# Mutagenesis and Mechanism-Based Inhibition of *Streptococcus pyogenes* Glu-tRNA$^{Gln}$ Amidotransferase Implicate a Serine-Based Glutaminase Site

Mark R. Harpel,*,‡ Kurumi Y. Horiuchi,‡ Ying Luo,‡ Li Shen,‡ Wenjun Jiang,‡ David J. Nelson,§ Kelley C. Rogers,|| Carl P. Decicco,§ and Robert A. Copeland‡

*Chemical Enzymology, Medicinal Chemistry, and Antimicrobials Departments, Bristol-Myers Squibb Company, Experimental Station, Route 141 and Henry Clay Road, Wilmington, Delaware 19880*

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ABSTRACT: The absence of Gln-tRNA synthetase in certain bacteria necessitates an alternate pathway for the production of Gln-tRNA$^{Gln}$: misacylated Glu-tRNA$^{Gln}$ is transamidated by a Gln-dependent amidotransferase (Glu-AdT) via catalysis of Gln hydrolysis, ATP hydrolysis, activation of Glu-tRNA$^{Gln}$, and aminolysis of activated tRNA by Gln-derived $NH_3$. As observed for other Gln-coupled amidotransferases, substrate binding, Gln hydrolysis, and transamidation by Glu-AdT are tightly coordinated [Horiuchi, K. Y., Harpel, M. R., Shen, L., Luo, Y., Rogers, K. C., and Copeland, R. A. (2001) *Biochemistry 40*, 6450−6457]. However, Glu-AdT does not employ an active-site Cys nucleophile for Gln hydrolysis, as is common in all other glutaminases: some Glu-AdT lack Cys, but all contain a conserved Ser (Ser176 in the A subunit of *Streptococcus pyogenes* Glu-AdT) within a sequence signature motif of Ser-based amidases. Our current results with *S. pyogenes* Glu-AdT support this characterization of Glu-AdT as a Ser-based glutaminase. Slow-onset ($\sim$50 $M^{-1}$ $s^{-1}$), tight-binding ($t_{1/2}$ > 2.5 h for complex dissociation), Gln-competitive inhibition of the Glu-tRNA$^{Gln}$/ATP-independent glutaminase activity of Glu-AdT by $\gamma$-Glu boronic acid is consistent with engagement of a Ser nucleophile in the glutaminase active site. Conversion to rapidly reversible, yet still potent ($K_i$ = 73 nM) and Gln-competitive, inhibition under full transamidation conditions mirrors the coupling between Gln hydrolysis and aminolysis reactions during productive transamidation. Site-directed replacement of Ser176 by Ala abolishes glutaminase and Gln-dependent transamidase activities of Glu-AdT (>300-fold), but retains a wild-type level of $NH_3$-dependent transamidation activity. These results demonstrate the essentiality of Ser176 for Gln hydrolysis, provide additional support for coordinated coupling of Gln hydrolysis and transamidase transition states during catalysis, and validate glutaminase-directed inhibition of Glu-AdT as a route for antimicrobial chemotherapy.

Correct incorporation of mRNA-specified amino acids into growing polypeptide chains is ensured by ribosomal recognition of a distinct aminoacyl-tRNA molecule for each encoded amino acid. In eukaryotic cytoplasm and certain Gram-negative eubacteria, a set of 20 aminoacyl-tRNA synthetases properly matches and covalently links each amino acid with its unique tRNA partner for delivery to the ribosome (the "direct" aminoacylation pathway). However, the absence of genes for Gln-tRNA[1] synthetase and/or Asn-tRNA synthetase in all Gram-positive eubacteria, many Gram-negative eubacteria, archaebacteria, chloroplasts, and eukaryotic mitochondria mandates an alternate means for producing these key intermediates (reviewed in ref *1*). This "indirect" pathway entails misacylation of tRNA$^{Gln(or\ Asn)}$ with Glu (or Asp) by a nondiscriminating aminoacyl-tRNA synthetase and subsequent transamidation by a Gln-dependent amidotransferase (Glu-AdT or Asp-AdT). The lethality of genetically disrupting Glu-AdT in *Bacillus subtilis* (*2*) highlights the essentiality of this enzyme for cell viability and its potential as a target of antibacterial chemotherapy.

Three distinct reactions are catalyzed by Glu-AdT (Figure 1). Similar to most Gln-dependent transamidases (reviewed in refs *3*−*5*), the synthesis of Gln-tRNA$^{Gln}$ ("transamidation") by Glu-AdT is chemically and kinetically coupled to Gln hydrolysis ("glutamination") through ATP-dependent activation of the ultimate acceptor molecule (i.e., Glu-tRNA$^{Gln}$) (*6*). For Glu-AdT, this process appears to involve ATP hydrolysis and formation of a $\gamma$-phosphoryl-Glu-tRNA$^{Gln}$ intermediate (*7*, *8*).

Glu-AdT is generally found as a heterotrimer ($\sim$117.5 kDa) consisting of "A" ($\sim$53 kDa), "B" ($\sim$53.5 kDa), and "C" ($\sim$10.9 kDa) subunits. Crystallographic structures of Glu-AdT have not yet been reported. However, all three subunits are required for transamidation activity. As shown by primary sequence comparisons and independent genetic expression of individual subunits, the A subunit provides the

* Address correspondence to this author at Bristol-Myers Squibb Co., Experimental Station, Route 141 and Henry Clay Rd., Wilmington, DE 19880. Phone: 302-467-5024; Fax: 302-467-6820; E-mail: mark.harpel@bms.com.

‡ Chemical Enzymology Department.

§ Medicinal Chemistry Department.

|| Antimicrobials Department.

[1] Abbreviations: Gln-tRNA, glutaminyl-charged transfer RNA; Asn-tRNA, asparaginyl-charged tRNA; tRNA$^{Gln}$, tRNA complementary to the Gln codon; Gln-tRNA$^{Gln}$, Gln-charged tRNA$^{Gln}$; Glu-tRNA$^{Gln}$, Glu-charged tRNA$^{Gln}$; Asn-tRNA$^{Asn}$, Asn-charged tRNA$^{Asn}$; Glu-AdT, Glu-tRNA$^{Gln}$ amidotransferase; $\gamma$-Glu boronic acid, L-2-amino-4-borono butanoic acid; S176$_A$A, Glu-AdT mutant in which Ser at position 176 of the A subunit is substituted by Ala.

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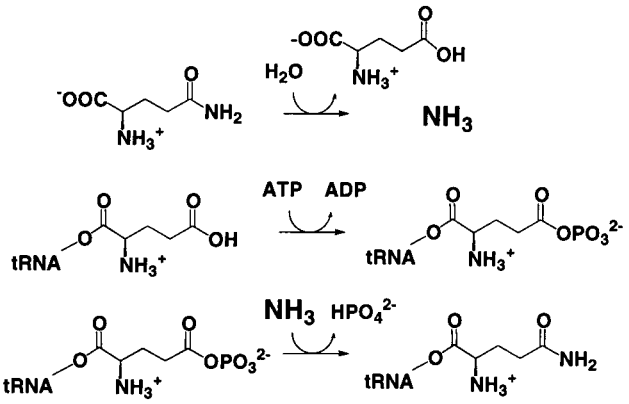


FIGURE 1: Reactions catalyzed by Glu-AdT. Top: Gln hydrolysis ("Glutaminase") reaction. Middle: Proposed ATP-dependent activation of Glu-tRNA$^{Gln}$. Bottom: Transamidation of activated Glu-tRNA$^{Gln}$ intermediate using $NH_3$ derived from Gln hydrolysis. The $NH_3$ used for transamidation may also be derived from $NH_4Cl$, but at lower efficiency than when provided from Gln hydrolysis (see Results).

site of glutaminase action, and the B subunit may provide the site of Glu-tRNA$^{Gln}$ recognition and delivery (*2*) (J. C. Berbaum and K. C. Rogers, unpublished data). The only proposed role for the C subunit is to promote stable expression of the A subunit (*2*).

To understand the mechanism of Gln hydrolysis for use in designing Glu-AdT inhibitors as antimicrobial agents, we focused on the recombinant enzyme from *Streptococcus pyogenes* as a representative surrogate pathogen system. Key to the hydrolysis mechanism of most glutaminases is attack of the Gln carboxamide by an active-site nucleophile and formation of a tetrahedral intermediate in preparation for hydrolysis and C−N bond scission. Although active-site Cys functions as the catalytic nucleophile in most glutaminases (reviewed in refs *3*−*5*), the identity of the corresponding catalytic residue in Glu-AdT is still contentious. *Chlamydomonas reinhardtii* Glu-AdT (*9*), but not the enzyme from *Bacillus subtilis* (*10*), is sensitive to 6-diazo-5-oxonorleucine, a Cys-specific modifier that inactivates Cys-dependent glutaminases (*4*). However, Glu-AdT from *S. pyogenes* is devoid of Cys residues[2] and is not sensitive to 6-diazo-5-oxonorleucine or general Cys-directed reagents (M. R. Harpel and K. Y. Horiuchi, unpublished results). Importantly, alignment of the primary sequence of the A subunit from multiple organisms reveals a conserved "amidase signature" motif characteristic of bacterial amidases and mammalian fatty acid amide hydrolase (*2*, *11*−*13*). Included in this signature sequence is a conserved Ser-Lys catalytic diad (Figure 2). Although the catalytic roles of the conserved Ser and Lys as nucleophile (*13*, *14*) and activating base (*15*, *16*), respectively, have been confirmed in the latter enzymes, the potential roles of the cognate residues in Glu-AdT have not been experimentally verified.

Herein, we describe site-directed mutagenesis and mechanism-based inhibition analyses that implicate Ser176$_A$ as the active-site nucleophile for facilitating Gln hydrolysis by Glu-AdT. Coupling of inhibitor modality and potency between $\gamma$-Glu boronic acid (as an analogue of Gln) and Glu-tRNA$^{Gln}$/ATP cosubstrates is consistent with mechanism-based inhibition by this compound and extends our previous finding of inter-site communication between the various substrates and distinct chemical transformations catalyzed by Glu-AdT (*6*). These results also demonstrate the utility of targeting the glutaminase reaction for potent inhibition of this essential bacterial enzyme.

[2] K. C. Rogers, unpublished data.

## EXPERIMENTAL PROCEDURES

*Materials*. Recombinant *S. pyogenes* Glu-AdT was used throughout. As described previously (*6*), the heterotrimeric enzyme was heterologously expressed in *Escherichia coli* strain BL21(DE3) (Novagen) from plasmid pgat10, which harbors the *gat*CAB operon from *S. pyogenes* ATCC 12344D, and purified by a modification of the published procedure (*2*). The resultant Glu-AdT used in most experiments (except for comparative studies with mutant enzyme, vide infra) was >94% homogeneous by electrophoretic analysis (*6*). Glu-tRNA$^{Gln}$ (anticodon UUG) was produced from recombinant *B. subtilis* tRNA$^{Gln}$, overproduced in *E. coli*, and charged with Glu using partially purified *B. subtilis* Glu-tRNA synthetase (*2*), as described previously (*6*).

Glutamate oxidase from *Streptomyces* sp. and horseradish peroxidase (Type XII) were obtained from Sigma. Amplex red was purchased from Molecular Probes. $\gamma$-Glu boronic acid was synthesized as described (*17*) and dissolved in DMSO for use in assays. Other reagents were obtained from various vendors at the highest quality commercially available.

*Site-Directed Mutagenesis and Purification of Mutant Glu-AdT*. Site-directed mutagenesis of plasmid pgat10 was accomplished with the QuikChange kit from Stratagene and synthetic primers from Sigma Genosys [forward primer = 5′-pGACACTGGTGGTGCTATTCGGCAG-3′; reverse primer = 5′-pCTGCCGAATAGCACCACCAGTGTC-3′; underlined bases correspond to the site of mutation and result in a change in protein sequence from Ser to Ala at position 176 of the A subunit (S176$_A$A)]. The anticipated mutation was verified by DNA sequencing at the sequencing facility of Bristol-Myers Squibb Co. at Wilmington; no additional mutations were noted.

S176$_A$A and wild-type Glu-AdT were produced in parallel for comparative studies of the two proteins. Briefly, *E. coli* strain BL21(DE3) cells, transformed with wild-type or mutant expression plasmid, were cultured as 1:200 dilutions of overnight cultures into LB broth containing 50 $\mu$g/mL ampicillin. Following growth at 37 °C with shaking for 4.5 h ($OD_{650} \sim 0.4$), the cultures were induced with 0.1 mM isopropyl $\beta$-D-thiogalactoside and allowed to grow for another 2.5 h. Cells were harvested by centrifugation and stored at −80 °C until use. Wild-type and mutant enzymes used for comparative analyses were isolated by a smaller-scale, single-column version of the standard purification scheme. All steps were performed at 4 °C. Briefly, cells (2.5 g) were resuspended in a pH 7 buffer containing 25 mM HEPES, 10 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride. Following disruption by sonication, the supernatant from ultracentrifugation of this material was loaded onto a preequilibrated Q-Sepharose Fast-Flow column (16/10; Amersham Pharmacia Biotech) and eluted with a linear gradient (16 column volumes) from 0 to 500 mM NaCl in resuspension buffer (lacking phenylmethylsulfonyl fluoride). Wild-type and mutant enzyme each

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    Spyo_AdTA  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
rhodo_amidase  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~MSS LTPPNSNQMS
        hFAAH  MVQYELWAAL PGASGVALAC CFVAAAVALR WSGRRTARGA VVRARQRQRA

    Spyo_AdTA  ~~~~~MSFNH KTIEELHDLL VAKEISATEL TQATLEDIKS R....EEAVG
rhodo_amidase  ALNNHFRFG. LTTPELEEF. .APALEATLA SSETVERLYE R.TAPEPPQR
        hFAAH  GLENMDRAAQ RFRLQNPD.L DSEALLALPL PQ.LVQKLHS RELAPEAVLF

    Spyo_AdTA  SFIT.VSE.. ..EVALKQAA A...IDAKGI DADNLMSGIP LAVKDNISTK
rhodo_amidase  SWTSPTAD.. ..ENPLSAWY V...TTSISE TDEGPLAGRT VAVKDNVAVA
        hFAAH  TYVGKAWEVN KGTNCVTSYL ADCETQLSQA PRQGLLYGVP VSLKECFTYK

    Spyo_AdTA  EILTTAASKM LTNYEPIFNA TSVANAYAKD MIVIGKTNMD EFAMGGSTET
rhodo_amidase  GVPMMNGSRT VEGFTPRYDA TVVRRLLDAG ATITGKAVCE DLCFSGASFT
        hFAAH  GQDSTLGLSL NEGVPAECDS VVVHVLKLQG AVPFVHTNVP QSMFSYDCSN

    Spyo_AdTA  SYFKKTKNAW DHTKVPGGSS GGSATAVASG QVRLSLGSDT GGSIRQPAAF
rhodo_amidase  SHPQPVRNPW DESRITGGSS SGSGALVASG QVDMAVGGDQ GGSIRIPAAF
        hFAAH  PLFGQTVNPW KSSKSPGGSS GGEGALIGSG GSPLGLGTDI GGSIRFPSSF

    Spyo_AdTA  NGVVGLKPTY GTVSRYGL.. ..IAFGSSLD QIGPFAPTVK ENAQLLNVIA
rhodo_amidase  CGIVGHKPTH GLVPYTGA.. ..FPIERTID HLGPMTRTVS DAAAMLTVLA
        hFAAH  CGICGLKPTG NRLSKSGLKG CVYGQEAVRL SVGPMARDVE SLALCLRALL

    Spyo_AdTA  SSDV..KDAT SA.PVRIADY TSKIGRDIKG MKIALPKEYL GEGI.DPEIK
rhodo_amidase  GTDG..LDPR QTHRIEPVDY LAALAEPASG LRVGVVTEGF DTPVSDAAVD
        hFAAH  CEDMFRLDPT ....VPPLPF REEVYTSSQP LRVGY.YETD NYTMPSPAMR

    Spyo_AdTA  ETVLASVKQF EALGATVEEV ...SLPHSKY GVAVYYIIAS SEASSNLQRF
rhodo_amidase  NAVRTAIDVL RSAGLTVEEV ...SIPWHLD AMAVWNVIAT EGAA..YQML
        hFAAH  RAVLETKQSL EAAGHTLVPF LPSNIPHALE TLSTGGLF.S DGGHTFLQNF

    Spyo_AdTA  DGIRYGFRAD DAKNLDEI.Y VNTRSQGFGD EVKRRI.... MLGTFSLSSG
rhodo amidase  DGNAYGMNTD GFYDPELIAH FSRQRLEHGH QLSKTVKLVG MSGRYTSEVG
        hFAAH  KGDFVDPCLG DLVSILKLPQ WLKGLLAFLV KPLLPRLSAF LSNMKSRSAG

    Spyo_AdTA  YYDAYFKKAG QVRTLIIQDF DKVFADYDLI LGPTT.PTVA FGLDTLNHDP
rhodo_amidase  .GGKYYAMAR QLVPEVRAAY DAALARYDVL VMPTL.PYTA TKIPTTD.IP
        hFAAH  KLWELQHEIE VYRKTVIAQW RAL..DLDVV LTPMLAPALD LNAPGRATGA

    Spyo_AdTA  VA.MYLADLL .....TIPV. NLAGLPGISI P......AGF VD........
rhodo_amidase  LA.DYLDTAL SMIINTAPF. DVTGHPACSV P......ADL VH........
        hFAAH  VSYTMLYNCL DFPAGVVPVT TVTAEDEAQM EHYRGYFGDI WDKMLQKGMK

    Spyo_AdTA  ...GLPVGLQ LIGPKYAEET IYQAAAAFEA VTDYHKQQPI IFGGDK
rhodo_amidase  ...GLPTGMM IIGKHFDDAT VLRVAQLYEH AVGNYPVPPA AAGTLT
        hFAAH  KSVGLPVAVQ CVALPWQEEL CLRFMREVER LMTPEKQSS~ ~~~~~~
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FIGURE 2: Amino acid sequence alignment of *S. pyogenes* Glu-AdT A subunit with representative members of the amidase signature family. Sequences: "Spyo_AdTA" = A subunit of *S. pyogenes* Glu-AdT (Rogers, K. C., unpublished results); "rhodo_amidase" = *Rhodococcus rhodocrous* amidase, GenBank accession no. BAA03744; "hFAAH" = human fatty acid amide hydrolase, GenBank accession no. NP001432. Note that Ala90, Thr95, and Gly185 (underlined) in the *S. pyogenes* Glu-AdT A subunit are confirmed differences from the original genome sequence (Pro, Cys, and Ser in the original translation from the University of Oklahoma database for the *S. pyogenes* genome initiative, as published in ref *2*). Sequence identities are denoted in boldface. The amidase signature motif contained in all three sequences is enclosed by the box. Conserved Lys77 and Ser176 and corresponding residues of the Lys-Ser catalytic diad of bacterial amidase and fatty acid amide hydrolase are shown in large, boldface text.

eluted as a dominant peak at ~225 mM NaCl. Fractions substantially enriched in Glu-AdT, according to SDS−PAGE analysis, were pooled and dialyzed at 4 °C versus a pH 7 buffer containing 50 mM HEPES, 10 mM KCl, 10 mM $MgCl_2$, 50% glycerol, and 1 mM DTT. Samples were stored at −20 °C. The Glu-AdT variants produced by this scheme were of equal purity (~65%), as evaluated by Coomassie-blue staining and optical scanning of denaturing SDS−PAGE gels; this level of purity was sufficient for the described kinetic analyses (vide infra).

*Protein and Activity Assays*. Protein concentrations were determined by the Bradford dye-binding assay (*18*) with reagent obtained from BioRad. Bovine serum albumin served as the standard for this assay.

The transamidase-independent Gln hydrolysis activity of Glu-AdT was monitored by enzyme-coupled, fluorescence-based detection of Glu in quenched reaction aliquots. Reactions were carried out at 23 °C in a pH 7 buffer containing 50 mM HEPES, 15 mM $MgCl_2$, 25 mM KCl, 1% DMSO (for control and sample reactions in inhibition studies), 10 $\mu$g/mL (85 nM) Glu-AdT, and variable levels of Gln and inhibitor as indicated. HEPES does not complex borate and is therefore a suitable buffer for these studies (*19*). Reactions were initiated by addition of Gln and inhibitor,

unless otherwise indicated. To define reaction time courses, aliquots were periodically quenched with 25 mM HCl, and then neutralized with 25 mM NaOH. The level of Glu in these samples was quantified as fluorescent product (resorufin) accumulated after 60 min incubation with 30 $\mu$M Amplex Red, 0.04 unit/mL Glu oxidase, and 1.1 units/mL horseradish peroxidase, as described by McElroy et al. (*20*) and Zhou et al. (*21*). Reactions were standardized with authentic Glu in parallel reaction mixtures.

The transamidase and transamidase-coupled glutaminase activities of Glu-AdT were measured by the HPLC-based method described by Horiuchi et al. (*6*). Reactions were run under similar conditions as above, but contained 8.5 nM Glu-AdT and variable amounts of Glu-tRNA$^{Gln}$, ATP, and Gln (or $NH_4Cl$), as indicated in the text and figure legends. Reactions were generally initiated by addition of Gln (and inhibitor, where indicated). Aliquots were periodically quenched with 3 volumes of ice-cold ethanol, incubated for 15 min at −80 °C, and centrifuged. The supernatants (containing unreacted Gln substrate and Glu product of glutaminase activity) were separated from pellets (containing unreacted Glu-tRNA$^{Gln}$ substrate and Gln-tRNA$^{Gln}$ product of transamidase activity). To quantify glutaminase activity, the supernatants were dried by vacuum centrifugation, resuspended in a defined volume of pH 9 borate buffer, fluorescently derivatized, and subjected to amino acid analysis by reversed phase chromatography using AccQ Tag reagents from Waters. Chromatographic conditions were optimized to effect separation of derivatized Gln and Glu (*6*). To quantify transamidation activity (i.e., Gln-tRNA$^{Gln}$ formation), the pellets were washed 3 times with cold 75% ethanol, dried briefly, resuspended in pH 9 borate buffer, and incubated at 65 °C for 15 min to effect release of tRNA-associated Glu (from remaining Glu-tRNA$^{Gln}$ substrate) and Gln (from Gln-tRNA$^{Gln}$ product). Known volumes of the samples were then treated as above for HPLC analysis. As an alternative to centrifugation, total tRNA from ethanol-quenched reactions was collected on a 96-well, glass-fiber, vacuum filter plate (Millipore type B, 1 $\mu$m) and thoroughly washed with cold ethanol prior to hydrolysis and derivatization for HPLC analysis as described.

Theoretical data fits were obtained by iterative, nonlinear regression using Kaleidagraph (Synergy Software) or Grafit (Erithacus Software).

## RESULTS

*Site-Directed Mutagenesis of Conserved Ser176$_A$.* Sequence-conserved Ser176 in the A subunit was replaced with Ala (S176$_A$A) by site-directed mutagenesis to address the catalytic essentiality of the seryl hydroxyl at this position. When assayed under standard reaction conditions (50 $\mu$M Gln, 5 $\mu$M Glu-tRNA$^{Gln}$, and 1 mM ATP) at high enzyme concentrations (170 nM S176$_A$A versus 10 nM wild type), the transamidase and transamidase-coupled glutaminase activities of S176$_A$A were ≤0.3% and ≤0.1%, respectively, of the wild-type level. These are baseline levels of activity for each assay, as determined from reactions lacking enzyme. Thus, S176$_A$A is equally impaired (>300-fold) in both activities during Gln-dependent transamidation. The substrate concentrations used in these assays correspond to approximately 2-fold, ≥10-fold, and 7-fold excess over the measured $K_m$ values of wild-type enzyme for Gln, Glu-tRNA$^{Gln}$, and ATP, respectively (*6*); neither activity is enhanced by further increases in Glu-tRNA$^{Gln}$ or Gln concentration. Given the extreme increase (>1000-fold) in $K_m$ values required to account for these observations, the lower catalytic capacity of S176$_A$A likely reflects impairment of $k_{cat}$ from loss of a catalytic seryl hydroxyl.

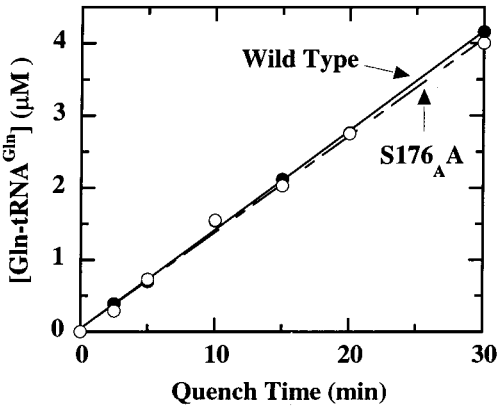


FIGURE 3: Use of exogenously supplied $NH_3$ for transamidation of Glu-tRNA$^{Gln}$ by wild-type (●) and S176$_A$A (○) Glu-AdT. Enzyme-catalyzed production of Gln-tRNA$^{Gln}$ from Glu-tRNA$^{Gln}$ (10 $\mu$M) was monitored by the HPLC method described under Experimental Procedures, except that Gln was replaced by 200 mM $NH_4Cl$ as amide donor. S176$_A$A and wild-type Glu-AdT concentrations were each 0.2 $\mu$M. The ATP concentration was 1 mM.

As previously shown for a number of glutaminases (*3*) and Glu-AdT from various sources (*2*, *9*, *10*), *S. pyogenes* Glu-AdT can utilize exogenously supplied $NH_3$ in place of Gln-derived $NH_3$ to support transamidation. This activity requires high levels of $NH_4Cl$ and elicits a lower catalytic rate than when Gln is provided as nitrogen donor; in our hands, the reaction velocity at 200 mM $NH_4Cl$ was 2−5% of the activity realized under $V_{max}$ conditions with Gln as the nitrogen donor. However, use of exogenous $NH_4Cl$ provides a means of bypassing the glutaminase reaction and thereby independently evaluating the catalytic competency of Glu-AdT mutants in other aspects of catalysis. S176$_A$A exhibits a level of activity indistinguishable from wild-type enzyme under these conditions (Figure 3). Therefore, the catalytic machinery for Glu-tRNA$^{Gln}$ activation and transamidation (including ATP and Glu-tRNA$^{Gln}$ binding) are intact in S176$_A$A, localizing the catalytic impairment of this mutant to Gln hydrolysis.

The competency of S176$_A$A in $NH_4Cl$-promoted transamidation demonstrates the structural integrity of the recombinant S176$_A$A enzyme. Coelution of S176$_A$A with wild-type Glu-AdT during denaturing and nondenaturing electrophoresis further indicates that the mutant enzyme is properly assembled into a heterotrimer of full-length subunits. Quantitative scanning of Coomassie-blue-stained denaturing gels shows that the ratio of C subunit to A and B subunits (the latter two are unresolved during electrophoresis) is the same for S176$_A$A as for wild-type Glu-AdT (data not shown).

*Inhibition of Transamidase-Independent Gln Hydrolysis by γ-Glu Boronic Acid.* Following a 15 min preincubation with Glu-AdT, γ-Glu boronic acid blocks the transamidase-independent glutaminase activity of Glu-AdT with $IC_{50}$ = 1.6 $\mu$M at a concentration of Gln approximately twice $K_m$ (*17*). Because boronic acid-based inhibitors of Ser-based

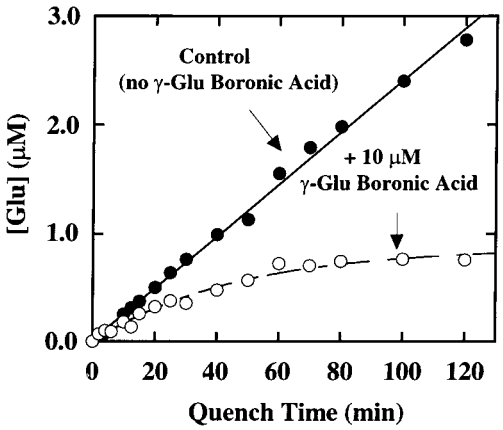


FIGURE 4: Representative reaction time courses for the Glu-tRNA$^{Gln}$/ATP-independent glutaminase activity of Glu-AdT in the absence (●) and presence (○) of $\gamma$-Glu boronic acid (10 $\mu$M). Reaction conditions and discontinuous detection of Glu formation by the coupled fluorometric assay were as described under Experimental Procedures. Both reactions contained 25 $\mu$M Gln. The fits to the data (described under Results) provided the following values: initial velocity = 0.024 ± 0.001 $\mu$M/min in the absence of $\gamma$-Glu boronic acid; initial velocity = 0.019 ± 0.001 $\mu$M/min and $k_{obs}$ = 0.022 ± 0.002 min$^{-1}$ (0.0004 s$^{-1}$) in the presence of $\gamma$-Glu boronic acid.

hydrolases frequently exhibit time-dependent inhibition due to conformational adjustments attendant to formation of a semistable tetrahedral adduct (e.g., refs *22*, *23*), we further characterized the kinetics of Glu-AdT inhibition by $\gamma$-Glu boronic acid to better gauge its potency and mechanism as an inhibitor of Gln hydrolysis.

In the absence of preincubation (i.e., initiation of reaction by simultaneous addition of Gln and inhibitor), the rate of Glu formation by Glu-AdT steadily declines with time in the presence of $\gamma$-Glu boronic acid. This decline in activity contrasts sharply with the linearity exhibited in a control reaction lacking inhibitor (Figure 4), and is described by a slow-onset inhibition function (eq 1), where $v_s$ is the terminal steady-state velocity, $v_i$ is the initial velocity, and $k_{obs}$ is the pseudo-first-order rate constant for onset of inhibition (*24*−*26*).

$$[P] = v_s t + [(v_i - v_s)/k_{obs}](1 - e^{-k_{obs}t}) \qquad (1)$$

The steady-state (final) phase for reactions containing $\gamma$-Glu boronic acid is characterized by an extremely low rate of Glu formation. Accordingly, the best fits to these data (at all concentrations of Gln and inhibitor tested; data not shown) are obtained by fixing $v_s = 0$. Functionally, this assumption is validated by the attainment of complete inhibition and very low reversal that occurs over these time frames (vide infra).

The time-dependency of $\gamma$-Glu boronic acid inhibition was verified and quantified by monitoring the loss of activity upon preincubation of Glu-AdT with inhibitor for various periods of time. As shown in Figure 5, postincubation glutaminase levels decrease with preincubation time according to a slow, single-exponential decay function. The final activity level (~11%) mirrors the expected amount of free enzyme remaining after formation of a stable, 1:0.9 complex between enzyme (10 $\mu$M) and inhibitor (9 $\mu$M) during preincubation. According to the data fit, formation of the final, inhibited complex occurs with a half-time of ~0.6 h and a slow, second-order rate constant, $k$/[I] = 35 M$^{-1}$ s$^{-1}$.

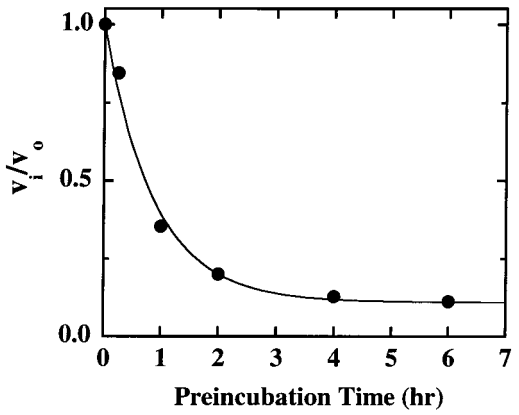


FIGURE 5: Onset of $\gamma$-Glu boronic acid inhibition of Glu-AdT. Glu-AdT (10 $\mu$M) was preincubated with inhibitor (9 $\mu$M) in the absence of Gln. At the indicated times, aliquots were diluted 1:100 into a reaction mixture containing Gln (100 $\mu$M final), mixed, and assayed for residual Glu-tRNA$^{Gln}$/ATP-independent glutaminase activity by the fluorometric analysis described under Experimental Procedures. The *y*-axis values, $v_i$ and $v_o$, refer to the initial velocities of parallel samples containing and lacking inhibitor, respectively. Dilution of enzyme and inhibitor resulted in carry-over of 90 nM inhibitor [~6% of $IC_{50}$ at 50 $\mu$M Gln following 15 min preincubation (*17*)] into the reaction. The line through the data corresponds to a single-exponential function with a residual component: $k_{obs}$ = 1.14 ± 0.13 h$^{-1}$ (0.0003 s$^{-1}$); residual activity = 10.9 ± 2.8%; $R^2$ = 0.992.

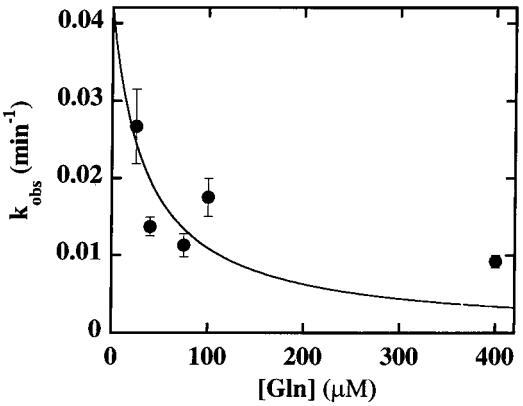


FIGURE 6: Competition of $\gamma$-Glu boronic acid inhibition by Gln (Glu-tRNA$^{Gln}$/ATP-independent glutaminase activity). Reaction time courses were initiated by simultaneous addition of Gln and $\gamma$-Glu boronic acid to Glu-AdT and monitored as in Figure 4. The inhibitor concentration was maintained at 10 $\mu$M throughout. The $k_{obs}$ values were obtained from fits to individual time courses according to eq 1 under Results, assuming a single-exponential process and zero steady-state (second-phase) velocity. The error bars combine the variation in $k_{obs}$ from exponential fits and range of values obtained in multiple determinations. The solid line through the data is a best fit to the data according to eq 2 under Results with the $K_m$ value for Gln fixed at 35 $\mu$M, as previously reported (*6*) and verified in parallel control reactions. According to this fit, $k_{obs,max}$ = 0.042 ± 0.006 min$^{-1}$ (0.0007 ± 0.0001 s$^{-1}$).

Competition with substrate was determined by measuring $k_{obs}$, the pseudo-first-order rate constant for onset of inhibition, from progress curve analyses at constant inhibitor and varying Gln concentrations. Despite data scatter due to technical difficulties in fully defining progress curves at all substrate concentrations and within time frames that maintain linearity in control reactions, $k_{obs}$ decreases systematically with increasing Gln concentration, as expected for competitive binding of Gln and $\gamma$-Glu boronic acid (Figure 6). The second-order rate constant obtained from fitting these data to the appropriate equation (eq 2) is $k_{max}$/[I] = 70 M$^{-1}$ s$^{-1}$.

$$k_{obs} = k_{max}/(1 + [Gln]/K_m) \quad (2)$$

At a subsaturating concentration of Gln (25 $\mu$M), $k_{obs}$ increases linearly with $\gamma$-Glu boronic acid concentration up to at least 25 $\mu$M (Figure 7). This is the highest concentration of inhibitor at which the onset rate could accurately be measured under these conditions. The slope of this line provides an apparent second-order rate constant of 60 $M^{-1}$ $s^{-1}$ (eq 3). Adjustment for competitive inhibition with respect to Gln (eq 4) raises this value to 102 $M^{-1}$ $s^{-1}$, which is higher, yet still in reasonable agreement with estimates from our other determinations.

$$k_{obs} = k_{off} + k_{on,app}[I] \quad (3)$$

$$k_{on} = k_{on,app}/(1 + [Gln]/K_m) \quad (4)$$

The *y*-intercept from Figure 7 should provide an estimate for the rate of inhibitor dissociation from the terminal complex with Glu-AdT. The high error of the linear fit and uncertainty in $k_{obs}$ values at high inhibitor concentration preclude such definitive assignment from these data, but places a maximum limit of $k_{off} < 6 \times 10^{-5}$ $s^{-1}$ and minimum $t_{1/2} > 2.3$ h for inhibitor dissociation.

In contrast to the dependence of $k_{obs}$ on inhibitor concentration, the corresponding initial velocities are invariant throughout the same concentration range (Figure 7, inset).

*Reversibility of Inhibited Complex.* Although $\gamma$-Glu boronic acid competes with Gln for initial binding to Glu-AdT, release of inhibitor from the enzyme occurs very slowly (if at all) in the absence of Glu-tRNA$^{Gln}$ and ATP. This slow reversal was confirmed by diluting a preformed complex of Glu-AdT and $\gamma$-Glu-boronic acid into a fresh reaction mixture containing a kinetically saturating concentration of Gln, as shown for a representative data set in Figure 8. Despite a low carry-over level of $\gamma$-Glu boronic acid upon dilution ($<0.1 \times IC_{50}$), the enzyme recovers little activity over the linear response time of a parallel control reaction incubated only with DMSO. Although the minor reversal of inhibitor binding is insufficient for an accurate fit to eq 1, it reveals that the $t_{1/2}$ for inhibitor dissociation is >2.5 h. The inhibitor-containing reaction can also be described by a straight line approximating 10% of control activity, indicative of minimal inhibitor release from the 1:0.9 complex formed during preincubation between excess Glu-AdT (10 $\mu$M) and $\gamma$-Glu boronic acid (9 $\mu$M). Either interpretation of these data validates the assumption of $v_s \sim 0$ used in obtaining $k_{obs}$ from progress curve analysis (e.g., Figure 4).

In contrast, complete restoration of activity occurs within 2 min of diluting the preformed complex between Glu-AdT and $\gamma$-Glu boronic acid into a reaction mixture that contains kinetically saturating levels of Glu-tRNA$^{Gln}$ and ATP in addition to Gln (data not shown). Neither Glu-tRNA$^{Gln}$ nor ATP alone was sufficient to reverse inhibitor binding at saturating Gln concentration.

*Steady-State Inhibition of Glu-tRNA$^{Gln}$ Transamidation.* Because $\gamma$-Glu boronic acid binding by Glu-AdT is readily reversed in the presence of all three substrates, steady-state analysis was used to verify the nature of inhibitor interaction during transamidation. Inhibition by $\gamma$-Glu boronic acid is competitive with respect to Gln (average $K_i = 73 \pm 13$ nM from multiple determinations) at saturating concentrations

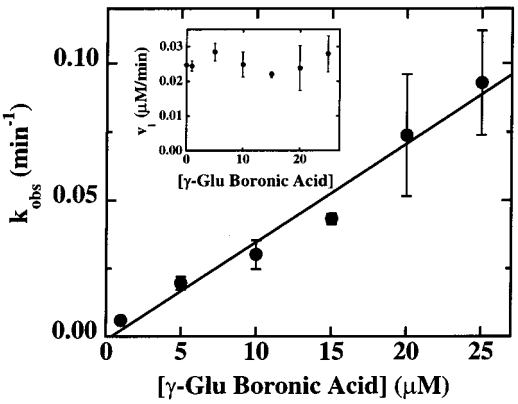


FIGURE 7: Dependence of inhibition onset rate ($k_{obs}$) and initial velocity (inset) on $\gamma$-Glu boronic acid concentration (Glu-tRNA$^{Gln}$/ATP-independent glutaminase activity). Reactions were initiated by simultaneous addition of inhibitor (variable concentration) and Gln (25 $\mu$M) to Glu-AdT in reaction buffer, and monitored for formation of Glu by the discontinuous fluorometric assay described under Experimental Procedures. The individual time courses were fit to a single exponential (eq 1 under Results) with $v_s = 0$ in order to obtain $k_{obs}$ and initial velocity. The error bars represent the inherent error in $k_{obs}$ and the initial velocity from these fits. The line through the $k_{obs}$ versus inhibitor data is a linear fit with slope = 0.0036 ± 0.0003 $\mu M^{-1}$ $min^{-1}$ (60 $M^{-1}$ $s^{-1}$) and intercept = −0.0013 ± 0.0047 $min^{-1}$ (−0.00002 ± 0.00008 $s^{-1}$).

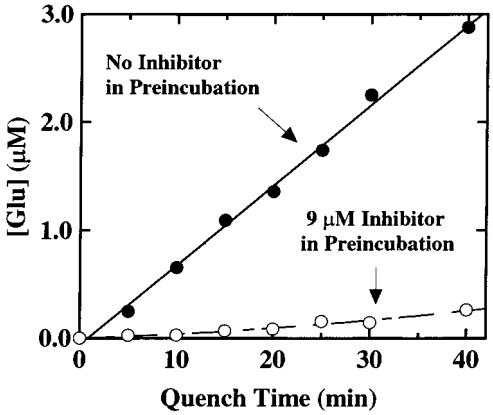


FIGURE 8: Reversibility of $\gamma$-Glu boronic acid binding to Glu-AdT. Glu-AdT (10 $\mu$M) was incubated for 4 h in the absence (●) and presence (○) of a substoichiometric amount of $\gamma$-Glu boronic acid (9 $\mu$M) at 1% DMSO throughout. Glutaminase activity was measured by the fluorometric method described under Experimental Procedures after 100-fold dilution into otherwise complete reaction mixtures containing 100 $\mu$M Gln, but lacking Glu-tRNA$^{Gln}$ and ATP. Linear regression provides activities of 0.074 $\mu$M/min (absence of inhibitor) and 0.006 $\mu$M/min (presence of inhibitor; fit not shown). The line shown through the inhibitor-containing sample is an attempted fit to eq 1 with the following parameters: initial velocity = 0.003 (±0.003) $\mu$M/min, steady-state velocity = 0.043 (±1.05) $\mu$M/min, $k_{off}$ = 0.005 (±0.13) $min^{-1}$, $R^2$ = 0.968. The large errors in these parameters reflect the slow dissociation of inhibitor from this complex.

of Glu-tRNA$^{Gln}$ and ATP (representative pattern shown in Figure 9). In contrast, less than a 2-fold difference in $IC_{50}$ was observed when ATP was varied from subsaturating ($0.3 \times K_m$) to saturating ($7 \times K_m$) levels with the other two substrates maintained at kinetically saturating concentrations (data not shown). Thus, inhibition with respect to ATP appears to be noncompetitive. Similar analysis with respect to Glu-tRNA$^{Gln}$ was complicated by its low $K_m$ ($\leq 0.2$ $\mu$M) and difficulties in measuring pseudo-first-order rates at subsaturating concentrations of this substrate (*6*). However,

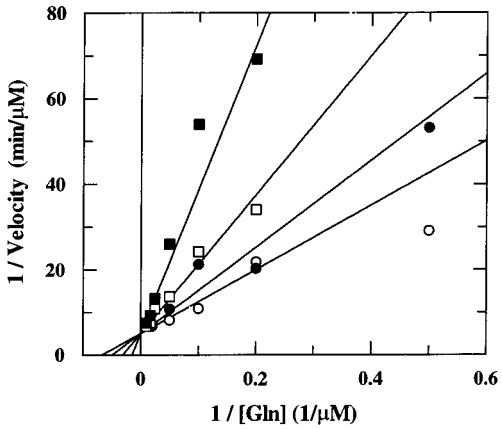


FIGURE 9: Representative pattern for steady-state inhibition of Glu-AdT transamidation activity by $\gamma$-Glu boronic acid. Glu-tRNA$^{Gln}$ transamidation by Glu-AdT was measured according to the HPLC method described under Experimental Procedures. All reactions were initiated by simultaneous addition of Gln (2−1000 $\mu$M) and $\gamma$-Glu boronic acid (○ = 0 nM; ● = 30 nM; □ = 100 nM; ■ = 300 nM) to a solution of Glu-AdT (8.5 nM), Glu-tRNA$^{Gln}$ (10 $\mu$M), and ATP (1 mM). Lines drawn through the data were obtained by global fitting of $v_i$, [Gln], and [$\gamma$-Glu boronic acid] to the Henri−Michaelis−Menten equation, modified for competitive inhibition, using Grafit (Erithacus Software). From this data set, $K_i = 86 \pm 16$ nM; the average of multiple determinations provides $K_i = 73 \pm 13$ nM. The global fit was transformed as a double-reciprocal plot for display purposes.

less than 2-fold variation in $IC_{50}$ was observed when Glu-tRNA$^{Gln}$ was varied between 0.15 $\mu$M ($\sim K_m$) and 5 $\mu$M ($\sim 25 \times K_m$), which could reflect either noncompetitive or uncompetitive inhibition.

## DISCUSSION

Bacteria that lack a functional Gln-tRNA synthetase require Glu-AdT to sustain protein synthesis. An essential component of this process is hydrolysis of Gln by Glu-AdT, which produces a sequestered source of $NH_3$ for kinetically coupled transamidation of Glu-tRNA$^{Gln}$. Specific inhibition of Gln hydrolysis should therefore disrupt transamidation and significantly impede in vivo protein synthesis. However, details of the Gln hydrolysis reaction have been sparse. Our current results lay the groundwork for understanding the molecular details of this reaction: site-directed mutagenesis pinpoints the catalytic essentiality of Ser176$_A$ to Gln hydrolysis, and inhibition by $\gamma$-Glu boronic acid implicates nucleophilic assistance by an active-site Ser and also validates targeted inhibition of Gln hydrolysis as an effective, mechanism-based means of blocking transamidation.

Although $NH_3$ is the formal nitrogen donor for transamidation of Glu-tRNA$^{Gln}$, its in vivo source is the hydrolysis of Gln by Glu-AdT. The enzyme exhibits a large kinetic preference for Gln, versus $NH_3$ or other nitrogen donors such as Asn (refs *2*, *7*, *9*, *10*; Y. Luo, L. Shen, K. Y. Horiuchi, and M. R. Harpel, unpublished data), and displays strong kinetic coupling between Gln hydrolysis and transamidation (*6*). Specifically, $k_{cat}$ for Gln hydrolysis is only maximized when all substrates are present for Glu-tRNA$^{Gln}$ transamidation, with minimal impact on the apparent $K_m$ for Gln. In this regard, Glu-AdT resembles other glutaminases that couple Gln hydrolysis to amidation of activated acceptor substrates. Most of these enzymes employ an active-site Cys for nucleophilic catalysis of the hydrolysis reaction (reviewed in refs *3*−*5*). However, *S. pyogenes* Glu-AdT lacks Cys residues[2] and contains a conserved Ser (Ser176$_A$) within an "amidase-family" sequence signature motif found in bacterial amidases and mammalian fatty acid amide hydrolase (Figure 2). Based on its analogous position with the active-site Ser of these enzymes, we anticipated that Ser176$_A$ acts as the catalytic nucleophile during Gln hydrolysis. Hence, this residue should be critical for Glu-AdT activity.

Our mutagenesis results with *S. pyogenes* Glu-AdT support such a role for Ser176$_A$. Replacement by Ala reduces $k_{cat}$ at least 300-fold (below detection limits) in both the transamidase-coupled Gln hydrolysis and the glutaminase-promoted transamidation activities, even at substrate concentrations exceeding saturation for wild-type enzyme. Therefore, the mutant is impaired in chemical steps associated with Gln hydrolysis, per se, rather than substrate capture. Furthermore, the high level of $NH_4Cl$-promoted transamidation exhibited by this mutant localizes the full impact of loss of the seryl hydroxyl to impairment of Gln hydrolysis. These observations are in accord with prior mutagenesis studies of the sequence-conserved, active-site Ser in other amidase family members. In particular, mutagenesis to Ala completely abolishes the hydrolytic activity of *R. rhodocrous* J1 amidase (*14*) and elicits a $10^6$-fold decrease in activity for fatty acid amide hydrolase (*13*) with no impact on the substrate $K_m$ in either case. For fatty acid amide hydrolase, assignment of the implicated Ser as catalytic nucleophile was additionally confirmed by kinetic and protein chemistry studies with a chemically reactive substrate analogue (*13*).

Another characteristic of the amidase signature family is the use of an active-site Lys as general base to activate the Ser hydroxyl for nucleophilic catalysis (*15*, *16*). This property is shared with a larger group of hydrolases that either employ a Ser (or Thr)-Lys catalytic diad or an N-terminal Ser/Thr nucleophile in combination with its α-amine as general base (reviewed in ref *27*). This larger family includes such varied enzymes as type I signal peptidase, UmuD protease, penicillin-binding proteins, $\beta$-lactamases (classes A, C, and D[3]), penicillin acylase, and the 20S proteasome (e.g., see refs *28*−*38*). Among this diverse group, Glu-AdT stands out as the only enzyme to couple substrate hydrolysis to an acceptor reaction and to use Gln as the primary hydrolytic substrate. Although Glu-AdT contains a conserved Lys (Lys77$_A$) at the appropriate spacing in its primary sequence (Figure 2), whether this Lys fulfills a similar catalytic role remains to be determined experimentally.

To gain additional insight into the mechanism of Gln hydrolysis and further probe the involvement of an active-site nucleophile, we characterized the kinetics of Glu-AdT inhibition by $\gamma$-Glu boronic acid. Boronic acid-substituted analogues have been widely used as potent inhibitors and mechanistic probes of hydrolases that employ an active-site Ser or Thr nucleophile (e.g., see refs *22*, *23*, *39*−*42*). With specificity driven by the degree of similarity with substrate, the potency of these inhibitors is driven by the propensity of the boronic functionality to form a tetrahedral adduct with

[3] Of note, the essential Lys is carbamylated in the active form of class D $\beta$-lactamase from *Pseudomonas aeruginosa* (*28*, *29*), but not in class A or class C $\beta$-lactamases (personal communication of Prof. Shahriar Mobashery, Wayne State University). Whether lysyl carbamylation occurs in other hydrolases that contain the Ser-Lys catalytic diad has yet to be fully explored.

the active-site hydroxyl and exploit "oxyanion hole" interactions normally used for transition-state stabilization during catalysis. Inhibition by some boronate-containing inhibitors has been correlated with a His−boronate instead of Ser−boronate interaction for proteases containing a Ser-Asp-His catalytic triad (*43*, *44*). However, such compounds are generally weak inhibitors, not close mimics of substrate, and lack the slow-onset, tight-binding characteristics observed for inhibitors that interact through a catalytic Ser (*22*, *23*). Of particular interest to our goal of developing specific inhibitors of Glu-AdT, boronates lack potency toward hydrolases that employ an active-site Cys nucleophile, reflecting less stabilization of the tetrahedral adduct formed with cysteinyl $\gamma$-thiolate versus seryl $\gamma$-alkoxide (*45*).

As we previously reported (*17*), $\gamma$-Glu boronic acid is the most potent inhibitor of Glu-AdT yet reported. It has also been characterized as an effective (*19*), mechanism-based (*46*) inhibitor of $\gamma$-glutamyl transpeptidase, a Thr-based hydrolase (*47*). For Glu-AdT, $\gamma$-Glu boronic acid exhibits slow-onset (50−100 $M^{-1}$ $s^{-1}$) and tight-binding ($t_{1/2}$ > 2.5 h for complex dissociation) inhibition in the absence of ATP and Glu-tRNA$^{Gln}$. Interestingly, the inhibition lacks time-dependency, is rapidly reversible, and maintains high potency under full transamidation conditions. Under both conditions, Gln protects the enzyme from inhibition. We feel that these observations represent a mechanism-based mode of inhibition effected through interactions at the glutaminase active site.

Slow-onset, tight-binding inhibition of the Glu-tRNA$^{Gln}$/ATP-independent ("uncoupled") glutaminase activity of Glu-AdT can be viewed in the context of two general mechanisms for time-dependent inhibition (reviewed in refs *25*, *26*, and *48*). In the first case, these effects are manifested through slow inhibitor association and dissociation, respectively, in a single-step binding reaction. Reflective of this slow equilibration, $k_{obs}$ (the pseudo-first-order rate constant for inhibition onset) increases linearly with respect to inhibitor concentration, whereas the initial velocity (phase prior to onset) is unaffected. In the second mechanism, rapid equilibration of enzyme and inhibitor produces an initial encounter complex (EI). The slow onset of inhibition then results from a subsequent, rate-limiting isomerization of EI to a stable EI* form, described by a terminal equilibrium constant, $K_i$*. Because inhibitor binding precedes a rate-limiting step, plots of $k_{obs}$ versus inhibitor concentration are hyperbolic, and initial velocity varies as a function of inhibitor concentration. For Ser- and Thr-hydrolases, this second, slow kinetic step has been attributed to enzyme "reorganization", conformational change, or other rate-limiting changes attendant to covalent complexation and stabilization of the boronic acid functionality by the active-site hydroxyl (e.g., see refs *22*, *39*, and *49*).

Our results with Glu-AdT best fit the simple equilibrium-binding mechanism, although $K_i$ is ill-determined due to the extremely slow off-rate of inhibitor.[4] However, the two inhibition mechanisms may not be distinguished under conditions germane to our studies. In particular, practical limitations in studying inhibitor at concentrations greater than 25 $\mu$M may have excluded observation of kinetic saturation for $k_{obs}$ or an effect on initial velocity. Under these conditions (i.e., $K_i \gg 25\ \mu M \gg K_i$*), the apparent $K_i$ of the initial encounter complex in a two-step isomerization mechanism would therefore be at least 100 $\mu$M. Whether resulting from equilibration via simple binding (in which initial binding may be sufficiently slow to mask an isomerization required for tight binding) or conformational adjustment after low-affinity binding, the slow attainment of a tight-binding inhibitory state is highly suggestive of inhibitor complexation by an active-site nucleophile (presumably Ser) in the glutaminase site.[5]

Under full transamidation conditions, the apparent potency of $\gamma$-Glu boronic acid inhibition is enhanced[4] and undergoes a switch from essentially irreversible to rapidly exchangeable binding. The lack of tolerance for steric and isoelectronic variants of $\gamma$-Glu boronic acid (*17*) precludes correlative analysis of inhibitor potency versus catalytic efficiencies for a comparable, structurally diverse group of $\gamma$-Glu boronic acid and Gln derivatives, as required to thoroughly demonstrate transition state mimicry (*50*). However, substrate- and reaction-dependent modulation of inhibitor effects is reminiscent of the kinetic coupling between Gln hydrolysis and transamidation that we noted previously (*6*), and suggests that the inhibitor may interact with Glu-AdT in a manner that is similar to an intermediate along the normal reaction pathway.

Gln, Glu-tRNA$^{Gln}$, and ATP (or the slowly hydrolyzed analogue ATP$\gamma$S) are all required for full realization of $k_{cat}$ in Gln hydrolysis (*6*). We offered two possible explanations for this kinetic coupling of reactions: (1) an allosteric communication between glutamination and transamidation sites that is optimal only in the presence of all substrates; and (2) an inter-site communication resulting from ATP hydrolysis or some subsequent event, such as formation of the reactive, phosphorylated Glu-tRNA$^{Gln}$ intermediate. Because neither Glu-tRNA$^{Gln}$ nor ATP alone (in combination with saturating Gln) induces reversal of $\gamma$-Glu boronic acid binding, inhibitor release may similarly be induced by synergistic binding of all three substrates (with Gln competing directly for the glutaminase-binding site) or by a catalytic event coupled to transamidation. We cannot rigorously exclude cumulative allosteric effects of ATP and Glu-tRNA$^{Gln}$ binding on inhibitor interactions in the active site. However, a link with catalytic effects is especially intriguing due to the presumed transition-state mimicry of the boronic acid inhibitor. In this case, the stable, exchange-inert complex formed in the absence of Glu-tRNA$^{Gln}$ and ATP might exploit optimized transition-state interactions that are coupled to events that occur on the transamidation reaction pathway beyond the point of Gln hydrolysis. Indeed, similar "over-stabilization" of these interactions for the transition state of Gln hydrolysis under uncoupled reaction conditions may account for the ~300-fold lower rate of hydrolysis in the absence of transamidation (*6*). The enzyme's kinetic com-

[4] Based on the $IC_{50}$ reported in our previous study (*17*) and the limiting estimates of $k_{on}$ (43−102 $M^{-1}$ $s^{-1}$) and $k_{off}$ (<$6 \times 10^{-5}$ $s^{-1}$) reported herein, we roughly estimate $K_i \sim 0.5-1.4$ $\mu$M for inhibition of uncoupled Gln hydrolysis according to a simple binding mechanism. This compares with $K_i = 73$ nM under transamidation conditions.

[5] An alternative mechanism for the time-dependent inhibition is rate-limiting isomerization of enzyme *prior* to inhibitor binding. However, the observed increase in $k_{obs}$ with inhibitor concentration for Glu-AdT is inconsistent with such a mechanism. Although we cannot eliminate rate-limiting isomerization of inhibitor as an additional possibility for slow-onset inhibition, we feel that such slow rearrangements of $\gamma$-Glu boronic acid are unlikely.

mitment to forward catalysis under transamidation conditions would be manifested in enhanced inhibitor potency and an increase in off-rate for $\gamma$-Glu boronic acid. Regardless of the mechanistic details, we feel that the coincidence of inhibition effects and the kinetic coupling observed between reactions is indicative of mechanism-based inhibition by $\gamma$-Glu boronic acid arising from regulated interactions within the glutaminase active-site.

*Concluding Remarks*. In providing a kinetically regulated source of $NH_3$ for transamidation of Glu-tRNA$^{Gln}$, the coupled glutaminase reaction of Glu-AdT is central to the critical physiological function of this enzyme. Not only are the multiple components of Gln-tRNA$^{Gln}$ formation linked from a chemical perspective, they are also linked kinetically and potentially modulated through reaction intermediates. Our current results suggest that $\gamma$-Glu boronic acid exploits this modulation to efficiently inhibit Glu-tRNA$^{Gln}$ transamidation by engaging the predicted catalytic Ser nucleophile of the glutaminase active site. We cannot definitively state that Ser176 is the catalytic nucleophile involved in Gln hydrolysis and inhibition by $\gamma$-Glu boronic acid. In the absence of a three-dimensional visualization of the enzyme caught in a covalent complex with inhibitor, we can only infer from our kinetic analyses that this is likely to be true. However, site-directed mutagenesis clearly shows the essentiality of this residue for Gln hydrolysis, and our kinetic results are consistent with the involvement of such a nucleophile in mechanism-based inhibition. Crystallographic determination of such a complex or protein-chemistry analysis with inhibitors able to form irreversible, covalent complexes with the enzyme will ultimately be required to unambiguously demonstrate that Ser176$_A$ is the primary nucleophile in this reaction. With respect to the potential for novel antibacterials, the realization of potent mechanism-based Glu-AdT inhibition, microbial growth inhibition, lack of mammalian cytotoxicity,[6] and suggestions of in vivo target specificity[6,7] by $\gamma$-Glu boronic acid are encouraging, despite a limiting structure−activity relationship among analogues (*17*). Future development of inhibitors centered on this strategy must address these limitations through design of compounds able to maintain the favorable interactions of this class of inhibitors while exploiting other unique aspects of the Glu-AdT glutaminase site to effect greater specificity and structural diversity.

[6] Consistent with our design tenet, *E. coli* carbamoyl phosphate synthetase (a Cys-based glutaminase) is not inhibited by 100 $\mu$M $\gamma$-Glu boronic acid (data not shown). Gln synthetase, which activates Glu by phosphorylation and catalyzes the aminolysis of $\gamma$-Glu phosphate intermediate with free $NH_3$ instead of $NH_3$ derived from a coupled Gln hydrolysis reaction, is only weakly ($K_i \sim 30$ $\mu$M for *E. coli* enzyme) or not at all (sheep brain enzyme) inhibited by $\gamma$-Glu boronic acid (unpublished data of Dr. Lynn M. Abell, Bristol-Myers Squibb). However, the compound inhibits the Thr-based hydrolase, $\gamma$-Glu transamidase ($K_i = 35$ nM) (*19*, *46*). Because the isolation of mammalian mitochondrial Glu-AdT has not yet been reported, we have not directly tested this enzyme for inhibition by $\gamma$-Glu boronic acid. Nonetheless, the inhibitor does not promote general eukaryotic cytotoxicity according to two distinct assays (*17*).

[7] $\gamma$-Glu boronic acid inhibits growth of bacterial species that require Glu-AdT for cell viability, including *S. pyogenes* (*17*). In preliminary experiments, we were unable to recover growth-competent mutants of *S. pyogenes* after culturing up to $10^9$ colony-forming units on medium containing up to 8 times the minimal inhibitory concentration of $\gamma$-Glu boronic acid (L. D. Ecret, K. C. Rogers, and M. J. Rogers, unpublished data). Although there could be a number of reasons for this result, it may indicate a low resistance frequency due to on-target in vivo specificity of inhibitor action. Given our proposed in vitro mode of action, mutations in Glu-AdT that are capable of overcoming critical binding interactions with $\gamma$-Glu boronic acid might also disrupt essential (and potentially identical) interactions with the transition state or reaction intermediate that is being mimicked. Such disruption could cause sufficient catalytic impairment to render the Glu-AdT mutant incapable of supporting cell viability. Proof of this postulate will require further study, in particular the isolation of resistant mutants, demonstration that resistance specifically maps to mutation of the *gat*CAB operon (presumably in the gene encoding the A subunit), and demonstration of reduced susceptibility of an in vitro produced mutant Glu-AdT to $\gamma$-Glu boronic acid.

## ACKNOWLEDGMENT

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## REFERENCES

1. Ibba, M., Curnow, A. W., and Söll, D. (1997) *Trends Biochem. Sci. 22*, 39−42.
2. Curnow, A. W., Hong, K.-W., Yuan, R., Kim, S.-I., Martins, O., Winkler, W., Henkin, T. M., and Söll, D. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 11819−11826.
3. Zalkin, H. (1993) *Adv. Enzymol. Relat. Areas Mol. Biol. 66*, 203−309.
4. Massière, F., and Badet-Denisot, M.-A. (1998) *Cell. Mol. Life Sci. 54*, 205−222.
5. Zalkin, H., and Smith, J. L. (1998) *Adv. Enzymol. Relat. Areas Mol. Biol. 72*, 87−144.
6. Horiuchi, K. Y., Harpel, M. R., Shen, L., Luo, Y., Rogers, K. C., and Copeland, R. A. (2001) *Biochemistry 40*, 6450−6457.
7. Wilcox, M., and Nirenberg, M. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 229−236.
8. Wilcox, M. (1969) *Eur. J. Biochem. 11*, 405−412.
9. Jahn, D., Kim, Y.-C., Ishino, Y., Chen, M.-W., and Söll, D. (1990) *J. Biol. Chem. 265*, 8059−8064.
10. Strauch, M. A., Zalkin, H., and Aronson, A. I. (1988) *J. Bacteriol. 170*, 916−920.
11. Mayaux, J. F., Cerebelaud, E., Soubrier, F., Faucher, D., and Petre, D. (1990) *J. Bacteriol. 172*, 6764−6773.
12. Chebrou, H., Bigey, F., Arnaud, A., and Galzy, P. (1996) *Biochim. Biophys. Acta 1298*, 285−293.
13. Patricelli, M. P., Lovato, M. A., and Cravatt, B. F. (1999) *Biochemistry 38*, 9804−9812.
14. Kobayashi, M., Fujiwara, Y., Goda, M., Komeda, H., and Shimizu, S. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 11986−11991.
15. Patricelli, M. P., and Cravatt, B. F. (1999) *Biochemistry 38*, 14125−14130.
16. Patricelli, M. P., and Cravatt, B. F. (2000) *J. Biol. Chem. 275*, 19177−19184.
17. Decicco, C. P., Nelson, D. J., Luo, Y., Shen, L., Horiuchi, K. Y., Amsler, K. M., Foster, L. A., Spitz, S. M., Merrill, J. J., Sizemore, C. F., Rogers, K. C., Copeland, R. A., and Harpel, M. R. (2001) *Bioorg. Med. Chem. Lett. 11*, 2561−2564.
18. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
19. London, R. E., and Gabel, S. A. (2001) *Arch. Biochem. Biophys. 385*, 250−258.

20. McElroy, K. E., Bouchard, P. J., Harpel, M. R., Horiuchi, K. Y., Rogers, K. C., Murphy, D. J., Chung, T. D. Y., and Copeland, R. A. (2000) *Anal. Biochem. 284*, 382−387.
21. Zhou, M., Diwu, Z., Panchuk-Voloshina, N., and Haugland, R. P. (1997) *Anal. Biochem. 255*, 162−168.
22. Kettner, C. A., and Shenvi, A. B. (1984) *J. Biol. Chem. 259*, 15106−15114.
23. Kettner, C. A., Bone, R., Agard, D. A., and Bachovchin, W. W. (1988) *Biochemistry 27*, 7682−7688.
24. Williams, J. W., and Morrison, J. F. (1979) *Methods Enzymol. 63*, 437−467.
25. Morrison, J. F. (1982) *Trends Biochem. Sci. 7*, 102−104.
26. Szedlacsek, S. E., and Duggleby, R. G. (1995) *Methods Enzymol. 249*, 144−180.
27. Paetzel, M., and Dalbey, R. E. (1997) *Trends Biochem. Sci. 22*, 28−31.
28. Maveyraud, L., Golemi, D., Kotra, L. P., Tranier, S., Vakulenko, S., Mobashery, S., and Samama, J. P. (2000) *Struct. Fold. Des. 8*, 1289−1298.
29. Maveyraud, L., Golemi-Kotra, D., Ishiwata, A., Meroueh, O., Mobashery, S., and Samama, J.-P. (2002) *J. Am. Chem. Soc. 124*, 2461−2465.
30. Dalbey, R. E., Lively, M. O., Bron, S., and van Dijl, J. M. (1997) *Protein Sci. 6*, 1129−1138.
31. Kotra, L. P., Samama, J. P., and Mobashery, S. (2001) in *Bacterial Resistances to Antimicrobials* (Lewis, A., Salyers, A., Taber, H. W., and Wax, R. G., Eds.) pp 123−159, Marcel-Dekker, Inc., New York.
32. Massova, I., and Mobashery, S. (1998) *Antimicrob. Agents Chemother. 42*, 1−17.
33. Choi, K. S., Kim, J. A., and Kang, H. S. (1992) *J. Bacteriol. 174*, 6270−6276.
34. Duggleby, H. J., Tolley, S. P., Hill, C. P., Dodson, E. J., Dodson, G., and Moody, P. C. E. (1995) *Nature 373*, 264−268.
35. McDonough, M. A., Klei, H. E., and Kelly, J. A. (1999) *Protein Sci. 8*, 1971−1981.
36. Mcvey, C. E., Walsh, M. A., Dodson, G. G., Wilson, K. S., and Brannigan, J. A. (2001) *J. Mol. Biol. 313*, 139−150.
37. Groll, M., Ditzel, L. Löwe, J., Stock, D., Bochtler, M., Bartunik, H. D., and Huber, R. (1997) *Nature 386*, 463−471.
38. Jap, B., Zwicki, P., Baumeister, W., and Huber, R. (1995) *Science 268*, 533−539.
39. Crompton, I. E., Cuthbert, B. K., Lowe, G., and Waley, S. G. (1988) *Biochem. J. 251*, 453−459.
40. Strynadka, N. C. J., Martin, R., Jensen, S. E., Gold, M., and Jones, J. B. (1996) *Nat. Struct. Biol. 3*, 688−695.
41. Adams, J., Behnke, M., Chen, S., Cruickshank, A. A., Dick, L. R., Grenier, L., Klunder, J. M., Ma, Y.-T., Plamondon, L., and Stein, R. L. (1998) *Bioorg. Med. Chem. Lett. 8*, 333−338.
42. Bachovkin, W. W., Plaut, A. G., Flentke, G. R., Lynch, M., and Kettner, C. A. (1990) *J. Biol. Chem. 265*, 3738−3743.
43. Bachovchin, W. W., Wong, W. F., Farr-Jones, S., Shenvi, A. B., and Kettner, C. A. (1988) *Biochemistry 27*, 7689−7697.
44. Tsilikounas, E., Kettner, C. A., and Bachovchin, W. W. (1992) *Biochemistry 31*, 12839−12846.
45. Martichonok, V., and Jones, J. B. (1997) *Bioorg. Med. Chem. 5*, 679−684.
46. Stein, R. L., Decicco, C. P., Nelson, D., and Thomas, B. (2001) *Biochemistry 40*, 5804−5811.
47. Inoue, M., Hiratake, J., Suzuki, H., Kumagai, H., and Sakata, K. (2000) *Biochemistry 39*, 7764−7771.
48. Copeland, R. A. (2000) in *Enzymes*, 2nd ed., pp 318−332, Wiley-VCH, Inc., New York.
49. Weber, P. C., Lee, S.-L., Lewandowski, F. A., Schadt, M. C., Chang, C.-H., and Kettner, C. A. (1995) *Biochemistry 34*, 3750−3757.
50. Stein, R. L., Strimpler, A. M., Edwards, P. D., Lewis, J. J., Mauger, R. C., Schwartz, J. A., Stein, M. M., Trainor, D. A., Wildonger, R. A., and Zottola, M. A. (1987) *Biochemistry 26*, 2682−2689.

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