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Crucial Role of the HIGH-loop Lysine for the Catalytic Activity of Arginyl-tRNA Synthetase*

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The presence of two short signature sequence motifs (His-Ile-Gly-His (HIGH) and Lys-Met-Ser-Lys (KMSK)) is a characteristic of the class I aminoacyl-tRNA synthetases. These motifs constitute a portion of the catalytic site in three dimensions and play an important role in catalysis. In particular, the second lysine of the KMSK motif (K2) is the crucial catalytic residue for stabilization of the transition state of the amino acid activation reaction (aminoacyl-adenylate formation). Arginyl-tRNA synthetase (ArgRS) is unique among all of the class I enyzmes, as the majority of ArgRS species lack canonical KMSK sequences. Thus, the mechanism by which this group of ArgRSs achieves the catalytic reaction is not well understood. Using three-dimensional modeling in combination with sequence analysis and site-directed mutagenesis, we found a conserved lysine in the KMSK-lacking ArgRSs upstream of the HIGH sequence motif, which is likely to be a functional counterpart of the canonical class I K2 lysine. The results suggest a plausible partition of the ArgRSs into two major groups, on the basis of the conservation of the HIGH lysine.

Aminoacyl-tRNA synthetases (amino acid-tRNA ligases, aaRSs)[1] catalyze the aminoacylation of cognate tRNAs in a two-step reaction. At the first step, the aaRSs specifically bind the amino acid and ATP to activate the amino acid through the formation of an aminoacyl-adenylate. At the second step, the aminoacyl moiety is transferred to the 3′-terminal adenosine (A76) of the tRNA. The 20 aaRSs are divided into two classes (10 members each) on the basis of their evolutionarily distinct ATP-binding catalytic domains (1, 2). The class I aaRSs are characterized by the Rossman-fold architecture of the catalytic domain and by the two short conserved sequence motifs, His-Ile-Gly-His (HIGH) and Lys-Met-Ser-Lys (KMSK). These motifs, which are far apart in the primary sequence, come together in the three-dimensional structure to constitute a portion of the ATP-binding site. The second lysine in the KMSK motif (K2) plays a key role in the catalysis, as its mutation causes the most severe defect in the enzymatic activity (3–6). In the crystal structure of the glutaminyl-tRNA synthetase (GlnRS)·$tRNA^{Gln}$·ATP complex, this lysine interacts with the α-phosphate of the ATP molecule (7). In the structures of the tryptophanyl-tRNA synthetase·Trp-AMP and GlnRS·$tRNA^{Gln}$·Gln-AMP analog complexes (8, 9), the K2 side chain forms a salt bridge with the phosphate group of the adenylate. Thus, it is now believed that K2 stabilizes the negatively charged transition state of the first reaction step in the class I synthetases (10). In agreement with the proposed function, K2 is strictly conserved in the class I aaRSs, with only a few exceptions. Arginyl-tRNA synthetase (ArgRS) is the only class I enzyme for which many species lack the apparent KMSK motif and, subsequently, the K2 counterpart. Recently, the atomic structures of the yeast *Saccharomyces cerevisiae* (11) and *Thermus thermophilus*[2] ArgRSs have been determined. In both enzymes, the KMSK motif is degenerate, but ^{407}GMST^{410} (yeast ArgRS) and ^{394}QMSG^{397} (*T. thermophilus* ArgRS) were shown to be its structural variants, in which the class I principle lysine (K2) residues are absent. As the ATP-bound structures are not available for both the yeast and *T. thermophilus* enzymes, the mechanism by which these ArgRSs compensate for the lack of K2 remains obscure.

EXPERIMENTAL PROCEDURES

General—[^{32}P]Sodium pyrophosphate and L-[^{14}C]arginine were purchased from PerkinElmer Life Sciences. The *Pyrobest* DNA polymerase for PCR and the restriction endonuclease *Nde*I were obtained from Takara Shuzo Co., Ltd. (Shiga, Japan). The restriction endonuclease *Bbr*PI was from Roche Molecular Biochemicals (Tokyo, Japan). T7 RNA polymerase was purified from an overproducing strain, kindly provided by Dr. W. Studier (Stonybrook, New York), according to Ref. 12.

Preparations of Proteins and RNA—The wild-type *T. thermophilus* ArgRS was expressed in *Escherichia coli* BL21(DE3) cells by using a T7-promotor-controlled expression vector. The protein was purified by a combination of heat treatment (70 °C for 30 min) and sequential chromatographies on DEAE-Sephacel (ϕ2.5 × 20 cm) and FPLC PhenylSuperose (Amersham Pharmacia Biotech, United Kingdom). The expression plasmid for the mutant *T. thermophilus* ArgRS (K116G) was generated from the vector for the wild-type enzyme. A 357-base pair-long DNA fragment (*Nde*I-*Bbr*PI sites) in the enzyme gene was replaced by a PCR-amplified fragment that contains the AAG to GGG mutation in the 116th codon (Lys → Gly). The target mutation and integrity of the gene outside of the mutation site were confirmed by DNA sequencing. The mutant ArgRS was expressed in the *E. coli* cells and was purified according to the procedure used for the wild-type enzyme. The purified mutant ArgRS shows the same mobility on the SDS-polyacrylamide gel electrophoresis as the wild-type enzyme. The wild-type and mutant ArgRSs exhibit the same circular dichroism (CD) spectra with the calculated α-helical content of 45%, which is consistent with the struc-

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[1] The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase; ArgRS, arginyl-tRNA synthetase; PP_i, pyrophosphate; GluRS, glutamyl-tRNA synthetase; IleRS, isoleucyl-tRNA synthetase; PCR, polymerase chain reaction.

[2] A. Shimada, O. Nureki, and S. Yokoyama, unpublished result.

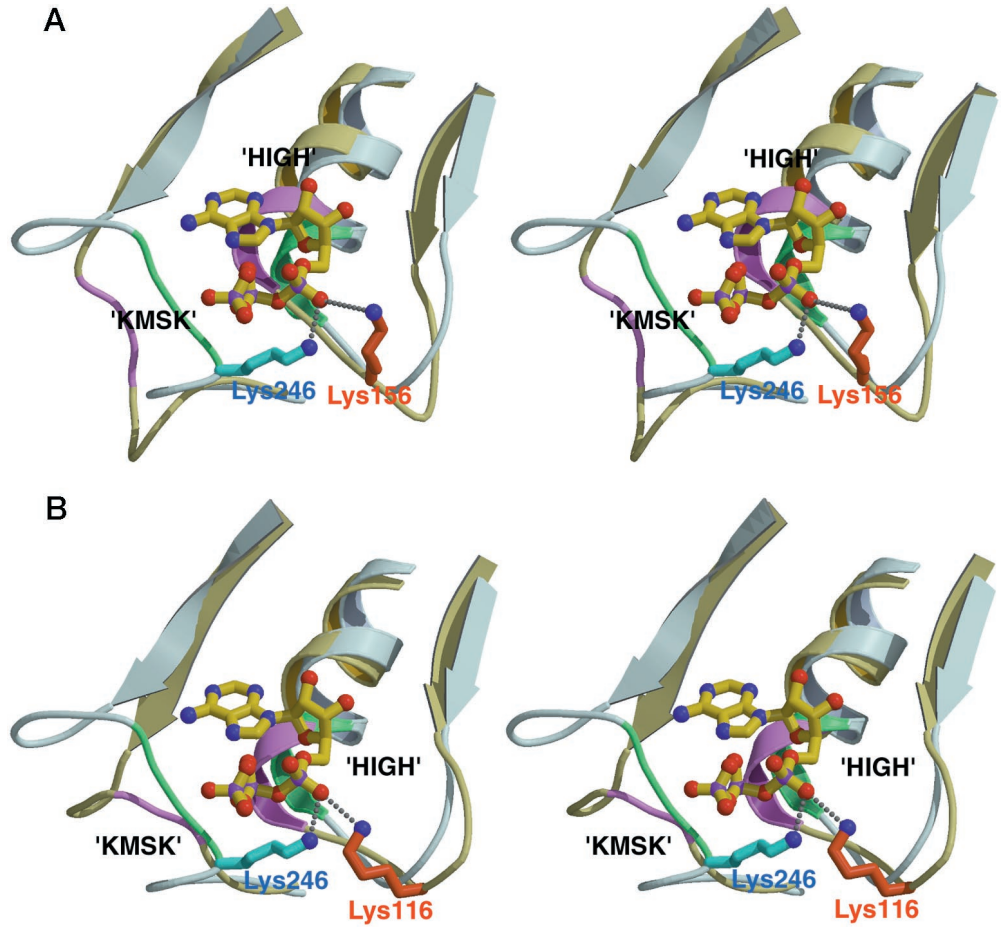

FIG. 1. **Position of the HIGH-loop Lys in the ArgRS structures.** *A*, a stereo view showing the yeast ArgRS catalytic site superposed on that of the ATP-bound *T. thermophilus* GluRS. In the GluRS active site (colored in *light blue*), the second Lys (Lys^{246}, *cyan*) of the KMSK motif (*green*) interacts with one of the α-phosphate oxygens of the ATP molecule (*yellow*). The ArgRS catalytic site (*dark orange*) is superposed on that of GluRS without any modification of the synthetase structure. Lys^{156} near the "HIGH" motif (*purple*) is displayed in orange. *B*, the catalytic site of *T. thermophilus* ArgRS (*dark orange*) is superposed on that of the ATP-bound GluRS, and the HIGH-loop lysine (Lys^{116}) is shown in *orange*. The figures were produced with the MOLSCRIPT (20) and RASTER3D (21) programs.

tural data. The two proteins are thermostable: the temperature profiles of the CD (222 nm) are linear and reversible in a wide temperature range (20–95 °C). *T. thermophilus* $tRNA^{Arg}$ was synthesized by *in vitro* transcription with T7 RNA polymerase and was purified as described previously (13, 14).

Measurements of Enzyme Activities—The arginine (and tRNA)-dependent PP_i-ATP isotopic exchange reaction was carried out at 65 °C in 50 mM Hepes-NaOH buffer (pH 7.2) containing 20 mM $MgCl_2$, 40 mM KCl, 2 mM arginine, 20 μM $tRNA^{Arg}$ transcript, 4 mM ATP, 4 mM [^{32}P]sodium pyrophosphate (1.8 TBq mol^{-1}), and 50 nM (wild-type or mutant) ArgRS. After various incubation times, a 10-μl aliquot of the reaction was withdrawn, and the amount of the synthesized [^{32}P]ATP was measured as described previously (15). The aminoacylation reaction was carried out at 65 °C in 100 mM Hepes-NaOH buffer (pH 7.5) containing 5 mM $MgCl_2$, 40 mM KCl, 4 mM ATP, 100 μM L-[^{14}C]arginine, 20 μM $tRNA^{Arg}$ transcript, and 100 nM (wild-type or mutant) ArgRS. At various incubation times, an 8-μl aliquot was removed, and the reaction was quenched by addition of ice-cold trichloroacetic acid. It was washed with 5% trichloroacetic acid on a filter paper (Whatman 3MM, ϕ24 mm), and the radioactivity of the acid-insoluble fraction (the synthesized [^{14}C]Arg-$tRNA^{Arg}$) was measured by a liquid scintillation counter.

RESULTS AND DISCUSSION

We have recently determined the structure of another class I synthetase, *T. thermophilus* glutamyl-tRNA synthetase (GluRS), bound to ATP, which is the first tRNA-free, ATP-bound structure of a class I synthetase.[3] In contrast to the ArgRSs, the overwhelming majority of GluRSs possess the highly conserved KMSK signature motif (^{243}KISK^{246} for *T. thermophilus* GluRS). In the GluRS·ATP complex, the side chain of Lys^{246} (the class I K2) forms a salt bridge with the α-phosphate of ATP (Fig. 1*A*), which is consistent with its catalytic function. The structures of the catalytic domains are well conserved between the two ArgRSs (yeast and *T. thermophilus*) and *T. thermophilus* GluRS (the root mean square displacements are 1.92 and 1.88 Å for 75 and 74 Cα atoms, respectively), which suggests the similarity in the ATP binding mode. To identify the functional counterpart of K2 in the ArgRSs, the ATP-binding sites of the ArgRSs were compared with that of the ATP-bound GluRS (Fig. 1). To our surprise, the superpositions showed that the ζ ammonium groups of the lysine residues, Lys^{156} in the yeast and Lys^{116} in the *T. thermophilus* ArgRSs, respectively, are located in close vicinity of the GluRS ATP α-phosphate (distances are 4.3 and 3.2 Å, respectively), without any modification of the synthetase structures (Fig. 1). These lysine residues protrude from the HIGH loops (the N-terminal loop of the HIGH motif) to form a putative contact with the ATP α-phosphate oxygen from the opposite direction of the GluRS KMSK lysine (K2). To investigate the functional significance of the HIGH-loop lysine residue, we prepared a *T. thermophilus* ArgRS with the mutation of Lys^{116} to Gly (K116G), and its catalytic activities were compared with those of the wild-type enzyme. The mutant ArgRS (K116G) exhibits considerably lower activity for the arginine (and $tRNA^{Arg}$)-dependent PP_i-ATP isotopic exchange, which is the

[3] S. Sekine, O. Nureki, D. G. Vassylyev, and S. Yokoyama, unpublished result.

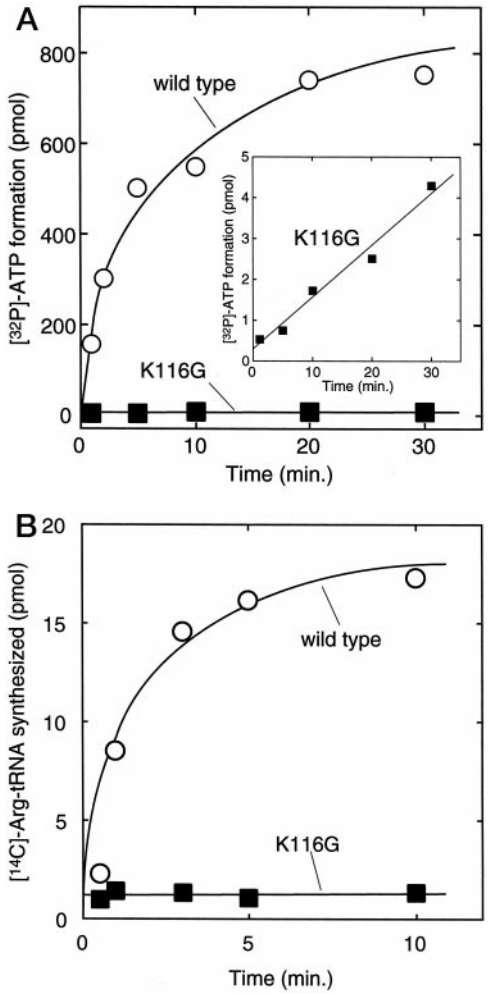

FIG. 2. **The effects of the mutation of HIGH-loop lysine on the ArgRS activities.** *A*, arginine and tRNA dependent PP_i-ATP isotopic exchange reactions catalyzed by the wild-type (○) and mutant (K116G, ■) *T. thermophilus* ArgRSs. *B*, profiles of $tRNA^{Arg}$ arginylation by wild-type (○) and mutant (K116G, ■) *T. thermophilus* ArgRSs.

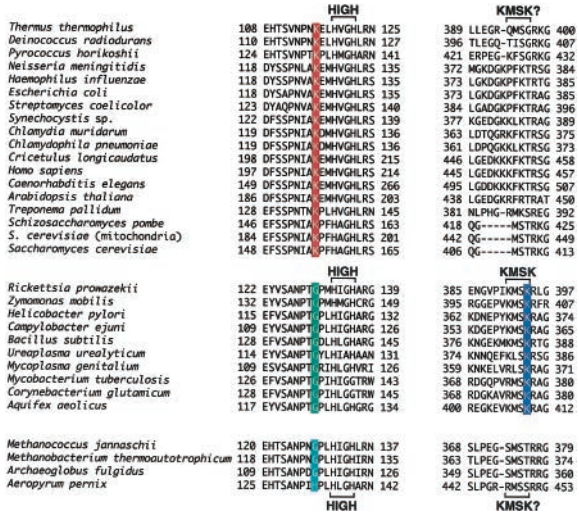

FIG. 3. **Alignment of the ArgRS sequences around the "HIGH" and "KMSK" regions.** The 36 available ArgRS sequences were compared, and 19, 13, and 4 of them were classified as HIGH, KMSK, and tentative groups, respectively. In the figure, only one species belonging to the same genus is listed. The proposed catalytic Lys in the HIGH group ArgRSs and its counterpart Gly in the KMSK group enzymes are shown in *red* and *green*, respectively. The second Lys in the canonical KMSK motif is indicated in *blue*.

reverse reaction of Arg-AMP formation (first step of aminoacylation) (Fig. 2*A*). The initial rate catalyzed by the mutant ArgRS is reduced by a factor of >1000, as compared with that of the wild-type enzyme. Consistently, the arginine charging on $tRNA^{Arg}$ catalyzed by the mutant ArgRS (K116G) is not detectable (Fig. 2*B*). Thus, we conclude that this lysine residue plays a crucial role during the aminoacylation reaction, in particular at the first step. It probably compensates for the lack of the second lysine (K2) in the KMSK motif.

A sequence analysis shows that the ArgRSs can be divided into two major groups, on the basis of the conservation of the HIGH-loop lysine (Fig. 3). The distribution of ArgRSs with the HIGH-loop lysine extends to Eukarya, Archaea, and several taxons of Bacteria (Proteobacteria β/γ subdivision, *Thermus-Deinococcus* group, Cyanobacteria, and so on) ("HIGH group"). Remarkably, these ArgRSs do not possess a canonical KMSK motif (11), which suggests that in these enzymes, the HIGH-loop lysine is likely to play the role of the class I K2. In contrast, in several bacterial classes (Proteobacteria α/ϵ subdivision, most Gram-positive bacteria, and Aquificales), a glycine residue substitutes for the HIGH-loop lysine in the ArgRSs (Fig. 3). Consistently, these bacteria preserve the KMSK motif, suggesting that the canonical class I K2 lysine mediates the adenylate formation ("KMSK group"). Exceptionally, a few archaea lack both the HIGH-loop and KMSK lysines, which suggests a somewhat different architecture of the active center and/or mechanism of the catalysis (tentative group). As far as we see from the sequences of the class I synthetases, the HIGH-loop lysine is a unique characteristic of the ArgRSs, and the purpose of the drastic migration of the catalytic residue in the primary sequences is not clear. ArgRS is one of the three class I synthetases that possess a tRNA-dependent mechanism of amino acid activation (16–18). Thereby, one possibility is that the appearance of the HIGH-loop lysine might be the result of the coevolution of the ArgRSs and $tRNA^{Arg}$ molecules from the subsequent organisms. It is worth mentioning that some ArgRSs from the HIGH group contain KMSK-like sites (*E. coli* etc.) (Fig. 3). Although the lysines of these sites are shifted in sequence from their positions within the canonical KMSK motifs (Fig. 3) (11), we cannot exclude here the possibility of their catalytic role.

The aaRSs are known to be potential targets for antibiotics. Mupirocin is a natural catalytic inhibitor that is specific to the isoleucyl-tRNA synthetases (IleRSs) from several Gram-positive and -negative bacterial pathogens. Several artificial Ile-AMP analogs have been developed to block the catalytic sites of the IleRSs of pathogens, but not to bind the human IleRS (reviewed in Ref. 19). Similarly, it might be possible to design Arg-AMP analogs that are sensitive to either the KMSK or HIGH-loop lysine in the ArgRSs. This would be especially useful, as the KMSK group includes the ArgRSs from many pathogens, such as *Rickettsia prowazekii*, *Helicobacter pylori*, *Mycobacterium*, and *Mycoplasma* species, etc., whereas all of the eukaryotic enzymes (including human) belong to the HIGH group. Thus, the diversity of the catalytic mechanisms in the ArgRS family could be utilized for the development of new selective anti-infectives.

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