Crystal Structures and Biochemical Analyses Suggest a Unique Mechanism and Role for Human Glycyl-tRNA Synthetase in Ap4A Homeostasis*

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Aminoacyl-tRNA synthetases catalyze the attachment of amino acids to their cognate tRNAs for protein synthesis. However, the aminoacylation reaction can be diverted to produce diadenosine tetraphosphate (Ap4A), a universal pleiotropic signaling molecule needed for cell regulation pathways. The only known mechanism for Ap4A production by a tRNA synthetase is through the aminoacylation reaction intermediate aminoacyl-AMP, thus making Ap4A synthesis amino acid-dependent. Here, we demonstrate a new mechanism for Ap4A synthesis. Crystal structures and biochemical analyses show that human glycyl-tRNA synthetase (GlyRS) produces Ap4A by direct condensation of two ATPs, independent of glycine concentration. Interestingly, whereas the first ATP-binding pocket is conserved for all class II tRNA synthetases, the second ATP pocket is formed by an insertion domain that is unique to GlyRS, suggesting that GlyRS is the only tRNA synthetase catalyzing direct Ap4A synthesis. A special role for GlyRS in Ap4A homeostasis is proposed.

Aminoacyl-tRNA synthetases (AARSs)4 are considered to be among the earliest proteins to have emerged during evolution. As a family of typically 20 members (one for each amino acid), AARSs catalyze the first step of protein synthesis by linking each amino acid onto the 3′-end of its cognate tRNA harboring the trinucleotide anticodon. Through evolution, the role of AARSs has also been broadened with expanded functions (reviewed in Refs. 1 and 2). These expanded functions often involve direct interaction partners. For example, human tyrosyl-tRNA synthetase interacts with chemokine receptor CXCR1 to induce cell migration (3); human glutaminyl-tRNA synthetase interacts with ASK1 to regulate apoptosis (4); human tryptophanyl-tRNA synthetase interacts with VE-cadherin to inhibit angiogenesis (5); human lysyl-tRNA synthetase interacts with the Gag protein of human immunodeficiency virus to facilitate viral assembly (6); and human glutamylprolyl-tRNA synthetase interacts with L13a and glyceraldehyde-3-phosphate dehydrogenase to form the GAIT complex for translational silencing to regulate inflammation (7). However, functional expansion also can be achieved indirectly via reaction products of AARSs. As examples, Lys-tRNA5ys and Ala-tRNA5hu are used to aminoacylate cytoplasmic membrane phosphatidylglycerol of Staphylococcus aureus and Pseudomonas aeruginosa, respectively, to enhance drug resistance in these microorganisms (8).

In addition to tRNA aminoacylation, the majority of AARSs have the capacity to catalyze a side reaction to form diadenosine oligophosphates (ApnA) in the absence of cognate tRNA (9). These reactions of AARSs are the most well known sources of ApnA in vivo (10). ApnA are made up of two adenosine moieties linked at the 5′-end of the ribose by a chain of two to six phosphates. In the 4 decades following the discovery of these molecules by Zamecnik et al. (10), ApnA have been linked to highly diverse physiological effects in prokaryotic and eukaryotic cells, including various types of mammalian cells and tissues, and to assorted functions associated with the nucleus, membrane receptors, and activities in the cytoplasm (reviewed in Refs. 11 and 12). The concentrations of ApnA molecules in vivo respond to numerous factors, including cell proliferation status, glucose level, heat shock, oxidative stress, and interferon stimulation. They have emerged as extracellular and intracellular signaling molecules (as pleiotropically acting “alarmones” (13) and second messengers (14)) implicated in the maintenance and regulation of vital cellular functions.

The aminoacylation reaction proceeds in two steps. First, the amino acid is activated by condensation with ATP to form aminoacyl-AMP, the enzyme-bound intermediate. The aminoacyl moiety is then transferred to the 3′-end of the cognate tRNA. When tRNA is absent, the enzyme-bound aminoacyl-AMP can be attacked by the γ-phosphate of a second ATP molecule to form diadenosine tetraphosphate (Ap4A), the most common diadenosine oligophosphate produced by a tRNA synthetase (see Fig. 1A). The presence of tRNA in most cases inhibits Ap4A synthesis (11). Therefore, a subgroup of tRNA synthetases that requires tRNA as cofactor for synthesis of aminoacyl-AMP is

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not capable of producing Ap4A. This group includes tRNA synthetases that are specific for arginine, glutamine, and glutamic acid and an unusual class I lysyl-tRNA synthetase (LysRS).

Although the amino acid recycles, the above mechanism requires the presence of the amino acid for the production of Ap4A via the aminoacyl-AMP intermediate. Using biochemical analyses and determinations of co-crystal structures, we demonstrate in this work that human glycyl-tRNA synthetase (GlyRS) produces Ap4A by direct condensation of two ATPs in the absence of glycine. Thus, the mechanism for GlyRS to synthesize Ap4A is decoupled from aminoacylation. Furthermore, GlyRS is likely to be the only synthetase that produces Ap4A by this mechanism. Our results raise the possibility that GlyRS plays a special role in Ap4A homeostasis.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Wild-type GlyRS was cloned, expressed, and purified as described previously (15). The molecular weights of the purified enzymes were verified by mass spectrometry, and purities (>95%) were measured by SDS-PAGE.

Crystallization and Data Collection—ATP, glycine, AMPCPP, and Ap4A were obtained from Sigma. Gly-AMP analog (5’-O-(N-1-glycyl)sulfamoyl)adenosine) was obtained from Integrated DNA Technologies. Sodium formate was obtained from Hampton Research. All commercial buffers and reagents were of the highest grade. For preparing the four GlyRS complex crystals, 2 µl of GlyRS/ligand solution (10 mg/ml GlyRS, 25 mM Tris-HCl, 150 mM NaCl, 10 mM MgCl2, and 10 mM different ligands, pH 7.5) was mixed with 2 µl of mother liquor (3.8–4.2 M sodium formate) and then equilibrated with 500 µl of mother liquor by the hanging drop method at room temperature. Crystals appeared in 5–7 days and grew to 0.5 × 0.5 × 0.3 mm. The crystals were then soaked with cryoprotectant (15–20% glycerol added to the mother liquor) for 3 s and frozen in liquid nitrogen.

X-ray diffraction data were collected at beam line 11-1 at the Stanford Synchrotron Radiation Laboratory. Diffraction data were processed and scaled using the program HKL2000 (16). The five GlyRS-ligand crystals belonged to the C2221 space group with typical unit cell parameters of a = 116–121, b = 138–140, and c = 132–134 Å. Each asymmetric unit contained one monomeric GlyRS molecule. Prior to use in structural refinements, 5% randomly selected reflections were set aside for calculating Rfree as a quality monitor (17).

Structure Determination and Refinement—The structures of the GlyRS complexes were determined using the native GlyRS structure (Protein Data Bank code 2PME) we solved previously because the new crystals are isomorphous. For these GlyRS complexes, the 2Fo – Fc difference Fourier map showed clear electron densities for most amino acid residues, including those in the substrate-binding site(s), but the N-terminal WHEP domain and insertion III were still disordered as the apo-GlyRS. Most substrate and ligand electron densities were obvious. Subsequent refinement with incorporation of the cofactors and water molecules yielded R and Rfree values of 0.22–0.24 and 0.24–0.29, respectively, at 2.5–3.4 Å resolution. Statistics for the final models are listed in Table 1. All manual modifications of the models were performed using the XtalView program (18). Structure refinements, which included maximal likelihood and simulated annealing protocols, were carried out using CNS (19). The PyMOL program was used in creating figures.

Ap4A Formation Assays and Thin Layer Chromatography—Concentrations of purified tRNA synthetases were measured by active-site titration assays as described previously (20). Purified enzyme (5 µM) was incubated in reaction buffer containing 50 mM HEPES, pH 7.5, 20 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 5 mM [α-32P]ATP, and 0.01 mg/ml inorganic pyrophosphatase at 25 °C. Where indicated, reactions contained 5 mM amino acid and 12.6 µM in vitro transcribed tRNA Gly. Assays in the presence or absence of zinc contained no dithiothreitol, 10 mM MgCl2, and either 150 µM ZnCl2 or 10 µM 1,10-phenanthroline. All aliquots were extracted with 25:24:1 phenol/chloroform/isoamyl alcohol, pH 8.0, at the indicated time points. Samples were spotted onto polyethyleneimine-cellulose TLC sheets (Macherey-Nagel), which were then developed in 3 M NH4(SO4)2 and 2% EDTA. Standards were located by UV shadowing. When development was complete, the chromatograms were immersed in methanol, dried, and exposed to a phosphor screen. Radioactive counts were visualized and quantitated on a PhosphorImager. Amounts were multiplied by 0.5 to reflect the incorporation of two labeled phosphates/Ap4A molecule.

RESULTS

Glycine-independent Formation of Ap4A Catalyzed by Human GlyRS—In the course of our ongoing studies of human GlyRS and its disease associations (21, 22) and wanting to have a complete understanding of its various activities, we chose to investigate the capacity of GlyRS in Ap4A synthesis. To determine whether human GlyRS can produce Ap4A, the enzyme was added to a mixture containing glycine and radiolabeled ATP in the absence of tRNA Gly. The production of radiolabeled Ap4A was monitored by TLC. Ap4A was clearly generated by GlyRS in a time-dependent manner (Fig. 1C). The identity of the reaction product as Ap4A was further confirmed by mass spectrometry (supplemental Fig. S1). A small amount of Ap3A (~15% of the total Ap3A and Ap4A) was also produced by human GlyRS. (Ap4A and Ap3A co-migrate in the TLC system used here.) Remarkably, when glycine was removed from the reaction, Ap4A was generated at the same rate with multiple turnovers (Fig. 1C), suggesting that the Ap4A synthesis by GlyRS is independent of glycine.

To rule out the possibility of potential glycine contamination in the GlyRS preparation, a standard amino acid activation assay was performed with no glycine or with incremental amounts of glycine added to the reaction. No activity was detected without glycine added, whereas clear ATP-PPi exchange activity was detected upon addition of glycine in 0.1 stoichiometry to GlyRS, thus suggesting no or very little glycine contamination in the GlyRS preparation (supplemental Fig. S2). On the other hand, adding saturating amounts of glycine (5 mM) had no effect on the rate of Ap4A production. Therefore, we concluded that Ap4A synthesis is completely glycine-independent and that the formation of Ap4A by human GlyRS requires only ATP (Fig. 1B).

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Glycine-independent Formation of Ap4A in the Co-crystal with Human GlyRS—Further insight into the mechanism of Ap4A formation by human GlyRS was obtained from crystal structure analysis. The enzyme was mixed with ATP in the absence of glycine and set up for crystallization. The enzyme was also incubated, in parallel, with Ap4A for crystallization. Interestingly, both setups yielded crystals having the same C2221 space group, with one subunit of the dimeric enzyme accommodated in the asymmetric unit (Table 1). (The space group is different from previous structures of the apoenzyme of wild-type GlyRS and two mutant forms (22, 23).) Both structures were solved by molecular replacement, and the two structures were almost identical (root mean square deviation of 0.56 Å for 530 Cα atoms), with each containing a clear electron density for Ap4A near the active site (Fig. 2). The density for the second adenosine (A2) of Ap4A was weak in the crystal that captured Ap4A synthesis (from ATP) compared with the density for the first adenosine (A1) in the same crystal and with the A2 density in the crystal containing preformed Ap4A (Fig. 2, B and C). This observation suggested that the Ap4A synthesis captured during crystallization may not be complete and that some enzyme-bound ATP remained unreacted. Nevertheless, the glycine-independent formation of Ap4A was captured in the crystal, and interestingly, these crystals diffracted to a higher resolution (2.5 Å) compared with the co-crystals of the preformed Ap4A (Table 1).

AP4A-binding Site of Human GlyRS Is Unique—We compared our GlyRS-Ap4A co-crystal structure with a previously solved co-crystal structure of Ap4A with seryl-tRNA synthetase (SerRS) from Thermus thermophilus (24). (GlyRS and SerRS are both class II tRNA synthetases and contain in their catalytic domain three conserved motifs (1–3) that are characteristic of all class II tRNA synthetases.) Interestingly, whereas the binding site for the first adenosine moiety of Ap4A is conserved and overlaps with the conserved ATP-binding site for all class II tRNA synthetases, the binding site for the second adenine is completely different between GlyRS and SerRS (Fig. 3A). In GlyRS, the second adenosine-binding site is sandwiched between the glycine-binding loop (see below) and insertion I of the catalytic domain (Fig. 3A). Insertion I (Phe144–Asn225), located between motifs 1 and 2 in the protein sequence, is specific to GlyRS and not found in any other tRNA synthetases (25). Therefore, both binding elements for the second adenosine moiety of Ap4A are unique to GlyRS. The binding site for the second adenosine is partially exposed to solvent. Two residues (Asp193 and Asn381) make H-bonding interactions with the N-7 and N-6 amino groups of adenine, respectively, to provide the specificity.
for ATP and to differentiate it from other nucleotides. Meanwhile, Asp^{146} and Arg^{159} of insertion I interact with the fourth phosphate group of Ap4A adjacent to the second adenosine group (Fig. 3B). Interestingly, comparing the Ap4A complex structure with our previously solved apo structure of human GlyRS (22), the largest conformational change occurs at insertion I (Fig. 4A and supplemental Fig. S3A). The root mean square deviation between the two structures is 2.44 Å for all 524 resolved Ca atoms; however, the root mean square deviation for the whole structure, exclusive of insertion I, is only 0.86 Å (455 Ca atoms), and that for insertion I alone is only 0.61 Å (82 Ca atoms). This observation suggests that Ap4A binding induces a hinge-like movement of insertion I, virtually as a rigid body, to form the binding site. The hinge is located where insertion I connects to the catalytic domain and is formed by loops immediately before/after two antiparallel strands (β5 and β7) at the beginning and end of insertion I, respectively.

Because the apo structure solved previously had a different space group (P4_122_1), it is possible that the conformational change in the complex structure was caused by differences in crystal lattice interactions. However, the crystal lattice interactions that involve insertion I are almost identical between the two crystals. In fact, we were able to obtain crystals of the free enzyme in the same C22_2 space group as found in the complex. However, even after optimization, these crystals diffracted only to <5 Å resolution compared with 2.5 Å resolution for the Ap4A complex, suggesting that there is an intrinsic conformational change associated with the complex structure that stabilizes the crystal packing in the C22_2 space group.

**ATP Binding-induced Formation of the Glycine- and Second ATP-binding Pockets**—To understand the determinants of the conformational change, we solved three additional co-crystal structures of human GlyRS with an ATP analog (AMPCPP), with ATP and glycine, and with a Gly-AMP analog (S'-O-(L-glycyl)sulfamoyl)adenosine). All of the crystals were obtained by co-crystallization and have the same space group (C22_2) as the Ap4A complex. Interestingly, all of the complex...
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structures superimpose well and have essentially the same conformation as the Ap4A complex as well as in contrast to the apo structure (Fig. 4A and supplemental Fig. S3A). (The root mean square deviation of all Cα atoms between structures of the complex is <0.5 Å.) This observation holds true even upon closer scrutiny of the active site, suggesting that ATP is the major allosteric effector for formation of the second ATP-binding pocket for Ap4A synthesis and for formation of the glycine-binding pocket for Gly-AMP synthesis (see below).

In comparing any one of the complex structures with the apo structure of human GlyRS, another major difference, in addition to the movement of insertion I, is the formation of a loop structure (Glu380–Cys388) that provides the binding site for glycine (Fig. 4, B–G, and supplemental Fig. S3, B–G). This glycine-binding loop was largely disordered in the apo structure, and as a consequence, the glycine-binding site was only partially formed in the free enzyme. Importantly, ATP (AMPCPP) binding alone is sufficient to induce formation of the glycine pocket, achieved mainly by engaging the conserved Tyr386 of the glycine-binding loop (Fig. 4D and supplemental Fig. S3D). Although, unlike in the human enzyme, the glycine-binding loop in T. thermophilus GlyRS was resolved in the apo form (26), a similar conformational change occurs upon binding of ATP (27), in which the glycine-binding loop and Tyr287 (equivalent to Tyr386 in human GlyRS) engage to form the pocket for glycine binding. Thus, from bacteria to humans, Tyr386, under the trigger of ATP, acts as the gatekeeper of the glycine-binding pocket in GlyRS.

As shown in the co-crystal structures with ATP and glycine (Fig. 4F and supplemental Figs. S3F and Fig. S4A) and with the Gly-AMP analog (Fig. 4G and supplemental Figs. S3G and S4B), Tyr386, together with Glu245 and Glu225, forms a van der Waals block against the Cα atom of glycine to prevent binding of any other amino acid (supplemental Fig. S4B). In addition, the carboxylate side chains of Glu245, Glu225, and Glu296 recognize the α-amino group of glycine, whereas the hydroxyl groups of Ser524 and Tyr386 form H-bonds with the carboxylate group of the amino acid (supplemental Fig. S4A). All of the residues for glycine binding are conserved in T. thermophilus GlyRS, with a very similar arrangement for glycine recognition (supplemental Fig. S4B).

Similarly, the binding site for the second adenosine is also formed following binding of the first ATP. Those residues (i.e. Asp146, Arg159, Asp193, and Asn381) that interact with the second adenosine share the same conformation between ATP and Ap4A binding (Fig. 4, B and C, and supplemental Fig. S3, B and C). Thus, in human GlyRS, ATP binding to the conserved ATP-binding site induces the formation of binding pockets for both glycine and a second adenosine.
Although the majority of the insertion I domain was disordered in the crystal structures of the free and ATP-bound GlyRS from *T. thermophilus*, the resolved region of insertion I that contains residues that are equivalent to Asp146 and Asn381 of human GlyRS superimposed well with the corresponding structure from the human enzyme (data not shown). As shown
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FIGURE 5. Inhibition of GlyRS-catalyzed Ap4A synthesis by tRNA<sub>Gly</sub>. A, GlyRS-catalyzed Ap4A synthesis is inhibited by tRNA<sub>Gly</sub> in both the absence and presence of glycine. B, stereo view of the structural model of the GlyRS-tRNA complex showing the steric block of the second adenosine-binding site by the 3′-end of the tRNA and the steric clash between insertion I and the tRNA acceptor stem. tRNA is shown in purple and orange, with the subunits of the GlyRS dimer shown in cyan and gray. The steric block/clash is highlighted by the red dashed circle.

in Fig. 3B, Arg<sup>159</sup> and Asp<sup>146</sup> interact with Ap4A (at the fourth phosphate group) through their specific side chains. Interestingly, both Arg<sup>159</sup> and Asp<sup>146</sup> are strictly conserved in <i>T. thermophilus</i> GlyRS and in all α2 GlyRS homodimers throughout evolution (26, 28). In contrast, neither Asp<sup>193</sup> nor Asn<sup>381</sup> is conserved in <i>T. thermophilus</i> GlyRS, consistent with the fact that these two residues interact with Ap4A through their main chain oxygen atoms (Fig. 3B). Therefore, the second adenosine-binding pocket and the capacity for direct Ap4A synthesis is likely to be conserved in <i>T. thermophilus</i> GlyRS and possibly in all α2 GlyRSs.

Structure-based Mechanism for Ap4A Formation Confirmed by Biochemical Analysis—Interestingly, the conformation of bound ATP or of AMPcpp completely overlaps with the first adenosine and the three successive phosphate groups of Ap4A (Fig. 3C). Furthermore, the γ-phosphate of ATP has a second conformation, which partially overlaps with the fourth phosphate of Ap4A, suggesting that the binding sites for the phosphate groups of ATP are conserved for Ap4A binding in human GlyRS. This observation is consistent with the mechanism of direct condensation of two ATP molecules (Fig. 1A). In the most likely scenario, the γ-phosphate of the ATP bound in the conserved binding site attacks the α-phosphate of the second ATP to form Ap4A (Fig. 3C). Results from our Ap4A synthesis assay are consistent with this scenario. When [α-<sup>32</sup>P]ATP was used as substrate, radioactive counts from the Ap4A product were almost exactly doubled compared with when [γ-<sup>32</sup>P]-ATP was used (supplemental Fig. S5), indicating that the reaction was asymmetric, with three phosphates originating from one ATP molecule and with one phosphate originating from the other. In contrast, the previously known amino acid-dependent mechanism involves the γ-phosphate of the second ATP attacking the α-phosphate of the aminoacyl-AMP reaction intermediate to form Ap4A (24). Consistently, there is less overlap between conformations of the bound ATP and Ap4A beyond the α-phosphate in <i>T. thermophilus</i> SerRS, which is known to carry out serine-dependent formation of Ap4A (Fig. 3D).

<i>tRNA Inhibits Ap4A Formation by GlyRS</i>—It is known that the presence of cognate tRNA inhibits amino acid-dependent Ap4A synthesis because the tRNA competes with the second ATP for the aminoacyl adenylate, the reaction intermediate both for charging and for Ap4A synthesis. As Ap4A formation in human GlyRS is decoupled from glycine and thus, presumably, the aminoacylation reaction, we wondered if the availability of tRNA would still influence Ap4A formation. We included tRNA<sup>Gly</sup> in the reaction in both the absence and presence of glycine and found that no or a very small amount of Ap4A was produced compared with the amount of Ap4A produced in the absence of the cognate tRNA (Fig. 5A). This observation suggests that tRNA binding also inhibits the amino acid-independent formation of Ap4A by GlyRS, most likely via a separate mechanism. Interestingly, a structural model of the GlyRS-tRNA complex can explain the observed inhibition. The model was generated by overlapping the catalytic domain of GlyRS (bound with Ap4A) with that of <i>Escherichia coli</i> threonyl-tRNA synthetase bound with its cognate tRNA (Protein Data Bank code 1QF6) (29). (Among the several class II tRNA synthetases whose structures were solved with their bound tRNA, <i>E. coli</i> threonyl-tRNA synthetase has the closest conformation to human GlyRS.) In this model, the CCA 3′-end of tRNA completely blocks the second ATP/adenosine-binding site (Fig. 5B). In addition, there is a steric clash between insertion I and the tRNA acceptor stem. Therefore, tRNA binding is likely to induce a conformational change that moves insertion I.
away from the current conformation found in the Ap4A complex (Fig. 5B). Thus, the inhibition of GlyRS-catalyzed Ap4A synthesis by tRNA comes from the disruption of the second ATP-binding site by direct blocking and/or by tRNA-induced conformational change.

GlyRS Is Unique among tRNA Synthetases in Amino Acid-independent Ap4A Formation—As binding of Ap4A (in particular, the second adenosine) involves elements that are unique to GlyRS (i.e. insertion I and the glycine-binding loop), it is possible that the amino acid-independent Ap4A formation is specific to GlyRS. To test this hypothesis, we assayed human LysRS and TyrRS, both of which have been reported to carry out Ap4A synthesis. LysRS, in particular, has been considered to play a major role in generating Ap4A in vivo (10, 30). For reasons that we do not understand, the rate of Ap4A synthesis catalyzed by human LysRS in our system was considerably lower (~100-fold) than what has been reported. (Active-site titrations of LysRS confirmed that the enzyme preparation was fully active.) Nevertheless, as expected, whereas both LysRS and TyrRS formed Ap4A (detected by TLC) in the presence of the cognate amino acid, neither produced Ap4A in the absence of amino acid (Fig. 1, D and E), supporting the idea that GlyRS is unique among the synthetases in its mechanism of Ap4A formation.

It is worth noting that a portion of Ap3A was also found to be produced by both LysRS and TyrRS. Because Ap4A and Ap3A co-migrate in the TLC system used here, the Ap4A detected by TLC is actually a mixture of Ap3A and Ap4A, as revealed by subsequent mass spectrometry analysis. Interestingly, whereas GlyRS and TyrRS produced mostly Ap4A (85 and 78%, respectively) with only a small fraction of Ap3A, LysRS produced mostly Ap4A (85 and 78%, respectively) with only a small fraction of Ap3A, which was almost exclusively Ap3A (90%) and a small fraction of Ap4A.

Further Demonstration of a Unique Mechanism for Ap4A Synthesis by Human GlyRS—It has been shown that Zn2+ enhances the rate of Ap4A synthesis by LysRS (as well as phenylalanyl-tRNA synthetase) (9, 31–33). To test the effect of Zn2+ on GlyRS-catalyzed Ap4A synthesis, the activity was assayed in the presence of 150 μM ZnCl2 or 10 μM 1,10-phenanthroline (a Zn2+ chelator to sequester possible trace Zn2+ in the buffer). As a control, we also tested human LysRS. Consistent with previous reports, addition of 150 μM ZnCl2 to the reaction catalyzed by LysRS (in the presence of lysine) enhanced Ap4A/Ap3A synthesis by ~40-fold as detected by TLC (Fig. 6, inset). In contrast, addition of Zn2+ completely inhibited the activity of human GlyRS for Ap4A synthesis (Fig. 6). When Zn2+ was completely removed with 1,10-phenanthroline, the activity of LysRS dropped to lower than that of GlyRS. Although free Zn2+ is not largely available in vivo, as it is usually sequestered by various Zn2+-binding proteins, the differential effects of Zn2+ on Ap4A synthesis in vitro further distinguish the mechanism by which GlyRS forms Ap4A from that of LysRS and likely other AARSs.

DISCUSSION

We have shown that human GlyRS uses direct ATP condensation to synthesize Ap4A and that this unique mechanism is likely to be conserved for GlyRSs from eukaryotes and archaea that are α2 homodimers. Whereas some bacterial GlyRSs (e.g. T. thermophilus) are eukaryote-like α2 homodimers, others (e.g. E. coli) form α2β2 heterotetramers. The tetrameric GlyRSs share low sequence similarity with the dimeric counterparts and have no conservation of insertion I (28) and therefore are not likely to use the same mechanism for Ap4A synthesis. Indeed, the formation of Ap4A by E. coli GlyRS was found to be glycine-dependent (34).

In E. coli, a mutation in the gene encoding the α-subunit of GlyRS stimulated Ap4A synthesis, causing a 15-fold increase in the intracellular level of Ap4A. Independently, an insertion in the gene encoding Ap4A hydrolase (the enzyme that degrades Ap4A) disrupted the hydrolase activity and again significantly increased the Ap4A level (35). In both cases, the high level of Ap4A caused early cell division, suggesting that the intracellular level of Ap4A needs to be tightly regulated. It is intriguing to speculate that GlyRS is responsible for maintaining the basal level of cellular Ap4A. Furthermore, the transition from the α2β2 to the α2 GlyRS may be related to the selective pressure to maintain a constant basal level of Ap4A, which can be more readily achieved when Ap4A synthesis is independent of amino acid.

Whereas GlyRS may be linked to Ap4A homeostasis, LysRS, on the other hand, could play a major role in regulating Ap4A concentration in response to selective stimuli or specific physiological conditions. Interestingly, E. coli LysRS exists as two isoforms, LysS and LysU. Whereas LysS is constitutively expressed and responsible for aminoacylation, LysU is expressed only under conditions of cellular stress, and the primary function of LysU is considered to be the synthesis of Ap4A (36).

In humans, unlike GlyRS, LysRS is a component of the multisynthetase complex that consists of nine different tRNA synthetases and three auxiliary factors. Significantly, LysRS can be dissociated from the multisynthetase complex and secreted upon tumor necrosis factor-α stimulation (37). The Ap4A synthesis activity of LysRS is 6-fold higher in the free form than in
the complex (31) and is further stimulated in the presence of Zn²⁺. Moreover, Ap4A synthesis by human LysRS has been linked to the regulation of the transcription factors MITF and USF2 in immunologically activated mast cells (38, 39).

The activity of human GlyRS in Ap4A synthesis may be lower than that of LysRS under most physiological conditions, and such a consideration is consistent with the idea that GlyRS maintains and LysRS regulates the cellular level of Ap4A. Alternatively, the two enzymes, as a complementary pair, may function preferentially in different environments. Ap4A and other diadenosine oligophosphates have been detected in neuronal cells (41, 42). Ap4A is released upon nerve terminal depolarization and, in turn, acts on specific purinoreceptors to trigger the release of neurotransmitters such as glutamate, γ-aminobutyric acid, and acetylcholine. Interestingly, there is a clear connection between GlyRS and the neurological system. At least 10 dominant mutations of the gene encoding GlyRS have been identified in the human population to cause Charcot-Marie-Tooth disease, the most common heritable peripheral neuropathy (reviewed in Refs. 43 and 44). Potentially, the Charcot-Marie-Tooth-causing mutations in GlyRS would have an advantage in Ap4A synthesis compared with LysRS, whose Ap4A synthesis activity is absolutely lysine-dependent. Interestingly, GlyRS and LysRS are the only two examples of human tRNA synthetases having a single gene encoding both the mitochondrial and cytosolic forms (40). Thus, the GlyRS we studied here is identical in both the cytosol and mitochondrion (except for the addition of a mitochondrial targeting sequence in the mitochondrial GlyRS). In contrast, the cytosolic and mitochondrial forms of other human tRNA synthetases are two different proteins encoded by two separate genes. This uniqueness of GlyRS and LysRS may be connected to their special roles in Ap4A synthesis, where GlyRS maintains and LysRS regulates the level of Ap4A. Alternatively, the two enzymes, as a complementary pair, may function preferentially in different environments.

Mitochondria are abundant in neuronal cells. Ap4A and other diadenosine oligophosphates have been detected in neurons and are stored in synaptic vesicles or synaptosomes of neuronal cells or tissues (41, 42). Ap4A is released upon nerve terminal depolarization and, in turn, acts on specific purinoreceptors to trigger the release of neurotransmitters such as glutamate, γ-aminobutyric acid, and acetylcholine. Interestingly, there is a clear connection between GlyRS and the neurological system. At least 10 dominant mutations of the gene encoding GlyRS have been identified in the human population to cause Charcot-Marie-Tooth disease, the most common heritable peripheral neuropathy (reviewed in Refs. 43 and 44). Potentially, the Charcot-Marie-Tooth-causing mutations in GlyRS may affect Ap4A synthesis and homeostasis.

A rich body of expanded functions of AARSs has been found that links tRNA synthetases with broad biological systems. The amino acid-independent Ap4A synthesis by human GlyRS not only broadens the mechanisms for generating this important signaling molecule but also strengthens the connection of tRNA synthetases with other biological pathways via Ap4A.

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REFERENCES
Supplemental Figure S1. Ap4A identification by mass spectrometry. Purified product from a GlyRS Ap4A formation assay (with no glycine added) was analyzed by MALDI-TOF mass spectrometry. Peaks at 837 and 859 correspond to expected masses of H\(^+\) and Na\(^+\) ions of Ap4A. The peak at 757 corresponds to the H\(^+\) ion of Ap3A.
Supplemental Figure S2. Amino acid activation assay to detect potential glycine contamination in GlyRS. ATP-PP\textsubscript{i} exchange was measured in the absence of glycine or with glycine added at the indicated concentrations.
Supplemental Figure S3. Stereo views of the ATP binding-induced formation of glycine and second ATP binding pockets in human GlyRS. A. Superposition of apo human GlyRS structure (gray, PDB # 2PME) with five complex structures determined in this work, illustrating the conformational change of insertion I in all complex structures (pink: complex with ATP analog AMPCPP, green: complex with ATP and glycine, purple: complex with Gly-AMP, cyan: complex with Ap4A generated *in situ* from ATP, yellow: complex with pre-formed Ap4A). Only the bound Ap4A is shown (blue). B,D. Superposition of apo (gray) and AMPCPP complex (pink) structures showing formation of the second adenosine (A2) binding site and the glycine binding site by engaging Y386 of the glycine binding loop (arrow) upon ATP (AMPCPP) binding. C,E. Superposition of apo and Ap4A complex (cyan) structures. F,G. Superposition of the apo structure with the ATP and glycine complex structure (green) and with the Gly-AMP complex structure (purple).
Supplemental Figure S4. ATP and glycine binding pockets of GlyRS. A. Stereo view of the ATP and glycine binding sites in human GlyRS and the residues involved in the interaction. Dashed lines indicate H-bonds. B. Comparison of glycine recognition in human and *T. thermophilus* GlyRS (PDB # 1GGM).
Supplemental Figure S5. Relative amounts of radiolabel incorporated into Ap4A when synthesized with [α-32P]ATP or [γ-32P]ATP. GlyRS was incubated with either [α-32P] ATP or [γ-32P]ATP in the absence of glycine, under the standard Ap4A assay conditions (near identical results were seen in the presence of glycine, data not shown). Radioactive counts in Ap4A spots are plotted as a percentage of total radioactive counts in a lane.
Supplemental Methods

Ap4A Purification and Identification.
A standard Ap4A formation assay with GlyRS and no glycine was set up and allowed to proceed at 37 °C for 48 hours. The reaction was extracted with phenol:chloroform:isoamyl alcohol, spotted on a PEI-cellulose TLC plate and developed in 3 M (NH₄)₂SO₄ and 2% EDTA. The spot co-migrating with Ap4A standard was located under UV light and excised from the plate. The resin was scraped into a microfuge tube and the sample was eluted with 0.25 M NH₄HCO₃. The purified sample was analyzed by MALDI-TOF mass spectrometry. Relative abundances of Ap3A and Ap4A were estimated based on a standard curve created from mass spectrometry of known mixtures of Ap3A and Ap4A standards.

Amino Acid Activation Assay.
A standard amino acid activation assay (ATP-PPi exchange assay) was carried out in 100 mM HEPES pH 7.5, 20 mM KCl, 10 mM MgCl₂, 2 mM DTT, 2 mM ATP and 1 mM Na[^32P]PPi. Glycine was included at the indicated concentrations. The reactions were initiated by addition of 10 μM GlyRS at 25 °C. Time points were quenched into MultiScreen filter plates (Millipore) containing 10% charcoal, 0.5% HCl and 200 mM sodium pyrophosphate; and washed as described²⁰. The charcoal was dried to a fine powder and counted in a scintillation counter.
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