Mechanistic Studies of Reaction Coupling in Glu-tRNA^{Gln} Amidotransferase

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ABSTRACT: Organisms lacking Gln-tRNA synthetase produce Gln-tRNA^{Gln} from misacylated Glu-tRNA^{Gln} through the transamidation activity of Glu-tRNA^{Gln} amidotransferase (Glu-AdT). Glu-AdT hydrolyzes Gln to Glu and NH_3, using the latter product to transamidate Glu-tRNA^{Gln} in concert with ATP hydrolysis. In the absence of the amido acceptor, Glu-tRNA^{Gln}, the enzyme has basal glutaminase activity that is unaffected by ATP. However, Glu-tRNA^{Gln} activates the glutaminase activity of the enzyme about 10-fold; addition of ATP elicits a further 7-fold increase. These enhanced activities mainly result from increases in k_{cat} without significant effects on the K_m for Gln. To determine if ATP binding is sufficient to induce full activation, we tested a variety of ATP analogues for their ability to stimulate tRNA-dependent glutaminase activity. Despite their binding to Glu-AdT, none of the ATP analogues induced glutaminase activation except ATP-γS, which stimulates glutaminase activity to the same level as ATP, but without formation of Gln-tRNA^{Gln}. ATP-γS hydrolysis by Glu-AdT is very low in the absence or presence of Glu-tRNA^{Gln} and Gln. In contrast, Glu-tRNA^{Gln} stimulates basal ATP hydrolysis slightly, but full activation of ATP hydrolysis requires both Gln and Glu-tRNA^{Gln}. Simultaneous monitoring of ATP or ATP-γS hydrolysis and glutaminase and transamidase activities reveals tight coupling among these activities in the presence of ATP, with all three activities waning in concert when Glu-tRNA^{Gln} levels become exhausted. ATP-γS stimulates the glutaminase activity to an extent similar to that with ATP, but without concomitant transamidase activity and with a very low level of ATP-γS hydrolysis. This uncoupling between ATP-γS hydrolysis and glutaminase activities suggests that the activation of glutaminase activity by ATP or ATP-γS, together with Glu-tRNA^{Gln}, results either from an allosteric effect due simply to binding of these analogues to the enzyme or from some structural changes that attend ATP or ATP-γS hydrolysis.

In most organisms the translation of genetic information into new protein synthesis proceeds through a direct pathway involving 20 aminoacyl-tRNA synthetases that produce the required set of 20 different aminoacyl-tRNAs. An alternate pathway to aminoacylation exists in a diverse group of lower organisms (including most Gram-positive bacteria, some Gram-negative bacteria, and archaea) and in eukaryotic organelles (i.e., mitochondria) lacking specific aminoacyl-tRNA synthetases (reviewed in ref 1). For example, organisms without glutaminyl-tRNA synthetase (GlnRS)[1] form Gln-tRNA^{Gln} by catalyzing two consecutive enzymatic reactions. In the first reaction, a nondiscriminating glutamyl-tRNA synthetase (GluRS) mischarges tRNA^{Gln} with Glu, forming Glu-tRNA^{Gln}. In the second reaction, the tRNA-bound Glu is transamidated to Gln by the enzyme Glu-tRNA^{Gln} amidotransferase (Glu-AdT), a tRNA-dependent Gln amidotransferase. For bacteria lacking a GlnRS, Glu-AdT activity is essential for survival and proliferation. Hence, inhibition of this enzyme by cell-permeable small molecules could provide a novel basis for antibacterial therapy.

Glu-AdT is a heterotrimeric enzyme encoded by the *gatCAB* operon (*2*). The A subunit shares sequence homology with an "amidase signature" family, which consists of a large number of amidases that contain a highly conserved 30-residue linear sequence rich in serine and glycine (*3*, *4*). The A subunit is therefore proposed to be the site of Gln hydrolysis, using an active site serine nucleophile and a non-histidine catalytic base (*5*, *6*) to mediate this reaction.[2] Despite similarities in reaction pathways, Glu-AdT holds no sequence similarity with other Gln-dependent amidotransferases, which rely on active site Cys nucleophiles for catalysis (reviewed in refs 7 and 8). Roles for the B and C subunits of Glu-AdT are unknown. However, the B subunit shares sequence similarity with a class of proteins known as "Pet112 (yeast homologue)-like proteins" and is thought to have a tRNA binding region (*2*). It has been speculated

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[1] Abbreviations: Glu-tRNA^{Gln}, glutamyl-charged transfer RNA complementary to the glutamine codon; Glu-AdT, Glu-tRNA^{Gln} amidotransferase; GluRS, Glu-tRNA synthetase; GlnRS, Gln-tRNA synthetase; AsnRS, Asn-tRNA synthetase; ATP-γS, adenosine 5′-[γ-thio]triphosphate; AMP·PCP, adenosine 5′-[β,γ-methylene]triphosphate; TNP·ATP, 2′- (or 3′-) *O*-(trinitrophenyl)adenosine 5′-triphosphate; ATP γ-AmNS, adenosine 5′-triphosphate P^3-(5′-sulfo-1-naphthyl)amide; FSBA, 5′-*p*-fluorosulfonylbenzoyladenosine; BzATP, 2′- (or 3′-) *O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate; HPLC, high-performance liquid chromatography; P-γ-Glu-tRNA^{Gln}, Glu-tRNA^{Gln} with a phosphate group at the γ-carboxyl of the glutamyl moiety.

[2] We have now confirmed by site-directed mutagenesis and selective inhibition experiments that the sequence-conserved Ser in Glu-AdT plays a critical role in the glutaminase reaction (M. R. Harpel et al., manuscript in preparation).

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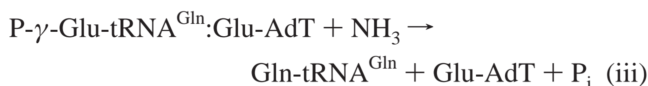
that the C subunit may be required for stabilization of the A subunit (*2*). The isolated enzyme from some organisms (e.g., *Bacillus subtilis*, *Deinococcus radiodurans*, and *Thermus thermophilus*) displays both Glu-tRNA^{Gln} and Asp-tRNA^{Asn} amidation activities in vitro, although the in vivo activity of these enzymes is generally restricted to one or the other activity (*2*, *9*−*11*). In other organisms, including *Streptococcus pyogenes* (the species origin of the enzyme used in the present study), the presence of an identifiable AsnRS gene, but not a corresponding GlnRS gene, suggests that the in vivo activity of Glu-AdT is likely to be restricted to amidation of Glu-tRNA^{Gln} only; however, we are not aware of any cellular biochemical studies that address this issue directly.

Three chemical events comprise the transamidation reaction catalyzed by Glu-AdT (*12*, *13*): (i) Hydrolysis of the amido donor, Gln, forms Glu and NH_3. The latter product is sequestered by the enzyme. (ii) ATP hydrolysis is proposed to activate the amido acceptor, Glu-tRNA^{Gln}, as an enzyme-bound intermediate with a phosphate group at the γ-carboxyl of the glutamyl moiety. (iii) Aminolysis of the activated acceptor by sequestered NH_3 forms the final product, Gln-tRNA^{Gln}.

$$\text{Gln} \xrightarrow{\text{Glu-AdT}} \text{Glu} + \text{NH}_3 \quad \text{(i)}$$

$$\text{Glu-tRNA}^{\text{Gln}} + \text{Glu-AdT} + \text{Mg-ATP} \rightarrow \text{P-}\gamma\text{-Glu-tRNA}^{\text{Gln}}\text{:Glu-AdT} + \text{Mg-ADP} \quad \text{(ii)}$$



$$\text{P-}\gamma\text{-Glu-tRNA}^{\text{Gln}}\text{:Glu-AdT} + \text{NH}_3 \rightarrow \text{Gln-tRNA}^{\text{Gln}} + \text{Glu-AdT} + \text{P}_\text{i} \quad \text{(iii)}$$

While the general scheme described above has been known for some time, a detailed kinetic mechanism for this enzyme has yet to be established. In this study, we begin to describe the mechanism of this complicated, three-substrate enzyme reaction. Employing amino acid analysis by HPLC, we have simultaneously determined the glutaminase and transamidase activities of the enzyme. Apparent kinetic constants for each substrate are reported under saturating condition for the other two substrates. We have found that the glutaminase, transamidase, and ATP hydrolysis activities of Glu-AdT are tightly coupled in terms of their kinetics and stoichiometries of product formation. Furthermore, we have used ATP analogues to suggest that ATP hydrolysis may be essential for full enzyme activation. These results are consistent with intersite communication between the glutaminase, transamidase, and ATP hydrolysis active sites of this enzyme.

EXPERIMENTAL PROCEDURES

Materials. ATP-γS was purchased from Boehringer Mannheim. AMP·PCP, FSBA, and BzATP were purchased from Sigma Chemical Co. TNP·ATP and ATP γ-AmNS were obtained from Molecular Probes. All studies were performed using recombinant *S. pyogenes* Glu-AdT expressed in *Escherichia coli* BL21λ DE3. The recombinant *S. pyogenes gatCAB* operon was amplified by PCR from genomic DNA (ATCC catalog no. 12344D) and cloned into pet15b as an *Xba*I/*Xho*I fragment in a construct analogous to that used for expression of the *B. subtilis* enzyme (*2*). The sequence used to construct primers for amplification was obtained from the University of Oklahoma database for the *S. pyogenes* genome initiative based on homology with the *B. subtilis gatCAB* operon (K. C. Rogers et al., manuscript in preparation). Extraction and purification of the recombinant enzyme were performed according to the procedure of Curnow et al. (*2*) using Q Sepharose Fast Flow, Superdex 200, and MonoQ chromatography. Enzyme purity was determined by SDS−PAGE with Coomassie Blue staining, and the final preparation was estimated to be >94% pure on the basis of densitometry measurements of the gel. Protein concentration was determined by the Bio-Rad protein assay (Bradford) or by the DC protein assay (Lowry's method) using bovine serum albumin as a standard, with both measurements giving similar results.

Recombinant *B. subtilis* tRNA^{Gln} was produced and isolated from *E. coli* and then charged with Glu using partially purified *B. subtilis* GluRS, essentially as described earlier (*2*). This tRNA^{Gln} was used because of its availability and because of its high sequence identity with the tRNA^{Gln} of *S. pyogenes*. The tRNA from these two organisms differ only in two reversed G−C base pairs, one in the acceptor stem at position 5:68 and the other in the anticodon stem at position 28:42. Potential differences in modified nucleotides are not known but are not expected to be significant given the similarities of the modified nucleotide content in glutamine tRNAs from other related eubacteria.

The charged tRNA was subsequently extracted with phenol and washed twice by precipitation with 2-propanol. These procedures were repeated at least twice to remove all protein and small molecule contaminants. The final pellet was resuspended in diethyl pyrocarbonate-treated water to around 100 μM. The preparations of Glu-tRNA^{Gln} thus prepared were free of contaminating transamidase activity, as judged by running mock enzyme reactions (vide infra) lacking Glu-AdT. The concentration of charged tRNA (Glu-tRNA^{Gln}) was quantified by amino acid analysis (Waters AccQ·Tag HPLC system and protocols) of the amount of Glu released upon deaminoacylation at 65 °C for 20 min in borate buffer (pH 9.0), relative to authentic Glu standards. Aliquots of Glu-tRNA^{Gln} were stored at −20 °C.

Because this tRNA is produced in *E. coli,* there are two host-derived tRNA species that could be potential contaminants of our substrate preparations and might affect activity measurements for Glu-AdT; these are *E. coli* tRNA^{Gln} and tRNA^{Glu}. The major *E. coli* tRNA^{Gln} isoacceptor (anticodon CUG) cannot be misacylated with Glu by the *B. subtilis* GluRS used here (*14*), but the minor *E. coli* tRNA^{Gln} (anticodon s^{2}UUG) and tRNA^{Glu} may be aminoacylated. To address this issue, we determined the maximum extent of Glu incorporation by *B. subtilis* GluRS into tRNA for the recombinant *B. subtilis* tRNA^{Gln} prepared as above and for tRNA similarly prepared from the same *E. coli* strain carrying the empty vector. Such studies indicated a maximum contamination level of <20%.

Enzyme Assays. Glutaminase and transamidase (NH_3 transfer) activities were measured by quantitating the formation of Glu or Gln with the AccQ·Tag amino acid HPLC analysis system (Waters). Reaction mixtures, containing 25 mM KCl, 15 mM $MgCl_2$, 50 mM Hepes (pH 7.0), and various amounts of Gln, ATP, and Glu-tRNA^{Gln}, were mixed with Glu-AdT to start the reaction. To establish time courses, reaction mixtures were incubated at room temperature, and

Table 1: Kinetic Parameters for Glu-tRNA[Gln] Amidotransferase

substrates[a]	glutaminase			transamidase		
	K_m[f] (μM)	k_{cat}[f] (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	K_m[f] (μM)	k_{cat}[f] (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Gln[b]	34.8 ± 14.5	0.019 ± 0.008	0.55	*g*	*g*	*g*
Gln (+ATP)[b]	27.8 ± 10.8	0.017 ± 0.007	0.61	*g*	*g*	*g*
Gln (+tRNA[c])[d]	14.2 ± 4.8	0.14 ± 0.01	9.9	*g*	*g*	*g*
Gln (+tRNA + ATP)[d]	25.1 ± 7.8	0.96 ± 0.22	38	15.9 ± 6.8	0.51 ± 0.16	32
ATP (+Gln + tRNA)[e]				117.7 ± 20.5	0.59 ± 0.03	5
tRNA (+Gln + ATP)[e]				∼0.2		

[a] The substrate is changed in concentration for K_m determination. [b]Conditions: 83 nM Glu-AdT ± 1 mM ATP. [c]Glu-tRNA[Gln]. [d]Conditions: 8.3 nM Glu-AdT, 10 μM Glu-tRNA[Gln], and ±1 mM ATP. [e]Conditions: 8.3 nM Glu-AdT, 100 μM Gln, and 5 μM Glu-tRNA[Gln] or 1 mM ATP. [f]Values represent means and standard errors from two to five experiments. [g]No transamidase activity.

aliquots were periodically quenched by mixing with 3 volumes of cold ethanol. Following centrifugation for 15 min at 4 °C, a fixed volume of supernatant was transferred into a clean tube for analysis of glutaminase activity while the pellet was retained for analysis of transamidase activity (vide infra). The supernatant was dried by vacuum centrifugation, suspended in borate buffer, and derivatized with the AccQ•Tag fluorescence reagent according to the manufacturer's protocol. Ten microliters of the derivatized sample was injected onto an AccQ•Tag HPLC column, and reversed-phase elution was performed according to the manufacturer's protocol. To optimize Glu and Gln separation, the pH of AccQ•Tag eluent A was adjusted to 5.8. The amount of Glu present in this supernatant sample was thus quantified as a measure of glutaminase activity.

The pellet from quenched reaction aliquots contained the tRNA, which was analyzed for the formation of Gln-tRNA[Gln] as a measure of transamidase activity. The pellet was washed three times with 75% cold ethanol by centrifugation to remove free Glu and Gln and then dried briefly. The dried pellet was resuspended with borate buffer (pH 9.0) and deaminoacylated by incubating for 15 min at 65 °C. After being cooled to room temperature, the sample was derivatized and analyzed by HPLC as described above; the amount of Gln in the pellet represents the formation of Gln-tRNA[Gln].

ATP hydrolysis was measured by the formation of ADP, as analyzed by HPLC using the method of Ally and Park (*15*). Briefly, reaction aliquots were quenched with cold ethanol and dried completely. The dried material was resuspended in 20 μL of elution buffer containing 125 mM EDTA, 35 mM KH_2PO_4 (pH 6.0), and 6 mM tetrabutylammonium hydrogen sulfate, and 10 μL of this was injected onto a C_{18} HPLC column followed by reversed-phase elution. Samples were kept at 4 °C until injection. ADP, ATP, and ATP-γS peaks were monitored by UV absorbance at 260 nm. No ADP formation was observed in parallel reactions run in the absence of enzyme.

All kinetic parameters were determined from initial velocity measurements obtained from HPLC data and analyzed by fitting to the Henri−Michaelis−Menten equation by nonlinear regression (*16*).

The potency of ATP analogues as inhibitors, indicative of binding affinity, was determined by measuring the fractional activity of the enzyme at fixed concentration of ATP (100 μM) with varying concentrations of the analogues and fitting the resulting data to the equation (*16*):

$$\frac{\nu_i}{\nu_0} = \frac{1}{1 + [I]/IC_{50}} \quad \text{(iv)}$$

where ν_i is the velocity in the presence of inhibitor at concentration [I], ν_0 is the velocity in the absence of inhibitor, and IC_{50} is the inhibitor concentration giving 50% inhibition.

RESULTS

Direct detection of Glu and Gln by fluorescent derivatization and HPLC analysis afforded us a sensitive means of studying simultaneously the kinetics of both the glutaminase and transamidase activities of Glu-AdT. Glu-AdT displays basal glutaminase activity in the absence of Glu-tRNA[Gln] and ATP substrates with an apparent k_{cat} of 0.019 ± 0.008 s^{-1} and apparent K_m for Gln of 34.8 ± 14.5 μM (Table 1). As summarized in Table 1, the kinetics of the glutaminase reaction were not affected significantly by the addition of ATP. In contrast, addition of the other substrate, Glu-tRNA[Gln], increased the apparent k_{cat} of glutaminase activity by about 10-fold with little effect on the apparent K_m for Gln. However, transamidation to form Gln-tRNA[Gln] did not occur unless both ATP and Glu-tRNA[Gln] were present in the reaction mixture. In the presence of all three substrates, the apparent k_{cat}/K_m for glutaminase activity was increased by 70-fold relative to the basal (Gln only) activity. The magnitudes of the apparent kinetic constants, k_{cat}, K_m for Gln, and k_{cat}/K_m were similar whether monitored by glutaminase or transamidase activities (Table 1). Thus ammonia production at the glutaminase active site of Glu-AdT is tightly coupled to transamidation at the (putatively) distal tRNA binding site.

By holding the other two substrates at constant, saturating concentrations, we also determined the apparent K_m values for ATP and Glu-tRNA[Gln] from analyses of both Gln-tRNA[Gln] (transamidase) and Glu (glutaminase) production in the full enzymatic reaction. These data are also summarized in Table 1. The apparent K_m values for ATP and Glu-tRNA[Gln] were 117.7 ± 20.5 μM and approximately 0.2 μM, respectively. The apparent K_m for Glu-tRNA[Gln] is less well determined than that for the other two substrates, for two reasons. First, at low concentrations of this substrate the amount of Gln-tRNA[Gln] formed is very small and near the detection limit of the HPLC assay, making accurate determination of initial velocity very difficult. Second, the tRNA used in these studies contained a small, variable population (<20% of the total Glu-charged tRNA; see Experimental Procedures) of *E. coli* tRNA along with the authentic substrate. These contaminants contribute to the total Glu concentration (used as an estimate of Glu-tRNA[Gln] concentration) determined by hydrolysis of the tRNA pellet from ethanol precipitation of the reaction mixtures. Thus,

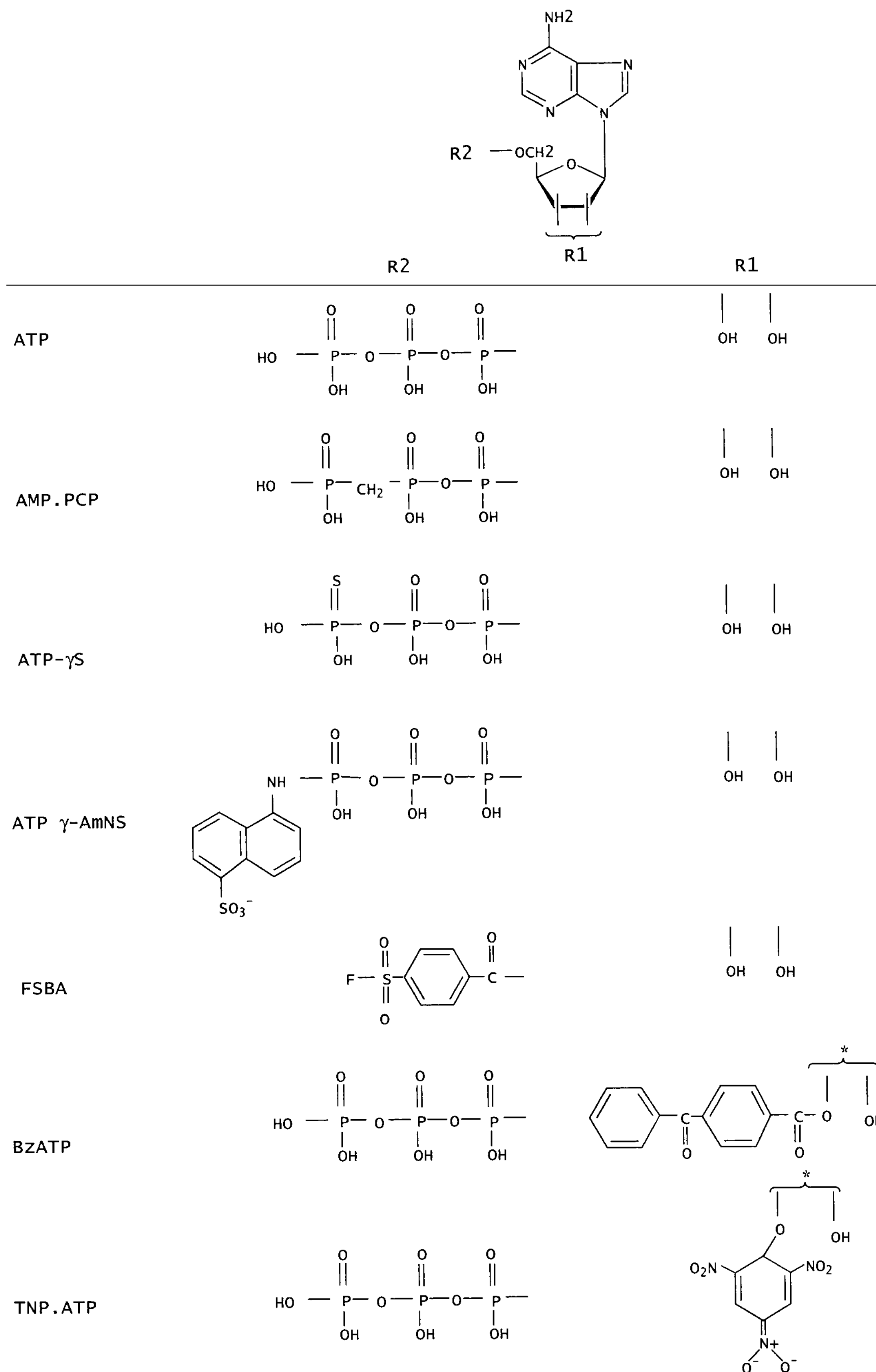


FIGURE 1: Chemical structures of ATP analogues used in this study. An asterisk indicates that modification is at either the 2′ or 3′ position.

the K_m value for Glu-tRNA^{Gln} reported in Table 1 must be considered an approximation of the true K_m value. This apparent K_m value did not vary considerably using different preparations of Glu-tRNA^{Gln}, so it is unlikely that the small levels of contaminating *E. coli* tRNA had a significant effect on our measurements. Nevertheless, for this reason, all of the kinetic constants reported in this paper must be viewed as experimentally determined *apparent* constants.

Table 2: Activation and/or Inhibition by ATP Analogues in the Presence of Glu-tRNA^{Gln}

	glutaminase % activity[a]	transamidase IC_{50}[c] (mM)
no ATP	100	
ATP	312	
AMP•PCP	28[b]	2.3
ADP	4	0.026
ATP-γS	339	0.19
TNP•ATP	71	2.4
ATP γ-AmNS	73	no inhibition
FSBA	100	no inhibition
(no tRNA)	4.5	

[a] Conditions: 50 μM Gln, 5 μM Glu-tRNA^{Gln}, 8.3 nM Glu-AdT, and 1 mM ATP or ATP analogues unless otherwise mentioned. [b] 5 mM AMP•PCP. [c] Conditions are the same as in footnote *a* except ATP is 100 μM (around the K_m value) and the ATP analogue is varied to obtain IC_{50} for transamidation.

Although the addition of Glu-tRNA^{Gln} alone increased k_{cat} by about 10-fold, the full activation of glutaminase activity was only seen when ATP was present. To distinguish between an activation event caused simply by ATP binding as opposed to one requiring ATP hydrolysis, various ATP analogues were tested as activators of Glu-AdT glutaminase activity. Figure 1 illustrates the structures of the ATP analogues tested here. The ability of these ATP analogues to activate the glutaminase activity of Glu-AdT was measured in the context of the full enzymatic reaction (i.e., in the presence of Gln and Glu-tRNA^{Gln}), and the percent activation was referenced to the glutaminase reaction measured in the absence of ATP (defined as 100% activity). The results of these studies are summarized in Table 2. As mentioned above, ATP increased the glutaminase activity about 3-fold under these conditions. Only ATP-γS caused a similar level of activation, whereas AMP•PCP, a simple nonhydrolyzable ATP analogue, could not activate. Similar to the case with ATP, neither of these analogues significantly influenced glutaminase activity in the absence of Glu-tRNA^{Gln}. ATP γ-AmNS and FSBA, which have large substituents at the γ-phosphate position (Figure 1), also showed no ability to activate the enzyme. Although TNP•ATP has an intact γ-phosphate group, it is not hydrolyzed by certain enzymes (*17*, *18*) and did not promote activation of Glu-AdT. ADP also failed to activate the enzyme, resulting in a return to basal activity. None of the ATP analogues, including ATP-γS, yielded Gln-tRNA^{Gln} as a reaction product (data not shown).

The ability of the ATP analogues to inhibit transamidation activity was also evaluated to eliminate the possibility that the lack of activation by these compounds reflected a lack of binding to the enzyme. These experiments were performed by varying the concentration of the ATP analogues while holding the concentration of ATP constant at its apparent K_m and determining the IC_{50} values for the analogues as described under Experimental Procedures. As shown in Table 2, the IC_{50} values for AMP•PCP and TNP•ATP were around 2 mM, indicating that both analogues were at least half-saturating in the experiments in which they failed to activate the enzyme. The IC_{50} value for ATP-γS was about twice the K_m for ATP, whereas that for ADP was about 10-fold lower. Thus, these analogues were present at saturating concentrations during the activation experiments. ATP γ-AmNS and FSBA failed to inhibit Glu-AdT, presumably due to a lack of binding. BzATP, a photo-cross-linkable ATP analogue with bulky substitution of the ribose ring, irreversibly inactivated Glu-AdT when irradiated with enzyme alone, presumably as a consequence of covalent photo-cross-linking. Interestingly, the enzyme was protected from inactivation upon irradiation in the presence of Glu-tRNA^{Gln} (data not shown).

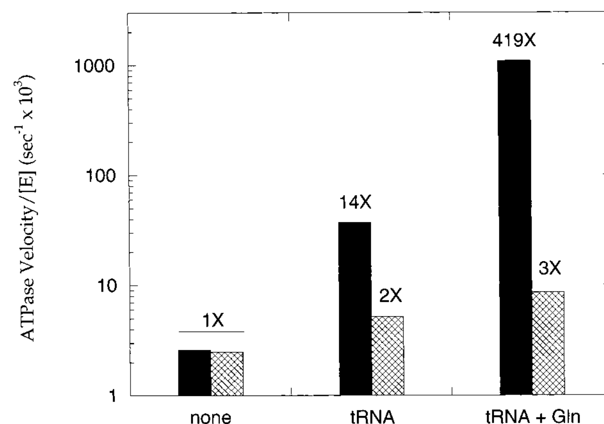


FIGURE 2: Effects of Glu-tRNA^{Gln} (tRNA) and a combination of Glu-tRNA^{Gln} and Gln (tRNA + Gln) on the Glu-AdT-catalyzed hydrolysis of ATP (solid bars) and ATP-γS (hatched bars). The label none refers to the hydrolysis rates in the absence of the other two substrates, Gln and tRNA. All hydrolysis rates were measured at fixed concentrations of ATP and ATP-γS of 1 mM, and Gln and Glu-tRNA^{Gln} were 100 μM and 10 μM, respectively, when they were present. The labels above the bars indicate the fold increase in activity relative to the basal hydrolysis rates in the absence of Gln and tRNA (1×). Note that the velocities have been multiplied by 10^3 and are plotted on a logarithmic scale so that the modest rates of ATP-γS hydrolysis can be clearly illustrated together with the more robust hydrolysis rates for ATP in the presence of tRNA and of tRNA + Gln.

ATP-γS was the only ATP analogue tested that was capable of fully activating the glutaminase activity of Glu-AdT. This result raised the question of whether glutaminase activation results from hydrolysis of this analogue by the enzyme or is simply a reflection of its greater binding affinity. To test this, we measured ATP or ATP-γS hydrolysis in the absence or presence of the other substrates. In the absence of Glu-tRNA^{Gln}, Glu-AdT had a low level of basal ATPase activity that was not affected by Gln (data not shown). Although ATP hydrolysis was stimulated 10-fold by the addition of Glu-tRNA^{Gln}, full activation (a >400-fold increase over the basal level) was achieved only when both Gln and Glu-tRNA^{Gln} were present (Figure 2). Interestingly, the ATP hydrolysis activity and the stimulation of this activity by Gln and Glu-tRNA^{Gln} were similar to what is observed for the glutaminase activity under similar conditions. In contrast, ATP-γS hydrolysis remained very low even in the presence of Glu-tRNA^{Gln} and Gln (maximally a 2−3-fold stimulation; Figure 2). Hydrolysis of ATP-γS was verified by experiments performed at high concentration of enzyme (1 μM), where we observed a Glu-tRNA^{Gln}-dependent burst of ATP-γS hydrolysis, which was followed by a very low steady-state level (data not shown).

Potential coupling between the glutaminase, transamidase, and ATP or ATP-γS hydrolysis activities was tested by simultaneously measuring the three activities in the presence of all substrates. Figure 3 shows the time courses for ADP, Glu, and Gln-tRNA^{Gln} formation, representing the ATPase,

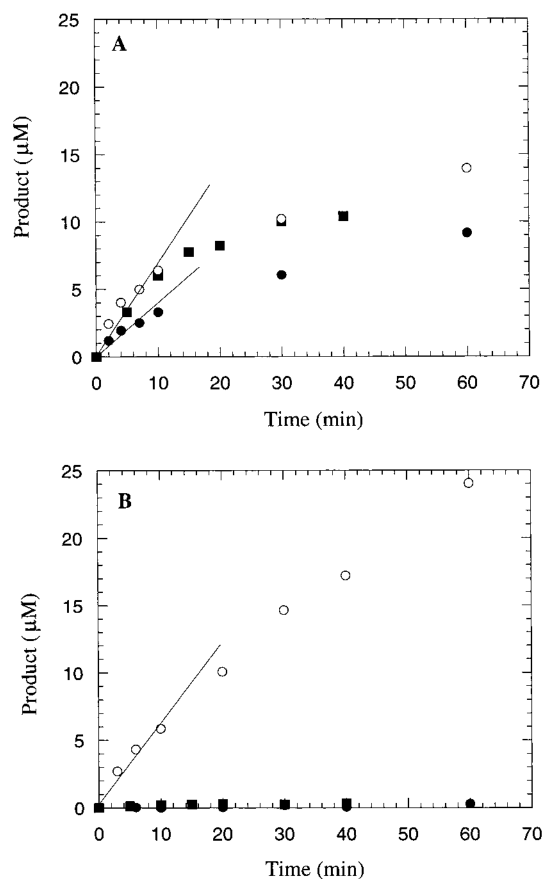


FIGURE 3: Time courses for the amidotransferase reaction in the presence of ATP (A) or ATP-γS (B). Conditions are 100 μM Gln, 10 μM Glu-tRNA^{Gln}, 1 mM ATP or ATP-γS, and 8.3 nM Glu-AdT in the buffer described in Experimental Procedures. Glutaminase (○), transamidase (●), and ATPase (■) activities were measured as described in Experimental Procedures. The solid lines drawn through the data represent the initial velocity obtained by linear regression of the early time point data (up to 10 min). The initial velocities for glutaminase activity are 1.18 $\pm$ 0.04 and 0.92 $\pm$ 0.02 s^{-1} with ATP and ATP-γS, respectively, the initial velocity for transamidase activity is 0.6 $\pm$ 0.02 s^{-1} with ATP, and the initial velocities for ATPase activity are 1.09 $\pm$ 0.1 and 0.013 + 0.03 s^{-1} with ATP and ATP-γS, respectively.

glutaminase, and transamidase activities of the enzyme, respectively. ADP production was tightly coupled with glutaminase activity in the presence of ATP (initial velocities of 1.09 $\pm$ 0.1 and 1.18 + 0.04 s^{-1} for ATPase and glutaminase activities, respectively). Although the initial velocity of transamidase activity was slightly lower than that of glutaminase, all three activities slowed considerably when the Glu-tRNA^{Gln} substrate was exhausted (Figure 3A). As expected, no transamidase activity and limited ADP formation was observed in the presence of ATP-γS (Figure 3B). However, the initial velocity of glutaminase activity was similar to that seen with ATP (1.18 $\pm$ 0.04 and 0.92 $\pm$ 0.02 s^{-1} with ATP and ATP-γS, respectively). Furthermore, Glu formation continued for a longer time in the presence of ATP-γS (and with molar conversion in excess of the Glu-tRNA^{Gln} concentration) since in this case the reaction was not limited by net Glu-tRNA^{Gln} consumption (Figure 3B).

DISCUSSION

In this study we determined apparent kinetic constants for each of the three substrates of Glu-AdT under a variety of conditions to evaluate the potential for kinetic interactions between these substrates. As described above, our estimate of the K_m value for Glu-tRNA^{Gln} is technically compromised by the low levels of transamidation seen in our assays at low Glu-tRNA^{Gln} concentrations. Additionally, the modest levels of contaminating populations of *E. coli* tRNA^{Glu} and *E. coli* tRNA^{Gln} in our Glu-tRNA^{Gln} preparations may have perturbed the measured values of K_m for this substrate. However, we have tested several different preparations of Glu-tRNA^{Gln}, having different levels of charged tRNA contamination, and obtained comparable K_m and k_{cat} values for all substrates, suggesting that any measurement error introduced by the presence of contaminating tRNA species is minimal.

Our data demonstrate significant kinetic coupling between the glutaminase and transamidase activities of Glu-AdT, similar to what has been reported for other Gln-dependent amidotransferases. In the absence of ATP and Glu-tRNA^{Gln}, the basal glutaminase activity of the enzyme is low. Addition of ATP alone has little impact on this activity. In contrast, binding of Glu-tRNA^{Gln} alone is sufficient to enhance the k_{cat} for Glu-AdT glutaminase activity some 10-fold, while having a minimal effect on the K_m for Gln. This result suggests some type of conformational communication between the Gln and Glu-tRNA^{Gln} binding sites, such that the glutaminase active site is altered in a way that augments its catalytic efficiency when the amido acceptor Glu-tRNA^{Gln} is bound. This result is reminiscent of what is seen for the enzyme Gln phosphoribosylpyrophosphate (PRPP) amidotransferase. In PRPP amidotransferase, binding of the amido acceptor PRPP also significantly activates glutaminase activity, and for this enzyme there is direct experimental evidence for a PRPP binding-induced conformational change (*19*). The augmentation of Glu-AdT glutaminase activity seen here reflects a high degree of tRNA substrate specificity; uncharged tRNA, Glu-tRNA^{Glu}, and Gln-tRNA^{Gln} are not capable of enhancing or inhibiting this activity (data not shown), consistent with previous reports (*12*, *20*). Since the effect is restricted to k_{cat}, the conformational changes in the glutaminase active site that attend tRNA binding must affect chemical steps after Gln binding per se.

While Glu-tRNA^{Gln} binding is sufficient to partially activate Glu-AdT glutaminase activity, full activation is only realized in the presence of ATP or the analogue ATP-γS. Our studies revealed that even subtle changes around the γ-phosphate were poorly tolerated (Figure 1 and Table 2). Thus, the enzyme did not bind ATP γ-AmNS or FSBA. Consistent with previous findings (*13*, *20*), replacement of the bridging oxygen between the γ- and β-phosphates with a methylene group (AMP•PCP) reduced the affinity for Glu-AdT by 10-fold relative to ATP. In contrast, the ATP binding pocket of Glu-AdT appears to be relatively tolerant of substitutions on the sugar. Despite bulky substitution on the ribose ring, TNP•ATP still bound to Glu-AdT but did not promote glutaminase activation. Additionally, the photo-cross-linkable analogue BzATP was able to bind and photochemically inactivate the enzyme in the absence of the tRNA substrate. Interestingly, however, the presence of Glu-tRNA^{Gln} protected the enzyme against photoinduced inactivation by BzATP. These results imply that Glu-tRNA^{Gln} binding induces a conformational change in Glu-AdT that affects the ATP binding pocket, which eliminates BzATP

binding or selects against an orientation of the benzophenone group of BzATP that is suitable for photo-cross-linking.

Although several ATP analogues could be shown to bind and inhibit Glu-AdT activity, only ATP and ATP-γS led to stimulation of glutaminase activity. In the case of ATP the glutaminase and transamidase activities of Glu-AdT were well coupled with the ATP hydrolyzing activity of the enzyme. With ATP-γS, at high enzyme concentration (1 μM), a rapid, but minimal burst of hydrolysis activity was followed by an extremely modest steady-state rate of hydrolysis. Nevertheless, ATP-γS augmented the glutaminase activity of Glu-AdT by an amount comparable to that seen for ATP.

There are two potential explanations for these observations. First, simple binding of either ATP or ATP-γS may be sufficient to induce a conformational change in the partially activated Glu-AdT•tRNA binary complex that is allosterically communicated to the glutaminase active site. In this case hydrolysis of the ATP is not required for glutaminase activation but is required for NH_3 transfer. The modest hydrolysis seen for ATP-γS is catalytically nonproductive in this model. The discrimination between ATP or ATP-γS and the other analogues tested then results merely from differences in specific binding interactions with the enzyme. The second possibility is that conformationally mediated augmentation of the glutaminase activity results not from binding of ATP or ATP-γS but rather from hydrolysis and/or subsequent formation of an intermediate species. Formation of the P-γ-Glu-tRNA^{Gln} (or the analogous thiophosphoryl-γ-Glu-tRNA^{Gln}) intermediate proposed by Wilcox (*13*) is the most logical candidate. Our data suggest that the binding of Glu-tRNA^{Gln} to Glu-AdT induces a conformational change that is transduced to the glutaminase active site and results in partial activation. Formation of the P-γ-Glu-tRNA^{Gln} intermediate could result in further conformational adjustments of the protein, leading to full activation of the glutaminase activity. In the case of ATP-γS, a small number of catalytic turnovers would produce the analogous, nonproductive intermediate and trap it on the enzyme, resulting in the stimulation of glutaminase activity and blocking transamidase activity. This proposed mechanism of glutaminase activation finds precedence in the studies of Miles and Raushel (*21*) on carbamoyl phosphate synthetase (CPS). In CPS, glutaminase activity is low in the absence of ATP and the amido acceptor, bicarbonate. The formation of the carboxy phosphate intermediate by ATP hydrolysis enhances Gln hydrolysis by a factor of 275. Hence, the phosphorylation event must be transmitted by conformational changes to the distal glutaminase active site, resulting in catalytic (conformational) coupling among these active sites. For Glu-AdT, the resulting coordination between formation of the activated acceptor molecule and formation of NH_3 would provide a means of ensuring in vivo efficiency and specificity with respect to tRNA and Gln substrates.

Both of these proposed mechanisms of glutaminase activation are consistent with the data presented in this paper, and a clear choice between them is not possible at this time. However, the apparent correlation between kinetic coupling and hydrolytic potential of ATP and ATP-γS makes the second proposal particularly intriguing. Support for this model may be provided by the reported trapping of a P-γ-Glu-tRNA^{Gln} species by Wilcox (*13*). The full activation of Glu-AdT glutaminase activity by ATP-γS in the presence of Glu-tRNA^{Gln}, along with a low level of ATP-γS hydrolysis, opens the possibility that the enzyme may also be able to form an analogous thiophosphoryl-γ-Glu-tRNA^{Gln} intermediate, as required for the second proposed mechanism of activation. Despite several attempts, however, we have been unable to trap this intermediate.[3] Given the inability of ATP-γS to promote transamidation, the putative thiophosphoryl intermediate would either be chemically unreactive to aminolysis or allow dissociation of NH_3 from the enzyme prior to transfer to the activated tRNA intermediate (effectively "uncoupling" Gln hydrolysis from transamidation). In either case, protection of the enzyme-bound intermediate from hydrolysis would be required to ensure continued activation of the glutaminase reaction, whereas facile hydrolysis could accompany release into solvent during turnover or under trapping conditions. The observation that the burst and subsequent low rate of ATP-γS hydrolysis elicit the same level of glutaminase activation as for ATP would necessitate a longer active site residency time for the putative thiophosphoryl-γ-Glu-tRNA^{Gln} intermediate than for the intermediate formed from ATP. In addition, Gln hydrolysis products accumulated to levels that exceed Glu-tRNA^{Gln}. Therefore, thiophosphoryl-γ-Glu-tRNA^{Gln} would promote glutaminase stimulation by coupling tRNA activation and Gln hydrolysis, without the productive forward commitment to catalysis observed in the presence of ATP.

Regardless of which of these alternative activation mechanisms is correct, the results reported here provide clear evidence of tight kinetic coupling between the glutaminase, transamidase, and ATP hydrolysis activities of Glu-AdT. This kinetic coupling is likely to reflect conformationally mediated communication between putatively distant active sites for these activities on the Glu-AdT molecule. Continued study of these proposed ligand binding-associated conformational changes may provide insights for inhibitory ligand design ultimately leading to clinically useful antibacterial agents based on specific inhibition of Glu-AdT.

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[3] We attempted to trap a thiophosphoryl-γ-Glu-tRNA^{Gln} intermediate from ATP-γS essentially as described earlier by Wilcox (*13*). Briefly, standard Glu-AdT reactions were performed with ATP[γ-^{35}S] in place of ATP and then quenched with an equal volume of 20% trichloroacetic acid (TCA) containing 1 mM unlabeled ATP-γS and 100 mM sodium phosphate. The TCA pellets were collected either on a nitrocellulose membrane or by centrifugation, washed with cold 5% TCA containing 100 mM sodium phosphate, and evaluated for radioactivity (indicative of trapped intermediate) by liquid scintillation counting. Reactions were run in the presence of catalytic or excess levels of Glu-AdT and quenched at various times. Under all conditions, detection of a Glu-AdT-dependent increase in radioactivity, which represents the expected complexes of thiophosphoryl-γ-Glu-tRNA^{Gln} intermediate and Glu-AdT, was precluded by high levels of nonspecific binding of ATP[γ-^{35}S], as evidenced by reactions lacking Glu-AdT. Our failure to experimentally verify the existence of this intermediate complex in these preliminary attempts may also reflect the instability of such a species in solution as described in the text, although we cannot dismiss an alternate mechanism for kinetic coupling than the one that we proposed.

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