that the enzyme has significant leaky glutaminase activity when the ATPPase domain is unliganded.[22] This is an exceptional example among two-domain-type GMP synthetases, as both *E. coli* and human enzymes have maintained tight regulation of glutaminase domain activity, thereby preventing any wasteful hydrolysis of glutamine in the absence of substrates bound to the ATPPase domain.[24,25] The focus of the present work is to understand ammonia channeling in PfGMPS, given that this enzyme has an unusual leaky glutaminase activity and that a detailed analysis on this aspect in GMP synthetases has not been previously carried out. The biochemical basis for the competition between free ammonia and ammonia from hydrolysis of glutamine for conversion of XMP to GMP has been examined. The fraction of GMP synthesized using ammonia from either source has been quantified using isotope-edited NMR spectroscopy. C102A mutant of PfGMPS that is devoid of glutaminase activity has been used to monitor the effect of glutamine binding on the utilization of external ammonia. The results of our studies are presented here.

MATERIALS AND METHODS

Restriction enzymes, DNA polymerases, and T_4 DNA ligase were either purchased from New England Biolabs or Bangalore Genei Pvt. Ltd., India, and were used according to the manufacturer's instructions. Primers for introducing point mutations were custom synthesized at Sigma-Aldrich. Media components were obtained from Himedia, India. XMP, ATP, $^{15}NH_4Cl$, $^{14}NH_4Cl$, and other biochemicals from Sigma-Aldrich were of the highest quality available. NMR tubes (5 mm internal diameter) and the matrices for MALDI-TOF mass spectrometry were purchased from Sigma-Aldich. All MALDI-TOF and LC-ESI MS spectra were acquired on Ultraflex II and ESI-Q-TOF mass spectrometers (Bruker Daltonics, Germany), respectively. All the PfGMPS activity assays were done on either Hitachi U-2010 or U-2810 spectrophotometers (Tokyo, Japan). CD spectra were recorded using a J-810 spectropolarimeter (Jasco Corp., Tokyo, Japan). Protein expression and purification protocols for PfGMPS were same as reported earlier.[22] Protein concentration was determined by Bradford assay[26] using bovine serum albumin as standard. Data fitting to different equations was done using GraphPad prism 5.

Assay for PfGMPS Activity. Activity of PfGMPS was monitored using assay conditions reported earlier.[22] Briefly, reaction rates were monitored as decrease in absorbance at 290 nm due to conversion of XMP (ε = 4800 M^{-1} cm^{-1}) to GMP (ε = 3300 M^{-1} cm^{-1}). A $\Delta\varepsilon$ value of 1500 M^{-1} cm^{-1} was used to calculate the amount of product formed. The standard assay consisted of 90 mM Tris HCl, pH 8.5, 150 μM XMP, 2 mM ATP, 5 mM glutamine, 20 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM DTT in a total reaction volume of 0.25 mL. Reactions were initiated with 0.37 μM of PfGMPS and monitored at 25 °C. Kinetic constants for ammonium were determined by varying the concentration of ammonium chloride from 4 to 100 mM, while keeping the other substrates at fixed saturating concentration. It should be noted that below pH 9.2 (the pK_a for $NH_3 \leftrightarrow NH_4^+$) ammonium ion is the predominant species.

pH Titration of Glutamine- and Ammonia-Dependent Activities. Assays to determine the effect of pH on the rate of conversion of XMP to GMP using either glutamine or ammonium chloride as the source of ammonia were carried out in buffers suitable for the pH under consideration. MES (2-[*N*-morpholino]ethanesulfonic acid) at pH 6.5 and Tris HCl and

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glycine in the pH ranges of 7.0−8.5 and 9.0−10.0, respectively, were used. At each individual pH, the concentration of either glutamine or ammonium chloride was varied while keeping the concentration of XMP, ATP and Mg^{2+} fixed. Data obtained at each pH were fitted to Michaelis−Menten equation for the determination of kinetic parameters k_{cat} and K_m (eq 1). Values for the parameter V/K obtained at different pHs were fitted to eq 2.

$$v = V_{max}[S]/K_m + [S] \quad (1)$$

$$Y = c/[1 + (H/K_1) + (K_2/H)] \quad (2)$$

where v represents the initial velocity, V_{max} the apparent maximal velocity, [S] the concentration of varying substrate, K_m the apparent Michaelis constant, Y represents V/K, c is pH-independent value of V/K, H is the hydronium ion concentration, and K_1 and K_2 are the dissociation constants of an acid and/or a base. Substrates were buffered to avoid pH change, and the pH was checked before and after the reactions. All measured values are reported as average of three parallel experiments. Control experiments were performed to check the stability of PfGMPS and the ionization of ATP and XMP, the two other substrates, under extreme pH conditions (6.5 and 10) used in this study.

Stoichiometry of Glutamate and GMP Formation. To measure stoichiometry across the glutaminase and the ATPPase domains, end-point measurements for the respective activities were performed. The reaction mixture contained 90 mM Tris HCl, pH 8.5, 150 μM XMP, 2 mM ATP, 5 mM glutamine, 20 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, and 0.37 μM of PfGMPS in a total volume of 1 mL. At different time points, the reaction was quenched by boiling for 3 min and clarified by centrifugation. The clarified solution was split into two and assayed for glutamate and GMP individually. Glutamate formation was measured using assay conditions reported earlier.[22] GMP formation was measured according to the method of Sokomato.[27] In this assay perchloric acid to a final concentration of 3.2% was mixed with the reaction mixture, and absorbance was measured at 290 nm and a value of 6×10^3 M^{-1} cm^{-1} was used for molar extinction coefficient of GMP. GMP concentration in the reaction mixture was also estimated by reverse phase HPLC (on a C_{18} column) using known concentrations of GMP as standard to validate the spectrophotometric assay. In a separate assay, an aliquot from the quenched reaction was used for monitoring the concentration of ammonia released if any to the medium, using α-ketoglutarate and NADPH as substrates for glutamate dehydrogenase that served as the coupling enzyme. The reaction assay contained 50 mM Tris HCl, pH 7.4, 3 mM α-ketoglutarate, 0.25 mM NADPH, 0.1 mM EDTA, and 2 units of glutamate dehydrogenase (GDH) in a total reaction volume of 0.3 mL, done at 25 °C for 5 min. Absorbance at 340 nm was measured after completion of the reaction and molar extinction coefficient of 6220 M^{-1} cm^{-1} was used for the estimation of ammonia. The GDH-linked assay could detect ammonia concentrations as low as 12 μM.

Steady-State Competition Assays in the Presence of Both Glutamine and External Ammonia

Estimation of Total GMP by UV Spectrophotometry. The assays contained 2.2 μM of PfGMPS, 20 mM ATP, 10 mM XMP, 100 mM $MgCl_2$, and 100 mM Tris HCl, pH 8.5, in a total reaction volume of 0.6 mL. NH_4Cl and/or glutamine were used at varied concentrations. In the assays where glutamine concentration was fixed (15 mM), NH_4Cl concentration was varied from 0 to 100 mM, and similarly, when NH_4Cl was maintained at 100 mM, glutamine was varied from 0 to 15 mM. The reactions were allowed to proceed at 37 °C for 10 min, stopped by boiling for 3 min followed by incubation on ice for 5 min. Estimation of total GMP formed in the reaction was done by UV spectrophotometry.[27]

Estimation of [^{15}N]GMP by NMR Spectroscopy. Competition assays between $^{15}NH_4Cl$ and glutamine were performed as described above, except that the reactions were started with 1.4 μM of PfGMPS. Products of the competition assays were quantified by ^{15}N-edited NMR spectroscopy. Standard spectra of GMP were obtained on samples that were enzymatically synthesized to contain ^{15}N at the 2-amino group. The enzymatically synthesized samples of [^{15}N]GMP were HPLC purified and characterized prior to NMR analysis (procedure for [^{15}N]GMP synthesis and characterization is provided in the Supporting Information). For accurate measurement of [^{15}N]-GMP in the competition assays, a standard curve was generated by plotting NMR peak intensities, normalized to the internal [^{15}N]uracil standard, against different known concentrations of [^{15}N]GMP quantified by UV spectrophotometry. The concentrations of [^{15}N]GMP used for the generation of standard curve were 0.11. 0.20, 0.37, 0.75, 1.12, 1.50, and 2.23 mM.

All NMR spectra were acquired on a Bruker Avance (AV500) NMR spectrometer using a triple resonance probe equipped with a single (Z-axis) pulse field gradient accessory. NMR data were acquired on samples prepared in the assay buffer that was adjusted to contain 5% D_2O and a final pH of 4.7 prior to data acquisition. All NMR spectra were acquired at 303 K. Nitrogen-15-labeled uracil (CIL, Andover, MA) dissolved in d_6-DMSO to a final concentration of 43 mM was placed in a coaxial capillary tube (2 mm internal diameter, Wilmad LabGlass, Vineland, NJ) as an internal standard. Nitrogen-15-edited NMR spectra were acquired using the one-dimensional version of the FastHSQC experiment.[28] Peak heights were measured using the NMR data processing and analysis software NMRPipe.[29] Peak heights of [^{15}N]GMP were normalized to the uracil internal standard and then quantified from the standard curve.

Chemical Modification of Glutaminase Domain. PfGMPS (10 μM) was incubated either with 0.2 mM acivicin (L-[αS,5S]-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid) or 0.25 mM DON (6-diazo-5-oxo-L-norlucine) at 25 °C for 30 min in 50 mM Tris HCl, pH 8.5, in two separate reactions. The ammonia- and glutamine-dependent activities of the modified enzyme were measured under standard assay conditions[22] using either 5 mM glutamine or 100 mM NH_4Cl as ammonia source. For analysis of the modified enzyme by MALDI-TOF mass spectrometry, excess of acivicin or DON and the buffer components were removed by dialyzing the sample against 5 mM Tris HCl, pH 8.0. Trypsin digestion was performed at 37 °C for 30 min using trypsin to PfGMPS ratio of 1:50. The digested sample was mixed with α-cyano-4-hydroxycinnamic acid in 1:1 ratio, spotted onto a MALDI-TOF plate and spectra acquired in the positive ion reflectron mode. For obtaining the sequence of the modified peak the digested sample was passed through a nano LC column attached to an ESI-Q-TOF mass spectrometer run in positive ion mode, followed by MS/MS analysis of all the eluted peptides. A linear gradient of 0−100% methanol was used for peptide elution.

Construction and Biochemical Characterization of C102A PfGMPS. Site-directed mutagenesis of cysteine 102 to alanine (C102A) was achieved by the quick change PCR method with a

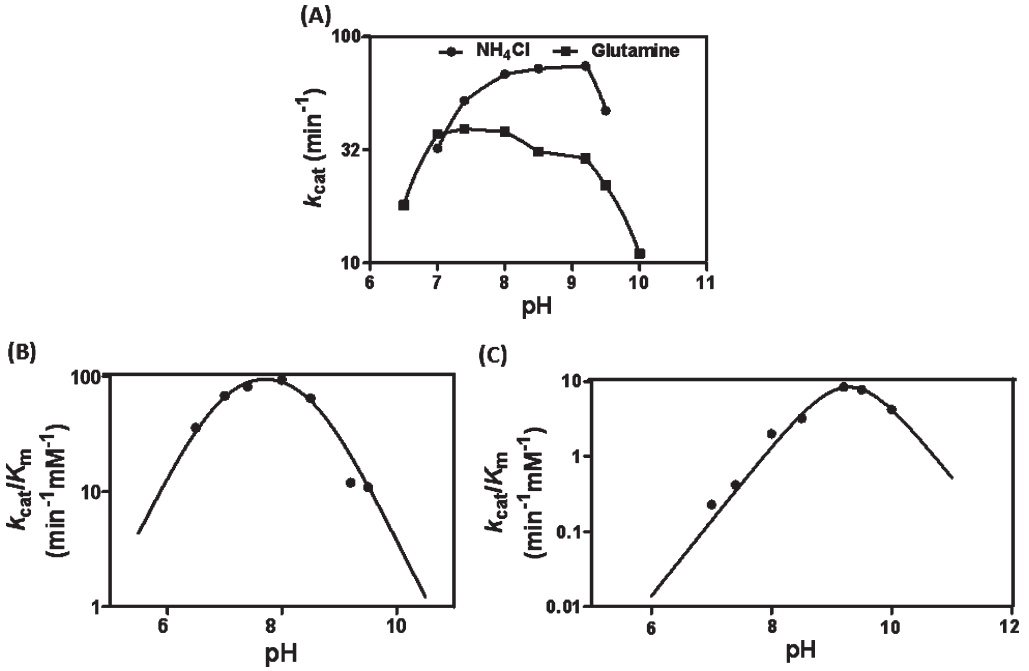

Figure 1. pH dependence of kinetic parameters of glutamine- and ammonia-dependent activities of PfGMP synthetase. (A) Plot of k_{cat} of NH_4Cl and glutamine-dependent activities versus pH, without a fit to any equation. (B) Plot of V/K versus pH where glutamine was used as ammonia source. (C) Plot of V/K versus pH where ammonium chloride was used as ammonia source. Plots in (B) and (C) represent the data fitted to eq 2. Both glutamine- and ammonia-dependent activities were measured in presence of saturating concentrations of the ligands, 2 mM ATP, 0.15 mM XMP, and 20 mM $MgCl_2$. Buffer concentration at all pHs was maintained at 0.15 M. At different pHs, activity values measured over a range of glutamine and ammonium chloride concentrations were used to estimate the values of K_m and V_{max} by fitting the data to eq 1. Stock solutions of both glutamine and ammonium chloride were buffered to avoid any pH change upon their addition to the reaction mixture, and the pH of the assay mixture was monitored before and after the reaction.

single mutagenic primer.[30] The procedure involved two steps: first, an *Eco* RV restriction site was introduced using wild type pQE 30-PfGMPS clone as a template and CCAATTTTTGGTATATGATATCGTATGCAAGAG as the primer, and in second step the restriction site was removed to introduce the desired mutation using CCAATTTTTGGTATAGCATATCGTATGCAAGAG as the primer. The mutant DNA generated was subcloned into pET 21b vector in which an N-terminal 6X-His sequence was introduced using GTCGCTAGCCATCACCATCACCATCACGGA and GCAGAGCTCTCATTCGAATTCAATCGT TGCTGG as forward and reverse primers, respectively. Introduction of the desired mutation was confirmed by DNA sequencing and the clone was expressed in Rosetta pLys S strain of BL21 (DE3) *E. coli* cells. Expression and purification conditions for the mutant protein were the same as that reported for the wild type PfGMPS,[22] except that the culture was grown at 37 °C for 6 h post IPTG induction. The kinetic parameters for the C102A mutant for ammonium, XMP, and ATP were determined by varying one substrate at a time, while maintaining the other two at saturating concentration, and the data were fitted to eq 1. For analyzing the glutamine inhibition kinetic data, eqs 3 and 4 were used.

$$v = V_{max}[S]/[K_m + [S](1 + I/\alpha K_i)/(1 + I/K_i)] \quad (3)$$

$$y_{intercept} = (1/V_{max})(1 + I/\alpha K_i)/(1 + I/K_i) \quad (4)$$

where definitions for V_{max}, [S], and K_m are same as in eq 1. [I] represents inhibitor (glutamine) concentration. αK_i and K_i represent the inhibition constants of glutamine for enzyme−substrate (ES) and free enzyme (E) forms, respectively. α is the factor by which affinity of glutamine for ES is changed.

RESULTS

Biochemical Evidence for Ammonia Channeling. Channeling of ammonia under conditions of complete liganding was addressed by two biochemical assays, pH dependence of glutamine- and ammonia-dependent activities, and the stoichiometry of glutamate and GMP formation. Though PfGMPS can use both glutamine and external ammonia (Scheme 1), the kinetic parameters k_{cat} and K_m for the two substrates are different[22] and also exhibit a pH-dependent change. The optimum pH for glutamine- and ammonia-dependent activities was 7.4 and 9.2, respectively (Figure 1a). Using the experimentally derived kinetic parameters, a theoretical k_{cat} value for a reaction, where there is leakage of ammonia, generated by glutamine hydrolysis, was computed and compared with actual k_{cat} value, to provide support for the presence or absence of channeling in PfGMPS. This was done by (1) employing the experimentally derived ammonium k_{cat} and K_m values of 56 min^{-1} and 132 mM, respectively, at pH 7.4 and (2) applying initial velocity conditions where 10% conversion of 5 mM glutamine (saturating concentration used) produces a total of 0.5 mM ammonium. The theoretical k_{cat} value computed for glutamine-dependent reaction at pH 7.4 should have been 0.21 min^{-1} (56 × 0.5/132 = 0.21), if ammonia generated from glutamine first mixes with the bulk solvent and is then reutilized. However, the observed k_{cat} (40 min^{-1}) is far higher than the theoretical value of 0.21 min^{-1}, ruling out the possibility

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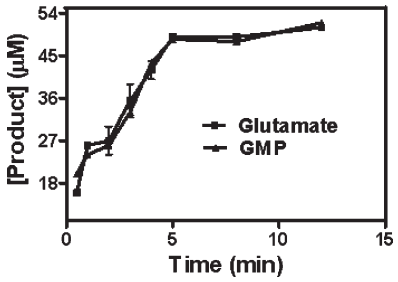

Figure 2. Measurement of concentration of products, glutamate and GMP in PfGMP synthetase reaction: (■) glutamate concentration measured as formation of NADH using glutamate dehydrogenase as a coupling enzyme; (▲) GMP concentration measured at 290 nm using extinction coefficient value of 6000 cm^{-1} M^{-1}. The details of assay are given in the Materials and Methods section.

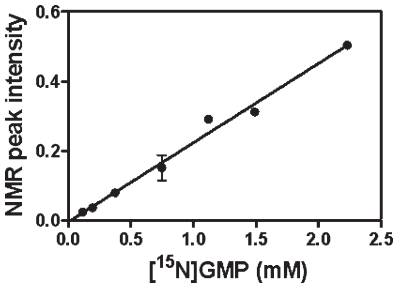

Figure 3. Standard curve relating NMR peak intensities to the known concentrations of synthesized [^{15}N]GMP. The conditions for ^{15}N-edited ^{1}H NMR spectral acquisition of the ^{15}N-labeled GMP at concentrations of 0.11. 0.20, 0.37, 0.75, 1.12, 1.50, and 2.23 mM are given in the Materials and Methods section. Peak heights are normalized relative to those of the internal [^{15}N]uracil standard.

that ammonia produced from glutamine equilibrates with the external medium.

When glutamine was used as ammonia source, the plot of V/K versus pH (Figure 1b) highlighted the presence of two different ionization constants (pK_{a1} = 6.9 ± 0.1 and pK_{a2} = 8.5 ± 0.1). However, when ammonium chloride replaced glutamine, two similar pK_a values (9.4 ± 0.1 and 9.2 ± 0.1) were obtained (Figure 1c), which were closer to the pK_a value of 9.2 for deprotonation of the ammonium ion (NH_4^+ to NH_3). This suggested that the observed pK_a values could be due to ammonium ion dissociation. A plot of V/K versus pH, with the K_m corrected for the actual concentration of ammonia at different pHs, yielded a horizontal line without any slope (data not shown), confirming that ammonia and not ammonium is the actual substrate for PfGMPS. This is further substantiated by the drop seen in ammonium K_m as the pH was increased from 7.0 to 9.5. It is important to note that below pH 9.2 the equilibrium of ammonia/ammonium shifts toward the latter, and hence, the majority of the species present is ammonium.

Stoichiometry of the glutaminase and the acceptor domain reactions in amidotransferases has been used as an indicator for ammonia channeling.[31] Channeling efficiency is defined as the ratio of GMP to glutamate formed in the ATPPase and the glutaminase domains when glutamine is used as the source of ammonia. Data in Figure 2 indicate that coupling between the two half-reactions, glutamine hydrolysis and GMP formation in the PfGMPS reaction, was almost stoichiometric. Also, the glutamate dehydrogenase coupled enzyme assays performed for estimation of ammonia formed in the PfGMPS reaction failed to detect any free ammonia. However, it should be noted that low concentrations of ammonia, if released into the surrounding medium, would escape detection by this assay. Overall, these results corroborated the conclusions drawn from pH-dependent studies of glutamine- and ammonia-dependent PfGMPS activities.

Steady-State Competition Assays between Glutamine and Ammonium. Although ammonia and glutamine are individually used by PfGMPS, as a source of ammonia albeit with different kinetic constants, the kinetics of utilization of external ammonia in the presence of glutamine has not been examined. Hence, a competition assay was performed wherein both glutamine and ammonium were present together. When NH_4Cl was varied from 0 to 100 mM with glutamine maintained at 15 mM (30 K_m), gradual increase in GMP formation was seen. However, when NH_4Cl was fixed at 100 mM (~5K_m) and glutamine titrated from 0 to 15 mM, no increase in activity was observed (Table 1 of the Supporting Information). These results suggested that PfGMPS is able to utilize external ammonia even in the presence of saturating concentration of glutamine.

To quantify, in the presence of glutamine, the incorporation of external ammonia into the final product GMP, NH_4Cl was replaced with $^{15}NH_4Cl$ in the competition assays, and product formation was monitored by NMR spectroscopy. Exchange of amino protons in GMP with the bulk solvent is known to be pH-dependent,[32] necessitating the optimization of a suitable pH at which the amino protons could be detected. Hence, ^{15}N-edited ^{1}H NMR spectra of the synthesized [^{15}N]GMP were acquired over a range of pH (1.5−5.5), and the presence of a sharp doublet for the 2-amino protons (6.15 ppm) between pH 4.5 and 5.5 permitted us to select pH 4.7 for all further experiments. The selected pH of 4.7 was also suitable because the ^{1}H peaks of $^{15}NH_4^+$/$^{15}NH_3$ present in the reaction mixture were completely exchange broadened and, hence, not detected. An earlier report on the exchange rates of amino protons in GMP had also shown the rate to be slowest in the pH range of 4.5−6.5.[32,33] Figure 3 represents the standard curve that relates NMR peak intensities, normalized to the internal uracil standard, to different concentrations (spectrophotometrically determined) of standard [^{15}N]GMP.

With the conditions set for accurately estimating [^{15}N]GMP, ^{1}H, ^{15}N-edited NMR spectroscopy was used to quantify [^{15}N]GMP in the steady-state competition assays where either glutamine or $^{15}NH_4Cl$ concentration was varied, while keeping the other at a fixed saturating concentration. Figure 4(II) (and Figure 3(II) of the Supporting Information) represents a typical ^{15}N-edited NMR spectrum obtained on samples where both glutamine and $^{15}NH_4Cl$ were together present in the reaction. The doublet at 6.15 ppm corresponds to the 2-amino group of [^{15}N]GMP. As shown in Table 1, in assays where glutamine concentration was constant (15 mM) and $^{15}NH_4Cl$ varied from 20 to 100 mM, the presence of glutamine could not prevent the incorporation of ^{15}N label into GMP. Similarly, when $^{15}NH_4Cl$ concentration was fixed (100 mM) and glutamine varied from 3 to 15 mM, an initial drop in ^{15}N incorporation into GMP was seen, which subsequently plateaued.

Apart from the presence of [^{15}N]GMP peaks, two doublets at 6.70 and 7.40 ppm consistently appeared in the ^{15}N-edited proton NMR spectra (Figure 4(II) and Figure 3(II) of the Supporting Information), except under the condition where glutamine was absent. A ^{15}N-edited proton NMR spectrum of 15 mM glutamine showed two low-intensity doublets at the same

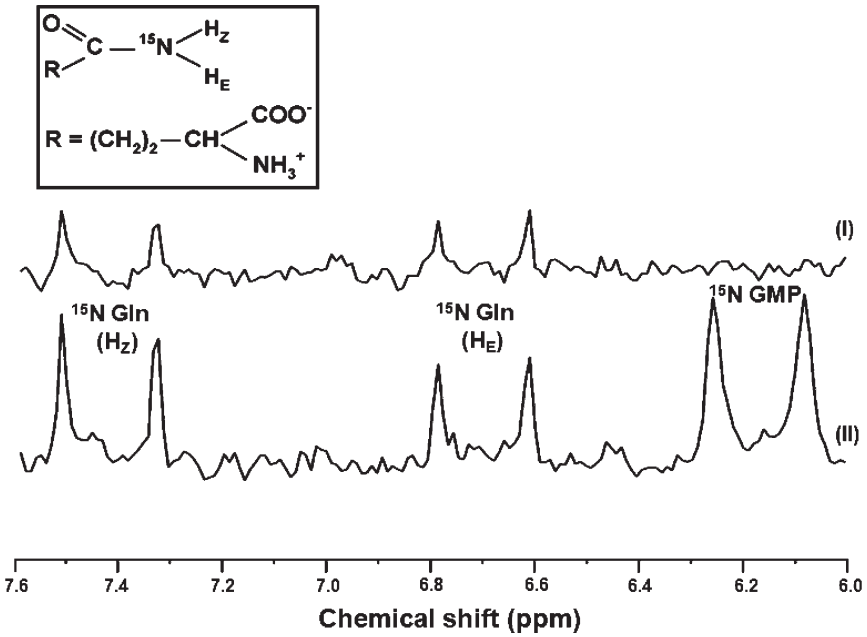

Figure 4. Comparison of one-dimensional ^{15}N-edited proton NMR spectra of glutamine and PfGMP synthetase-catalyzed reaction. (I) One-dimensional ^{15}N-edited proton NMR spectrum of glutamine. The assay contained 15 mM glutamine, 20 mM ATP, 10 mM XMP, 100 mM $MgCl_2$, and 100 mM Tris HCl, pH 8.5, in a total reaction volume of 0.6 mL without PfGMPS, and the spectrum was acquired after adjustment of pH to 4.7. (II) One-dimensional ^{15}N-edited proton NMR spectrum of PfGMPS-catalyzed reaction containing 15 mM glutamine, 100 mM NH_4Cl, 20 mM ATP, 10 mM XMP, 100 mM $MgCl_2$, 100 mM Tris HCl, pH 8.5, and 2.2 μM PfGMPS in a total reaction volume of 0.6 mL. Spectrum was acquired after the reaction was quenched, deproteinized, and pH adjusted to 4.7. The spectra in chemical shift range 6.0−7.6 ppm show the signals arising from [^{15}N]GMP (doublet, δ_{NH} = 6.15 ppm), [^{15}N]-L-glutamine H_E (doublet, δ_{NH} = 6.7 ppm) and [^{15}N]-L-glutamine H_z (doublet, δ_{NH} = 7.4 ppm). All the chemical shifts are reported with reference to TSP. Inset shows the structure of L-glutamine to highlight the H_E and H_Z protons.

Table 1. Steady-State Competition Assays for the Measurement of ^{15}N Incorporation into GMP Catalyzed by *P. falciparum* GMP Synthetase[a]

$^{15}NH_4Cl$ (mM)	Gln (mM)	[^{15}N]GMP[b] (mM)	[^{14}N]GMP (mM)	total GMP[c] (mM)	[^{14}N]GMP/[^{15}N]GMP ratio
0	15		0.37 ± 0.04	0.37 ± 0.04	
20	15	0.13 ± 0.01	0.34 ± 0.01	0.47 ± 0.01	2.56 ± 0.34
40	15	0.17 ± 0.01	0.36 ± 0.02	0.53 ± 0.08	2.05 ± 0.06
60	15	0.21 ± 0.01	0.36 ± 0.07	0.57 ± 0.08	1.68 ± 0.31
80	15	0.23 ± 0.03	0.37 ± 0.04	0.61 ± 0.06	1.60 ± 0.01
100	15	0.25 ± 0.02	0.41 ± 0.07	0.66 ± 0.06	1.67 ± 0.39
100	0	0.52 ± 0.07		0.68 ± 0.01	
100	3	0.39 ± 0.02	0.34 ± 0.06	0.74 ± 0.04	0.88 ± 0.18
100	6	0.27 ± 0.01	0.38 ± 0.03	0.66 ± 0.04	1.41 ± 0.05
100	9	0.25 ± 0.04	0.42 ± 0.00	0.67 ± 0.04	1.67 ± 0.26
100	12	0.27 ± 0.04	0.42 ± 0.03	0.69 ± 0.01	1.56 ± 0.30
100	15	0.25 ± 0.02	0.45 ± 0.02	0.70 ± 0.00	1.78 ± 0.17

[a] All assays were done under similar conditions, as mentioned in the Materials and Methods section. All other substrates, ATP, XMP, and $MgCl_2$, were maintained at 20, 10, and 100 mM, respectively. [b] Estimated by ^{15}N-edited proton NMR spectroscopy using known quantities of enzymatically produced [^{15}N]GMP as standard, and all the values are corrected for the natural abundance of [^{15}N]glutamine. Values are reported as mean ± SD of two independent experiments done with two separate preparations of PfGMPS. [c] Quantified by UV spectrophotometry.[27] Errors reported are ±SD of mean of two independent measurements with each having three replicates.

chemical shifts as seen in the spectra of assay mixtures (Figure 4(I) and Figure 3(I) of the Supporting Information). These peaks arise from the side-chain amide protons of L-glutamine at natural abundance.[17,34] These results indicated that the peaks at 6.70 and 7.40 ppm could arise due the formation of [^{15}N]-L-glutamine in the competition assays. To check whether complete liganding of PfATPPase domain is needed for [^{15}N]-L-glutamine formation, two separate competition assays were performed that contained both glutamine and $^{15}NH_4Cl$, but with either ATP or XMP or both excluded. The NMR spectra recorded for these assay mixtures indicated that [^{15}N]-L-glutamine was not formed, as the peak intensity did not exceed that obtained for the natural abundance of ^{15}N in 15 mM L-glutamine.

Effect of Chemical Modification and Mutagenesis of Cysteine 102 on Glutamine- and Ammonia-Dependent Activities. To examine the possible role that the glutaminase

domain may have on the entry of external ammonia, we chemically modified the glutaminase catalytic pocket with acivicin and DON, the two structural analogues of glutamine known to specifically inhibit the glutamine-dependent activity, by irreversible binding.[22,24,25,35] To ascertain the covalent binding of acivicn to PfGMPS, a tryptic digest of acivicin-treated protein was subjected to MALDI-TOF and ESI-Q-TOF MS analysis, followed by sequence identification of the selected peptides by employing MS/MS methods (Figure 2 of the Supporting Information). This study allowed us to identify a peptide K^{95}-K^{118} (KIPIFGICYGMQEIAVQMNGEVKK) of m/z 2695.39 Da that contained a single cysteine (C102), modified with acivicin (m/z 143 Da). The results listed in Table 2 show that the glutamine-dependent activity of the acivicin- or DON-modified PfGMPS was almost completely abolished. However, 90% of the activity was retained in both the samples when glutamine was replaced with ammonium in the assay. These results suggested that the modification of the glutaminase domain with acivicin and DON does not impede the ammonia-dependent activity.

Table 2. Effect of Acivicin and DON on the Glutamine- and Ammonia-Dependent Activities of *P. falciparum* GMP Synthetase[a]

	activity (nmol min^{-1} mg^{-1})	
inhibitor	ammonia-dependent	glutamine-dependent
none	714 ± 10	607 ± 7
acivicin	678 ± 15	48 ± 1
DON	631 ± 14	75 ± 7

[a] PfGMPS was incubated either with acivicin (0.2 mM) or DON (0.25 mM) for 30 min at 25 °C. Aliquots were assayed for both glutamine- and ammonia-dependent activities, under standard assay conditions (detailed in the Materials and Methods section), with either glutamine (5 mM) or ammonium chloride (100 mM) used as source of ammonia. The values are reported as mean ± SD estimated from three independent measurements.

A mutant of PfGMPS having Cys-102 replaced with alanine was generated with the aim of having an enzyme that can bind glutamine but not catalyze its hydrolysis. The kinetic constants for NH_4Cl, ATP, and XMP obtained for the ammonia-dependent activity of the purified C102A mutant of PfGMPS (Table 2 of the Supporting Information) were similar to that of the wild-type enzyme. As expected, the mutant C102A was totally devoid of glutamine-dependent activity, in agreement with the results from chemical modification. This also provides confirmatory evidence for the catalytic role of C102 in the glutaminase domain. The high degree of similarity, in the CD spectra of the C102A mutant and the wild-type enzymes (Figure 5a), rules out the possibility of structural changes in the mutant contributing to the loss of glutamine-dependent activity.

The effect of glutamine on the ammonia-dependent activity of C102A was evaluated by either varying the concentration of glutamine (0−15 mM) or ammonium chloride (0−100 mM) while keeping the second substrate fixed, 15 mM in the case of glutamine or 100 mM in the case of ammonium chloride. The presence of 15 mM glutamine in the reaction inhibited the ammonia-dependent GMP formation to ∼50%, compared to the reaction where glutamine was excluded (Figure 5b,c). This suggested that a partial inhibition of the ammonia-dependent activity in C102A mutant of PfGMPS arose from the binding of glutamine. Figure 6 shows double-reciprocal plots of the inhibition kinetics of C102A PfGMPS where [glutamine] was varied at different fixed concentrations with respect to the three substrates ATP, XMP, and ammonium. Hyperbolic replots obtained from the

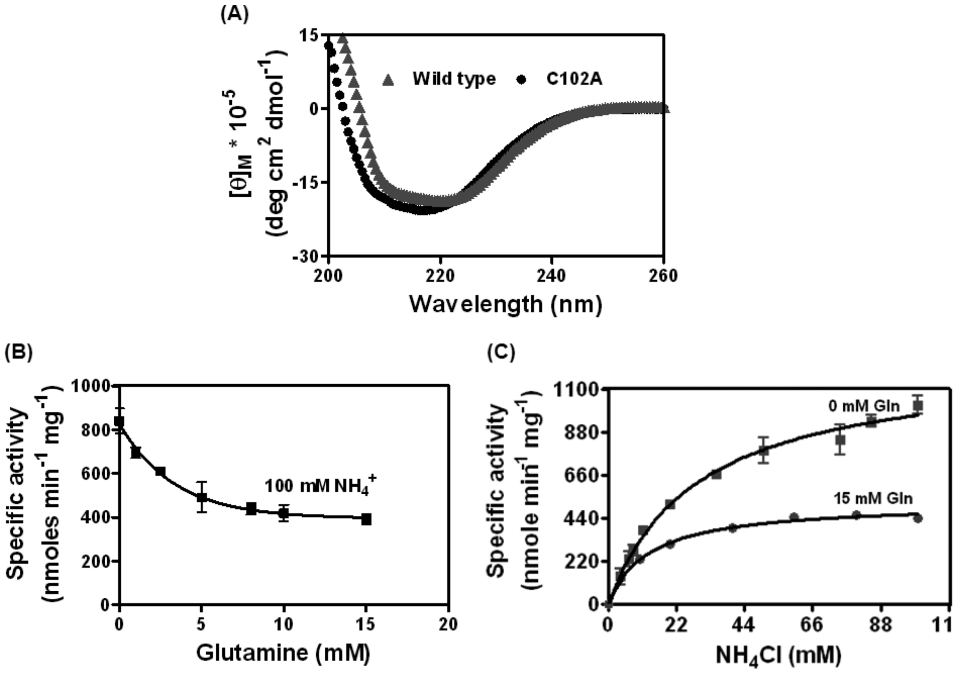

Figure 5. Characterization of C102A mutant of PfGMPS. (A) Comparison of far-UV CD spectra of mutant C102A and wild-type PfGMPS. Cuvettes with 0.1 cm path length were used and each spectrum is average of three scans. (B) Inhibition of ammonia-dependent activity of C102A at different concentrations of glutamine, with ammonium chloride fixed at 100 mM. (C) Inhibition of ammonia-dependent activity of C102A at fixed concentration of glutamine. All the assays were performed under standard assay conditions as detailed in the Materials and Methods section.

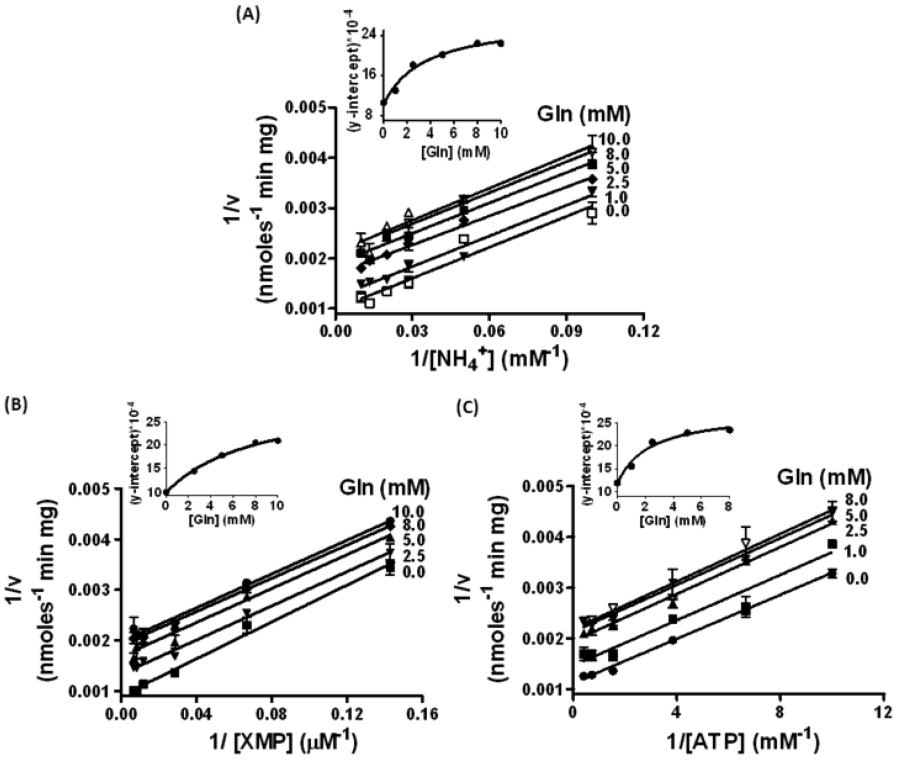

Figure 6. Double-reciprocal plots of initial velocities ($1/v$) versus different substrate (ammonia, ATP, and XMP) concentrations at various fixed concentrations of glutamine. (A) $1/v$ versus $1/[NH_4^+]$ at 0, 1, 2.5, 5, 8, and 10 mM of glutamine. ATP and XMP were at fixed concentrations. (B) $1/v$ versus $1/[XMP]$ at 0, 2.5, 5, 8, and 10 mM of glutamine with ATP and NH_4^+ maintained at fixed concentrations. (C) $1/v$ versus $1/[ATP]$ at 0, 1, 2.5, 5, and 8 mM glutamine with XMP and NH_4^+ at fixed concentrations. Assay conditions were same as those used for standard assays mentioned in the Materials and Methods section. The fixed concentrations of substrates used were 0.2 mM ATP, 0.15 mM XMP, and 100 mM NH_4Cl.

Table 3. Inhibition Constants of Glutamine for the Ammonia-Dependent Activity of C102A PfGMPS against Different Substrates[a]

substrate	α[b]	K_i (mM)	K_m (mM)
XMP	0.30 ± 0.03	9.5 ± 2.5	0.02 ± 0.00
ATP	0.41 ± 0.03	2.7 ± 0.7	0.18 ± 0.01
NH_4^+	0.35 ± 0.03	4.2 ± 1.0	23 ± 2

[a] All the values were obtained by global nonlinear fit of the inhibition data to eq 3. [b] α is the factor by which affinity of glutamine for ES is changed.

parallel linear primary plots are indicative of mixed hyperbolic or partial uncompetitive inhibition, where infinite concentration of an inhibitor (glutamine) leads to a finite inhibition, and hence, the velocity of the reaction, unlike the normal uncompetitive inhibition, can never be driven to zero. The parallel nature of the Lineweaver–Burk plots arises from the inhibitor (glutamine) modulating the affinity (K_m) of the substrate for the enzyme and V_{max} to an identical degree. A global fit of the initial velocity data to eq 3 yielded kinetic inhibition parameters for glutamine with respect to ATP, XMP, and ammonium (Table 3), and the values agreed well with those obtained from the fit of *y*-intercept values (from Lineweaver–Burk plots) to eq 4 for the respective substrates (data not shown).

DISCUSSION

The present investigation was aimed at understanding aspects of ammonia transfer from glutaminase to ATPPase domain in PfGMPS, the only two-domain-type GMP synthetase reported until now to possess significant leaky glutaminase activity[22] when the ATPPase domain is not completely liganded. However, during the complete catalytic cycle, the enzyme appears to have evolved a mechanism for preventing leakage of ammonia from the enzyme into the outside medium (Figure 2). While a 1:1 stoichiometry is assumed for the purposes of discussion, the possible release of low concentrations of ammonia into the solvent cannot be fully ruled out as such levels would be below the detection limit of the coupled enzyme assay. A 1:1 stoichiometry across domains has also been reported for glutamine phosphoribosylpyrophosphate amidotransferase and IGP synthase.[31,36] Whether the leaky glutaminase activity seen, when the ATPPase domain is unliganded in PfGMPS, is necessary under physiological conditions remains to be investigated. A recent report on two-subunit-type GMP synthetase from *Pyrococcus horikoshii* has shown that the enzyme possesses a low level of glutaminase activity in the absence of ATP and XMP, which is ~5-fold lower compared to that when ATP and XMP are present.[23] Also, PLP synthase exhibits substrate-independent glutaminase activity[37] while asparagine synthetase shows a stoichiometry of 1:1.5 for the activities of the synthetase and glutaminase domains.[38] The pH–activity relationship studies with glutamine and ammonium as substrates (Figure 1) agreed with the occurrence of 1:1 stoichiometry and suggested that ammonia generated by glutamine hydrolysis is channeled within PfGMPS from the glutaminase to the ATPPase domain. Similar conclusions have been made from the pH kinetics done on CTP synthetase[39] and *E. coli* GMP synthetase.[25] The two different

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ionization constants (6.9 ± 0.1 and 8.5 ± 0.1) obtained from the plot in Figure 2b could reflect the side-chain ionization constants for histidine and cysteine, respectively, which have been proposed to form a part of the catalytic triad involved in glutamine hydrolysis in the glutaminase domain. Similar ionization constant values of 6.0 and 8.6 have been proposed for His and Cys, the catalytic residues in glutamate synthase.[40]

The ^{15}N-edited proton NMR spectroscopic experiment was sensitive enough to detect the different levels of ^{15}N-labeled GMP produced in the competition assays permitting us to accurately quantify the molecule. As evident from Table 1 even in the presence of saturating concentration of glutamine (15 mM) and at a low concentration of $^{15}NH_4Cl$ (20 mM), $^{15}NH_3$ could still access the adenyl-XMP intermediate in the ATPPase domain. However, the $^{14}N/^{15}N$ ratio in total GMP will be dictated by k_{cat}/K_m for the two ammonia sources, after a correction is made for their initial concentrations.[9] Such a calculation done for a condition where 20 mM $^{15}NH_4Cl$ and 15 mM glutamine were present together suggested that the theoretical $^{14}N/^{15}N$ value of 13.5 was higher than the observed value of 2.56 ± 0.34. This difference in the values of the theoretical and observed $^{14}N/^{15}N$ ratios also persisted in other titrations including the one in which both $^{15}NH_4Cl$ and glutamine were present at saturating concentrations. The large difference seen between the theoretical and observed values of $^{14}N/^{15}N$ ratios highlighted that the [^{15}N]GMP fraction in the total pool of GMP is greater than what would be expected on the basis of k_{cat}/K_m. In the competition assays where glutamine concentration was fixed at 15 mM, the value for $^{14}N/^{15}N$ ratio after an initial decrease remained constant when $^{15}NH_4Cl$ concentration was increased, and similarly, when $^{15}NH_4Cl$ was present at 100 mM, increasing concentration of glutamine led to an initial increase in $^{14}N/^{15}N$ ratio and subsequently saturated. The above ratios also indicated that the absolute quantity of [^{15}N]GMP in the assays never exceeded $53 \pm 5\%$ of the total GMP, suggesting that the ammonia generated from glutamine does not equilibrate with the external medium, as in that case the ^{15}N incorporation should have been greater than 85% (calculation based on k_{cat}/K_m ratios of the two substrates). The channeling behavior in PfGMPS resembles that of AS-B[17] where glutamine could not impede the incorporation of external ammonia into asparagine, the final product of the reaction. However, asparagine competitively inhibits the glutamine-dependent AS-B reaction, with no effect on the utilization of external ammonia, and this differential inhibition has been proposed to be contributing to the unusual channeling behavior seen in the enzyme. In contrast, a similar role for GMP in PfGMPS cannot be entertained, as GMP inhibits the reaction competitively with respect to XMP and manifests similar inhibitory effects (similar K_i) on both glutamine- and ammonia-dependent activities.[22] In this context, it is interesting to highlight that the glutaminase domain of PfGMPS contains a unique 20 residue insertion, and whether the insertion has any role in the observed channeling behavior remains to be investigated. In contrast to PfGMPS and AS-B, glutamine in CPS has been shown to fully impede the entry of external ammonia into the enzyme.[9] Pertinently, though a biochemical evidence for ammonia channeling in *E. coli* GMP synthetase was provided earlier, no preformed channel was seen in the crystal structure.[41]

The formation of [^{15}N]glutamine from [^{14}N]glutamine and $^{15}NH_4Cl$ in competition assays stems from the exchange process between [^{14}N]glutamine and $^{15}NH_4Cl$ involving the thioester intermediate formed in the glutaminase domain, onto which $^{15}NH_3$ gains access leading to exchange with the side chain $-NH_2$ of L-glutamine. Similar exchange process during thioester intermediate formation has been reported earlier in asparagine synthetase.[17] Our inability to detect [^{15}N] glutamine in assays containing both glutamine and $^{15}NH_4Cl$ but lacking either ATP or XMP, or both, suggested the absence of exchange process in the glutaminase domain when the ATPPase domain is unliganded. This could be due to (1) inaccessibility of glutaminase active site to external ammonia or (2) external ammonia preventing the thioester formation and, thereby, glutamine hydrolysis in the glutaminase domain when the ATPPase domain is unliganded. The latter mechanism has been reported earlier in CPS[42] where high concentration of ammonium in the presence of glutamine abolished the formation of thioester when ATP and bicarbonate, the two other substrates, were excluded from the assay. Detection of the PfGMPS proteolytic fragment with C102 covalently attached to acivicin using mass spectrometry supports the formation of thioester intermediate in the glutaminase domain. Similar acivicn-labeled peptide has also been detected in human GMP synthetase tryptic digest.[24] To our knowledge the nitrogen-15-edited NMR spectroscopic evidence presented here is the first direct proof for the formation of thioester intermediate in catalysis by GMP synthetases.

Partial uncompetitive nature of glutamine inhibition with respect to ATP, XMP, and NH_4^+ in C102A mutant of PfGMPS indicates that glutamine can bind to either E or ES ($E.NH_3$) form of the enzyme with different dissociation constants (K_i and αK_i, respectively), with the ternary complex, ESI ($E.NH_3$.Gln) being catalytically productive, albeit with less efficiency than the ES ($E.NH_3$) form. The differences observed in the dissociation constant (K_i) values for glutamine for PfGMPS with respect to the different substrates (Table 3) reflects on the modulation of inhibitor binding affinity for the enzyme by the substrates. These results corroborated our previously proposed kinetic mechanism (two-site ping-pong) for PfGMPS where glutamate release is the irreversible step between the binding of either ATP or XMP and glutamine.[22] Therefore, glutamine, a mimic of glutamate when bound to the mutant C102A, switches the enzyme to a catalytically less active state. Though the irreversible binding of acivicin and DON to wild type PfGMPS may resemble the binding of glutamine to the C102A mutant of PfGMPS, inhibition of ammonia-dependent activity was not seen in the chemically modified enzyme. These observations suggest that the glutamine-bound C102A mutant of PfGMPS and the inhibitor (acivicn or DON)-modified PfGMPS may exist in different conformational states, or the inhibitor (DON and acivicin) packing in the catalytic pocket is different from that of glutamine and, hence, the differences in ammonia-dependent activities. It is pertinent to note that glutamine has also been shown to inhibit the ammonia-dependent activity of C1A mutants of human[43] and *E. coli*[44] asparagine synthetases. However, in both the enzymes, the primary reciprocal plots, $1/v$ versus either 1/[ammonia], 1/[aspartate], or 1/[ATP], were intersecting, except for the parallel nature of $1/v$ versus 1/[ATP] plot seen for the *E. coli* enzyme. Such a pattern has been attributed to the formation of an abortive complex during the ammonia-dependent catalysis in the presence of glutamine that leads to its inhibition.

In conclusion, this report provides the first detailed analysis of ammonia channeling in PfGMP synthetase that can serve as a model for comparative investigation of this phenomenon in other GMP synthetases. This study highlights the notion that

the optimization of channeling efficiency during evolution in different glutamine-dependent amidotransferases varies across this class of enzymes. Detailed catalytic and kinetic analysis of individual steps of PfGMPS reaction needs to be carried out to find answers for the many unresolved questions highlighted by this study. Furthermore, studies on other GMP synthetases would throw light on whether the specific aspects of channeling seen in PfGMPS are conserved or a unique feature of the parasite enzyme. The nonavailability of a crystal structure for PfGMPS precluded us from deducing the actual residues that form the ammonia translocation path in the enzyme. Also, *E. coli* GMP synthetase as a template was not useful due to the absence of any preformed conduit in the structure of the enzyme. It should also be emphasized that ammonia tunnels in different glutamine-dependent amidotransferases are diverse in architecture, making a direct primary sequence comparison of the residues involved in the formation of ammonia tunnel complicated.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Procedure for enzymatic synthesis, purification, and characterization of ^{15}N-labeled GMP; steady state competition assay for the formation of GMP (Table 1); steady state kinetic parameters of PfGMPS C102A mutant (Table 2); characterization of purified [^{15}N]GMP (Figure 1); elucidation of the covalent modification of PfGMPS by acivicin through mass spectrometry (Figure 2); comparison of one-dimensional ^{15}N-edited proton NMR spectra of glutamine and PfGMP synthetase-catalyzed reaction (Figure 3). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; NADPH, nicotinamide adenine dinucleotide phosphate; HPLC, high performance liquid chromatography; ESI, electrospray ionization; LC, liquid chromatography; Q-TOF, quadrupole time-of-flight; NMR, nuclear magnetic resonance.

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