Arginyl-tRNA synthetase with signature sequence KMSK from *Bacillus stearothermophilus*

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ArgRS (arginyl-tRNA synthetase) belongs to the class I aaRSs (aminoacyl-tRNA synthetases), though the majority of ArgRS species lack the canonical KMSK sequence characteristic of class I aaRSs. A DNA fragment of the ArgRS gene from *Bacillus stearothermophilus* was amplified using primers designed according to the conserved regions of known ArgRSs. Through analysis of the amplified DNA sequence and known tRNA^{Arg}s with a published genomic sequence of *B. stearothermophilus*, the gene encoding ArgRS (*argS'*) was amplified by PCR and the gene encoding tRNA^{Arg} (ACG) was synthesized. ArgRS contained 557 amino acid residues including the canonical KMSK sequence. Recombinant ArgRS and tRNA^{Arg} (ACG) were expres-

sed in *Escherichia coli*. ArgRS purified by nickel-affinity chromatography had no ATPase activity. The kinetics of ArgRS and cross-recognition between ArgRSs and tRNA^{Arg}s from *B. stearothermophilus* and *E. coli* were studied. The activities of *B. stearothermophilus* ArgRS mutated at Lys³⁸² and Lys³⁸⁵ of the KMSK sequence and at Gly¹³⁶ upstream of the HIGH loop were determined. From the mutation results, we concluded that there was mutual compensation of Lys³⁸⁵ and Gly¹³⁶ for the amino acid-activation activity of *B. stearothermophilus* ArgRS.

Key words: ATPase, compensation, cross-recognition, KMSK.

INTRODUCTION

The aaRSs (aminoacyl-tRNA synthetases; EC 6.1.1) catalyse the aminoacylation of cognate tRNAs [1]. They are specific to both amino acid and tRNA substrates, thus ensuring high-fidelity translation [2,3]. Except ArgRS, GluRS and GlnRS (arginyl-, glutamyl- and glutaminyl-tRNA synthetase), the other 17 aaRSs catalyse tRNA aminoacylation through two steps: amino acid activation followed by aminoacylation of the cognate tRNA [3]. The 20 synthetases in *Escherichia coli* can be classified into two groups with distinct catalytic structures according to their conserved sequence motifs and the specific three-dimensional structural elements. Class II aaRSs are characterized by three homologous motifs, designated motifs 1, 2 and 3. Class I aaRSs contain two signature peptides, His-Ile-Gly-His (HIGH) and Lys-Met-Ser-Lys (KMSK), located in the active site with a characteristic nucleotide-binding fold (Rossmann fold) [4]. Although these two signature sequences are far apart in the primary sequence, the three-dimensional structure brings them near each other at the ATP-binding site involved in the amino acid-activation step. Mutation of the second lysine in the KMSK motif (K2) causes severe defects in enzymic activity [5–8], indicating that this lysine plays a key role in catalysis.

ArgRS (EC 6.1.1.19) belongs to class I aaRSs [4] and requires the presence of the cognate tRNA for amino acid activation [9,10]. Alignment of available ArgRS sequences revealed that the majority of them lacked the canonical KMSK sequences [11]. The genes encoding ArgRSs from *E. coli* [12], *Saccharomyces cerevisiae* [13] and *Thermus thermophilus* [11] have been cloned and the corresponding recombinant proteins, which all lack the KMSK sequence, have been purified from *E. coli* overproduction strains [11,13,14]. The tertiary structures of the *S. cerevisiae* [13] and *T. thermophilus* [11] ArgRSs have been

solved, and ⁴⁰⁷GMST⁴¹⁰ in *S. cerevisiae* ArgRS and ³⁹⁴QMSG³⁹⁷ in *T. thermophilus* ArgRS were shown to be structural variants of KMSK [11,13]. In fact, in the KMSK-lacking ArgRSs, a conserved lysine upstream of the HIGH sequence motif (HIGH loop) seems to be a functional counterpart of the second lysine in KMSK of the canonical class I [15]. Sekine et al. [15] classified the ArgRSs from various sources into two major groups according to the conservation of this HIGH-loop lysine. ArgRSs in the HIGH group possess the HIGH-loop lysine but no canonical KMSK motif. In these ArgRSs, the HIGH-loop lysine probably compensates for the lack of K2 in the KMSK motif. In contrast, ArgRSs in KMSK group, found in several bacterial classes, contain the KMSK motif. In such cases, a Gly residue always takes the place of the HIGH-loop Lys [15].

Bacillus stearothermophilus is a Gram-positive bacterium, and its ArgRS has been studied [16–19], although its kinetic parameters have not been elucidated fully and it remains unclear whether the enzyme has ATPase activity [19]. Alignment of *B. stearothermophilus* ArgRS with 32 available ArgRSs showed that it was similar to *Bacillus subtilis* ArgRS [15], which contained the KMSK motif. Therefore, it is interesting to see whether the *B. stearothermophilus* ArgRS also contains the KMSK sequence and, if so, how K2 is related to the residue upstream of the HIGH motif. However, the gene encoding ArgRS from *B. stearothermophilus* bacterium (*argS'*) has not been studied until now.

Here we report the cloning and expression of the genes encoding *B. stearothermophilus* ArgRS and tRNA^{Arg}, followed by purification of the ArgRS and isolation of tRNA^{Arg}. Whether the purified recombinant ArgRS has ATPase activity was examined, and its kinetic properties and thermal stability were studied. Cross-recognition between ArgRSs and tRNA^{Arg}s from *E. coli* and *B. stearothermophilus* were also studied. *B. stearothermophilus*

Abbreviations used: aaRS, aminoacyl-tRNA synthetase; ArgRS, arginyl-tRNA synthetase; GluRS, glutamyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase; DTT, dithiothreitol; NTA, nitriloacetate.

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Table 1 Oligonucleotides used for PCR amplification

The sites created for restriction enzymes *NdeI* and *XhoI* are underlined in oligonucleotides Bst6 and Bst7 respectively.

Primer	Sequence	Direction
Bst1	5'-ATHGCNGGNCNGGNTTYATHAA-3'	Forward
Bst2	5'-RTANGTRTANGTNCCTCYTTYT-3'	Reverse
Bst3	5'-RTARAANCRTGRAANAGANGCAGNAGRTC-3'	Reverse
Bst4	5'-NCGNGCCTGNGCCTATG-3'	Reverse
Bst5	5'-NCGNGCNGCGAYTTYTCYTCRTTYTC-3'	Reverse
Bst6	5'-ATAACATATGAACATTGTCGGACAAATCAAGG-3'	<i>NdeI</i> forward
Bst7	5'-CTCCTCGAGTTACATTGTTCGGCGCTGAAA-3'	<i>XhoI</i> reverse

ArgRS has been shown to contain the KMSK sequence, so the relationship between the second Lys (K2) in KMKS and Gly upstream of HIGH can be investigated. Site-directed mutagenesis showed that a Lys residue at either position 136 or 385 was needed for amino acid activation by the ArgRS from *B. stearotheophilus*.

EXPERIMENTAL

Materials

L-Arginine, DTT (dithiothreitol), ATP and tetrasodium pyrophosphate were purchased from Sigma (St. Louis, MO, U.S.A.). DEAE-Sepharose CL-6B fast flow was purchased from Sigma, and Ni-NTA Superflow was from Qiagen (Hilden, Germany; where NTA is nitriloacetate). [³H]Arginine and [³²P]tetrasodium pyrophosphate were obtained from Amersham Biosciences (Little Chalfont, Bucks., U.K.). GF/C filters were from Whatman Biosystems (Maidstone, Kent, U.K.). Kinase, ligase and restriction endonucleases were obtained from Promega (Hercules, CA, U.S.A.). Expression plasmids pET22b(+) and pET28a(+) were purchased from Novagen Biosciences (Darmstadt, Germany). *E. coli* tRNA^{Arg} was isolated in our laboratory [20]. Automated DNA sequencing was performed by the BIOASIA Company (Shanghai, China). *B. stearotheophilus* was generously provided by Dr Bruce Roe (Department of Chemistry and Biochemistry, Advanced Center for Genome Technology, University of Oklahoma, Norman, OK, U.S.A.).

Isolation of *B. stearotheophilus* genomic DNA

DNA isolation was performed using the tissue/cell chromosome DNA Fast Extract kit from Watson BioTechnologies (Shanghai, China) according to the routine procedure [21].

Cloning of *argS'*

The complete genome of *B. stearotheophilus* has not yet been sequenced, so primers were based on five conserved regions identified in 31 other ArgRS genes (Table 1). Four primer pairs (Bst1-Bst2, Bst1-Bst3, Bst1-Bst4 and Bst1-Bst5) were used to amplify corresponding fragments from genomic DNA (Figure 1). Alignment of the fragments with a partial *B. stearotheophilus* genomic DNA sequence (gnlUOKNOR_1422lbstea_Contig172) deposited by Roe identified an open reading frame for *argS'*. According to the open reading frame sequence, primers (Bst6 and Bst7) with *NdeI* and *XhoI* sites at the 5' and 3' ends respectively were designed for PCR amplification of *argS'*. The PCR product was inserted into pET22b(+). *E. coli* BL21(DE3) was transformed with the recombinant plasmid pET22b(+)-*argS'* and

Figure 1 Position of the primers corresponding to different regions in *B. stearotheophilus argS'*

Right-pointing arrows (→) represent forward primers and left-pointing arrows (←) represent reverse primers. See Table 1 for details of the primers.

argS' was confirmed by DNA sequencing. As ATPase activity of *B. stearotheophilus* ArgRS was suggested in previous studies [19], we constructed recombinant plasmid h-*argS'*-pET28a(+) to produce N-terminal His-tagged ArgRS for removing any enzyme contamination by nickel affinity chromatography (Ni-NTA Superflow).

Purification of ArgRS

Transformants containing pET22b(+)-*argS'* were grown at 37 °C to a *D*₇₀₀ value of 0.5 in 1 litre of LB medium with 100 µg/ml ampicillin and induced by adding isopropyl β-D-thiogalactoside to a final concentration of 0.5 mM. After 6 h induction, cells were harvested and washed with disruption buffer (100 mM Tris/HCl, pH 7.5, 10 mM MgCl₂ and 1 mM EDTA). Wet cells (5 g) suspended in 30 ml of disruption buffer containing 0.4 mM PMSF and 0.5 mg of RNase were sonicated for 6 × 20 s at 15 W with a High-Intensity Ultrasonic Processor (375 W model; Sonics and Materials, Danbury, CT, U.S.A.). The crude extract was cleared of cellular debris by centrifugation at 12000 *g* for 20 min, and the supernatant was heated at 60 °C for 30 min and ultracentrifuged at 160000 *g* for 1 h. The supernatant was loaded on to a DEAE-Sepharose CL-6B column (3 cm × 18 cm) equilibrated with lysis buffer and eluted with a K₂HPO₄/KH₂PO₄ gradient of 50–600 mM. The fractions containing ArgRS were collected, concentrated and dialysed against 10 mM potassium phosphate buffer, pH 7.5, containing 50% glycerol.

His-tagged ArgRS was produced in *E. coli* BL21 (DE3) transformants containing the plasmid h-*argS'*-pET28a(+) at 25 °C. The product was purified by Ni-NTA Superflow as described previously [22].

Cloning and expression of the gene encoding *B. stearotheophilus* tRNA^{Arg}

There was only one tRNA^{Arg} gene in the published partial genomic DNA sequence (gnlUOKNOR_1422lbstea_Contig172), i.e. CG-CGCTCGTAGCTCAATTGGATAGAGCATCTGACTACGGA-TCAGAAGGTTAGGGGTTTCAATCCTCTCGAGCGCGCCA (the anticodon ACG is shown in bold). The cloning and expression of the *B. stearotheophilus* tRNA^{Arg} gene was performed as described previously [20].

Site-directed mutagenesis

PCR mutagenesis was carried out as described previously [23] using h-*argS'*-pET28a(+) as a template. The mutations were confirmed by DNA sequencing and *E. coli* BL21(DE3) was transformed to overproduce the mutant enzymes. The ArgRS mutants were purified by Ni-NTA Superflow as described previously [22].

Determination of molecular mass

The molecular mass of *B. stearotheophilus* ArgRS was determined by HPLC on a Superdex 200 HR 10/30 column.

CD spectroscopy

Protein samples (0.6 mg/ml) in 10 mM potassium phosphate buffer (pH 7.5) were measured on a Jasco J-715 spectropolarimeter at room temperature. A 0.1 cm-pathlength cuvette was used, and the spectra were accumulated in four scans [24].

Enzymic and kinetic assays

ATP-PP_i exchange and aminoacylation activities were measured at 37 or 55 °C, as described previously [25]. For assay of ATP-PP_i exchange, the reaction mixture contained 50 mM Tris/HCl, pH 8.0, 12 mM MgCl₂, 80 mM KCl, 2 mM arginine, 0.5 mM DTT, 4 mM ATP, 2 mM [³²P]tetrasodium pyrophosphate (specific activity 9000–10000 c.p.m./nmol) and 20 μM purified tRNA^{Arg}. For aminoacylation activity determination, the reaction mixture contained 50 mM Tris/HCl, pH 8.0, 12 mM MgCl₂, 4 mM ATP, 80 mM KCl, 0.5 mM DTT, 0.1 mM EDTA, 0.05 mg/ml BSA, 20 μM tRNA^{Arg} and 0.1 mM [³H]arginine (25 μCi/μmol). In both cases, the reaction was initiated by adding 10–90 nM ArgRS.

The enzyme kinetic constants were determined using various concentrations of the relevant substrates. To compare the kinetic parameters of *B. stearothermophilus* ArgRS with those of *E. coli* ArgRS, the assays were carried out at 37 °C. The same assays were carried out at 55 °C to study the thermal properties of ArgRS. Variation of *B. stearothermophilus* ArgRS activity was measured at various temperatures under the above assay conditions. Protein concentrations were measured by Bradford method using BSA as in [26].

Determination of ATPase activity

The reaction procedure was similar to that of Li and Altman [27]. The reaction mixture contained 50 mM Tris/HCl, pH 8.0, 80 mM KCl, 12 mM MgCl₂, 1 mM DTT and 0.1 μCi [^γ-³²P]ATP. The reaction was initiated by adding 1–10 μM enzyme.

Determination of the thermal stability of ArgRS

B. stearothermophilus ArgRS (40 μg/ml) in 50 mM Tris/HCl buffer, pH 8.0, containing 0.4 mg/ml BSA, was incubated at various temperatures for 10 min. The aminoacylation activity was then assayed at 55 °C after diluting the reaction mixture to an ArgRS concentration of 2 μg/ml with ice-cold 50 mM Tris/HCl buffer, pH 8.0.

RESULTS

Cloning and expression of *argS'* and purification of *B. stearothermophilus* ArgRS

The primary structure of *B. stearothermophilus* ArgRS deduced from *argS'* has 72.9% identity with respect to *B. subtilis* ArgRS, but only 22.2% identity with respect to *E. coli* ArgRS, perhaps because of the differences between Gram-positive and -negative bacteria. The *B. stearothermophilus* and *B. subtilis* ArgRSs are similar, but the G/C contents of their genes are different (55.32 and 48.59%, respectively). The *B. stearothermophilus* ArgRS consists of 557 amino acid residues with a theoretical molecular mass of 62.771 kDa. Interestingly, the *B. stearothermophilus* ArgRS possesses the ³⁸²KMSK³⁸⁵ sequence and Gly¹³⁶ upstream of the ¹³⁹HIGH¹⁴² loop (Figure 2).

Recombinant *B. stearothermophilus* ArgRS was purified to 60% homogeneity by heating the crude extract at 60 °C for

131 SANPTGDLHLGHARGAAVGD SLCNILAKAG 160
371 MVNLYQNGER VKMSKRTGKA VTMRELMEEV 400

Figure 2 Locations of HLGH and KMSK signature sequences of *B. stearothermophilus* ArgRS

Gly¹³⁶, ¹³⁹HLGH¹⁴² and ³⁸²KMSK³⁸⁵ are shaded.

30 min. In ion-exchange chromatography, it was eluted above 400 mM potassium phosphate, and was homogeneous in SDS/PAGE. We obtained 20 mg of the purified ArgRS from 5 g of cells. The molecular mass of ArgRS was 62.8 kDa measured by HPLC (results not shown), indicating that it existed as a monomer.

Cloning and expression of the tRNA^{Arg} gene

Total tRNA was isolated from the tRNA^{Arg} overproduction strain and purified on a DEAE-Sephacel column. The charging of [³H]arginine on the total tRNA reached 1300 pmol/A₂₆₀ unit. tRNA^{Arg} should be about 80% of the total tRNA, which was used to assay the ArgRS activity. The content of endogenous *E. coli* tRNA^{Arg} was negligible in comparison with the over-expressed *B. stearothermophilus* tRNA^{Arg}.

ArgRS has no ATPase activity

Although recombinant ArgRS was purified to homogeneity according to SDS/PAGE, the rate of ATP-PP_i exchange was slower than that of the aminoacylation reaction. This result was contrary to previous reports that the rate of the ATP-PP_i exchange reaction catalysed by *E. coli* ArgRS was faster than that of aminoacylation [12,25]. When we used [^γ-³²P]ATP to assay the hydrolysis of ATP in the absence of tRNA and arginine, ATP was still consumed. This result agreed with the work of Godeau and Charlier [18] and Godeau [19], who suggested that the *B. stearothermophilus* ArgRS possessed ATPase activity, so the parameters of *B. stearothermophilus* ArgRS in the ATP-PP_i exchange reaction could not be determined. Here N-terminal His-tagged ArgRS was produced in transformants containing the recombinant plasmid h-argS'-pET28a(+) to remove any contaminating proteins. The ArgRS purified by nickel-affinity chromatography did not have any ATPase activity. His-tagged ArgRS had almost the same specific activity of aminoacylation as ArgRS without the His tag. Taken together, these results suggest that the preparations of *B. stearothermophilus* ArgRS purified by DEAE-Sephacel CL-6B column chromatography may be contaminated with ATPase, which can be removed by nickel-affinity chromatography, and that *B. stearothermophilus* ArgRS itself has no ATPase activity.

Kinetic constants of *B. stearothermophilus* ArgRS

The kinetic constants of *B. stearothermophilus* ArgRS in amino acid activation and aminoacylation reaction were assayed at 37 °C to compare them with those of *E. coli* ArgRS (Table 2). In the amino acid-activation reaction, the *K_m* values for arginine and ATP were 7.4 and 1315 μM respectively, and *k_{cat}* values were 6.3 and 6.5 s⁻¹ respectively. In the aminoacylation reaction, the optimal pH was 8.0. The *K_m* values for arginine, ATP and *B. stearothermophilus* tRNA^{Arg} were 5.7, 1300 and 1.7 μM, which are comparable with those determined at pH 7.4 by Godeau [19]; and the *k_{cat}* value was 6.6 s⁻¹, which is four times greater than that reported by Parfait and Grosjean [16] (Table 2). Kinetic

Table 2 Kinetic constants for ArgRSs from *B. stearothermophilus* and *E. coli* at 37 °C

The kinetic constants of enzymes were determined as described in the Experimental section. Each assay was carried out at least for three times under the same conditions. Kinetic parameters (k_{cat} and K_{m}) were calculated from Eadie–Hofstee plots; all data were fitted to the Michaelis–Menten equation. When the concentration of one substrate was changed, that of the other was kept at a saturating concentration. *E. coli* data are from [25].

Substrate	Constant	ATP-PP _i exchange		Aminoacylation		
		<i>B. stearothermophilus</i> ArgRS	<i>E. coli</i> ArgRS	<i>B. stearothermophilus</i> ArgRS	<i>E. coli</i> ArgRS	<i>E. coli</i> ArgRS
Arginine	K_{m} (μM)	7.4 ± 0.4	150	5.7 ± 0.2	1.9*	12
	k_{cat} (s^{-1})	6.3 ± 0.2	40	6.5 ± 0.1	1.5*	26
	$k_{\text{cat}}/K_{\text{m}}$ ($\text{s}^{-1} \cdot \text{mM}^{-1}$)	851	267	1140	789*	2166
ATP	K_{m} (μM)	1315 ± 45	2000	1300 ± 80	540*	900
	k_{cat} (s^{-1})	6.5 ± 0.2	40	6.7 ± 0.2	1.5*	26
	$k_{\text{cat}}/K_{\text{m}}$ ($\text{s}^{-1} \cdot \text{mM}^{-1}$)	4.9	20	5.2	2.78*	28.9
<i>B. stearothermophilus</i> tRNA ^{Arg} (ACG)	K_{m} (μM)			1.7 ± 0.1	0.31*	4.9
	k_{cat} (s^{-1})			6.6 ± 0.1	1.5*	27
	$k_{\text{cat}}/K_{\text{m}}$ ($\text{s}^{-1} \cdot \text{mM}^{-1}$)			3882	4839*	5510
<i>E. coli</i> tRNA ^{Arg} (ACG)	K_{m} (μM)					2.5
	k_{cat} (s^{-1})					26
	$k_{\text{cat}}/K_{\text{m}}$ ($\text{s}^{-1} \cdot \text{mM}^{-1}$)					10400

* Data are from [16].

Table 3 Kinetic constants for ArgRS from *B. stearothermophilus* at 55 °C

Details are as for Table 2.

Substrate	Constant	ATP-PP _i exchange	Aminoacylation
Arginine	K_{m} (μM)	10 ± 0.9	9.4 ± 0.3
	k_{cat} (s^{-1})	19 ± 0.2	17 ± 0.1
	$k_{\text{cat}}/K_{\text{m}}$ ($\text{s}^{-1} \cdot \text{mM}^{-1}$)	1900	1808
ATP	K_{m} (μM)	1294 ± 43	1000 ± 55
	k_{cat} (s^{-1})	19 ± 0.2	17 ± 0.1
	$k_{\text{cat}}/K_{\text{m}}$ ($\text{s}^{-1} \cdot \text{mM}^{-1}$)	14.7	17
<i>B. stearothermophilus</i> tRNA ^{Arg} (ACG)	K_{m} (μM)		2.9 ± 0.1
	k_{cat} (s^{-1})		17 ± 0.1
	$k_{\text{cat}}/K_{\text{m}}$ ($\text{s}^{-1} \cdot \text{mM}^{-1}$)		5862
<i>E. coli</i> tRNA ^{Arg} (ACG)	K_{m} (μM)		2.4 ± 0.2
	k_{cat} (s^{-1})		1 ± 0.05
	$k_{\text{cat}}/K_{\text{m}}$ ($\text{s}^{-1} \cdot \text{mM}^{-1}$)		417

parameters (k_{cat} and K_{m}) were calculated from Eadie–Hofstee plots. Except the K_{m} value for ATP in the aminoacylation reaction, all the values were lower than those for *E. coli* ArgRS. Although tRNA^{Arg} bound *B. stearothermophilus* ArgRS tightly, the catalytic rates in both reactions only reached 25% of the rates catalysed by *E. coli* ArgRS at 37 °C. The kinetic constants of *B. stearothermophilus* ArgRS were also studied at 55 °C. All k_{cat} values increased about 3-fold compared with those at 37 °C. In both reactions, the K_{m} value for ATP was slightly decreased, whereas other K_{m} values were increased. Although at 55 °C the rate of reactions catalysed by *B. stearothermophilus* ArgRS increased, it was still lower than those catalysed by *E. coli* ArgRS at 37 °C (Tables 2 and 3).

Cross-recognition of ArgRSs and tRNA^{Arg}s from *B. stearothermophilus* and *E. coli*

In the aminoacylation assays, at 37 °C *E. coli* ArgRS aminoacylated *B. stearothermophilus* tRNA^{Arg} (ACG) at a similar rate with lower tRNA affinity than *E. coli* tRNA^{Arg} (ACG), whereas

B. stearothermophilus ArgRS did not detectably aminoacylate *E. coli* tRNA^{Arg} (ACG). *B. stearothermophilus* ArgRS at 55 °C and *E. coli* ArgRS at 37 °C showed comparable $k_{\text{cat}}/K_{\text{m}}$ values for their cognate tRNAs, the k_{cat} of *E. coli* enzyme being higher and the *B. stearothermophilus* enzyme having a higher tRNA affinity (Tables 2 and 3). In the cross-recognition of tRNAs, at 55 °C the *B. stearothermophilus* enzyme exhibited a 17-fold decrease of k_{cat} in the aminoacylation of the *E. coli* tRNA, although the affinities were similar. The resulting 14-fold decrease of $k_{\text{cat}}/K_{\text{m}}$ suggested that the *B. stearothermophilus* ArgRS–*E. coli* tRNA^{Arg} complex did not have an optimal conformation for catalysis.

Thermal properties and CD spectrum of ArgRS

As expected, the ArgRS from the thermophilic bacteria *B. stearothermophilus* was thermostable up to 65 °C. In addition, the specific activities of amino acid activation and aminoacylation increased with temperature. The two activities reached the highest at 55 °C, approx. 3-fold greater than those at 37 °C. Above 55 °C the two activities decreased slowly with increased temperature (Figure 3). The activation energy was calculated from the slope of the line when $\log v$ was plotted against $1/T$. The activation energy for both reactions was 14.6 kcal/mol. The secondary structure of *B. stearothermophilus* ArgRS was estimated from its CD spectrum. As compared with that of *E. coli* ArgRS, the secondary structure of *B. stearothermophilus* ArgRS contains slightly more β -sheet and β -turn structures, and slightly fewer α -helix and random structures.

ArgRS activity after mutating the two Lys residues in KMSK and the Gly upstream of HIGH

To investigate the importance of the Lys³⁸² and Lys³⁸⁵ residues in KMSK, and Gly¹³⁶ upstream of HIGH (Figure 2), three single-site mutants were created: ArgRS-K382A, ArgRS-K385A and ArgRS-G136K. It was shown that ArgRS-K382A had only 50% of the amino acid-activation activity of the native enzyme, while the other two mutants lost their amino acid-activation activities

Figure 3 Variations of *B. stearothermophilus* ArgRS activity with temperature

ATP-PP_i exchange activity (○) and the aminoacylation reaction (●) were assayed at increasing temperatures.

Table 4 CD estimates of *B. stearothermophilus* and *E. coli* ArgRSs

Protein samples (0.6 mg/ml) in 10 mM potassium phosphate buffer (pH 7.5) were measured on a Jasco J-715 spectropolarimeter at room temperature. A 0.1 cm-pathlength cuvette was used, and the spectra were accumulated in four scans.

Protein secondary structure	Secondary structure content	
	<i>B. stearothermophilus</i> ArgRS	<i>E. coli</i> ArgRS
α-Helix	44.6 %	47.6 %
β-Sheet	29.6 %	26.1 %
β-Turn	4.1 %	3.9 %
Random	21.8 %	22.4 %

completely (Figure 4, top panel), suggesting that Lys³⁸⁵ and Gly¹³⁶ are crucial for the first step of the reaction catalysed by *B. stearothermophilus* ArgRS. To investigate the relationship between Gly¹³⁶ and Lys³⁸⁵, an ArgRS-G136K/K385A double mutant was constructed. As expected, the double mutant partially restored the amino acid-activation activity (Figure 4, bottom panel). The above results suggested that *B. stearothermophilus* ArgRS mutants with two or no Lys residues at positions 385 and 136 lose amino acid-activation activity, while Lys at either one of these two positions is essential for the activity of *B. stearothermophilus* ArgRS. This result is the first direct evidence for the compensation of the second Lys in KMSK by Gly upstream of HIGH on amino acid-activation activity of ArgRS.

DISCUSSION

Here we report the cloning of the gene encoding *B. stearothermophilus* ArgRS and the purification of *B. stearothermophilus* ArgRS with or without a His tag. Purified *B. stearothermophilus* ArgRS had a molecular mass consistent with that deduced from the DNA sequence and existed as a monomer, agreeing with previous findings [16]. When *B. stearothermophilus* ArgRS was purified by DEAE-Sephacolumn chromatography under the experimental conditions, ArgRS was eluted above 400 mM potassium phosphate and showed ATPase activity. In contrast, *E. coli* ArgRS, which has a more positive charge, was

Figure 4 Activity of ArgRS mutants

Top panel: the ATP-PP_i exchange activity catalysed by ArgRS-K382A (Δ), ArgRS-G136K (▼) and ArgRS-K385A (○) decreased to various extents compared with that by *B. stearothermophilus* ArgRS (▲). Bottom panel: the ATP-PP_i exchange activity of ArgRS-G136K/K385A (●) was recovered compared with that of ArgRS-G136K (▼) and ArgRS-K385A (○).

eluted at 300 mM potassium phosphate and was free of the contaminating ATPases. Therefore, although *B. stearothermophilus* ArgRS was purified to more than 95% homogeneity by regular one-step DEAE-Sephacolumn chromatography after incubation at 60 °C for 30 min, contamination with ATPase activity could not be avoided. Accordingly, His-tagged ArgRS was expressed and purified on a Ni-NTA column. ArgRS purified by nickel-affinity chromatography did not possess any ATPase activity.

Because His-tagged ArgRS had almost the same specific activity of aminoacylation as that of the ArgRS without the His tag, it could be used to study the kinetic properties of *B. stearothermophilus* ArgRS. The aminoacylation activity of *B. stearothermophilus* ArgRS at 37 °C was higher than that found in several previous studies [16–19], but K_m is greater perhaps because of the difference in the strains of *B. stearothermophilus* (Table 2). In the present work *B. stearothermophilus* ArgRS was purified from an *E. coli* overproduction strain by a simple procedure, whereas in previous work the enzyme was purified directly from *B. stearothermophilus* through five-step column chromatography. The

Figure 5 Position of the HIGH-loop Lys in the ArgRS module structure

A stereo view showing the yeast ArgRS catalytic site superimposed on that of the ATP-bound *E. coli* GlnRS.

kinetic parameters in amino acid activation and aminoacylation were measured, and the k_{cat} values were equal for reactions at 37 and 55 °C. This result is in contrast to previously reported k_{cat} values of *E. coli* ArgRS in the first-step reaction, which were 4- or 2-fold greater than those in aminoacylation [12,25]. This difference may reflect the primary and tertiary structural differences between ArgRSs from Gram-positive (*B. stearothermophilus*) and Gram-negative (*E. coli*) bacteria. The tertiary structures of *S. cerevisiae* and *T. thermophilus* ArgRSs have been solved and both lack the KMSK signature sequence. [11,13]. However, the *B. stearothermophilus* ArgRS differs from them, since it contains a KMSK motif. Therefore, it will be interesting to study the crystallographic structure of *B. stearothermophilus* ArgRS.

AaRSs often display species-specific recognition of tRNAs [28,29]. So cross-recognition of *E. coli* and *B. stearothermophilus* ArgRSs to their cognate tRNA^{Arg} was investigated. *S. cerevisiae* ArgRS can catalyse the aminoacylation of *E. coli* tRNA^{Arg}; however *E. coli* ArgRS cannot arginylate *S. cerevisiae* tRNA^{Arg} [28]. In the case of *E. coli* and *Aquifex aeolicus* LeuRSs (leucyl-tRNA synthetases), *E. coli* LeuRS leucylated *A. aeolicus* tRNA^{Leu} efficiently, while *A. aeolicus* LeuRS was inefficient in leucylating *E. coli* tRNA^{Leu} [29]. In the present work, *E. coli* ArgRS charged *B. stearothermophilus* tRNA^{Arg} and *E. coli* tRNA^{Arg} with almost the same efficiency, whereas *B. stearothermophilus* ArgRS could only aminoacylate its own cognate tRNA. Even in the presence of *E. coli* tRNA^{Arg} *B. stearothermophilus* ArgRS could not catalyse the first-step reaction (results not shown), indicating that *B. stearothermophilus* tRNA^{Arg} may contain an identifying base that is important to the species-specific aminoacylation of *B. stearothermophilus* tRNA^{Arg}.

As mentioned in the Introduction, ArgRSs from various sources can be divided into two major groups based on the conservation of the HIGH-loop lysine [15]. The KMSK-lacking ArgRSs with a conserved Lys upstream of the HIGH sequence motif includes those from *S. cerevisiae*, *E. coli* and *T. thermophilus*. ArgRSs with the KMSK motif from several bacterial classes have a Gly residue replacing the Lys upstream of the HIGH loop. *B. stearothermophilus* ArgRS, which contains a KMSK motif and a HIGH loop-associated Gly residue, belongs to this class of ArgRS [15]. Thus this provided an opportunity to investigate the role of

the second Lys residue in KMSK as well as the importance of the Gly residue upstream of the HIGH loop. Site-directed mutagenesis revealed that the second Lys³⁸⁵ in KMSK and Gly¹³⁶ are both crucial for the activity of ArgRS, because single mutants at each of these positions resulted in a complete loss of amino acid-activation activity. However, the double-site mutant ArgRS-G136K/K385A regained some, though not all, of the amino acid-activation and aminoacylation activities, indicating that certain compensation exists between the two sites. In the *E. coli* GlnRS-ATP complex (a class I aaRS), the side chain of the Lys²⁷⁰ residue in ²⁶⁷VMSK²⁷⁰ forms a salt bridge with the α -phosphate of ATP to stabilize the negatively charged transition state of the first step [30]. Lys¹⁵⁶ in the yeast ArgRS is located in the vicinity of the ATP α -phosphate of the GlnRS-ATP complex with little modification of the synthetase structures (Figure 5). The structures of the catalytic domains are well conserved in *S. cerevisiae* ArgRS, *T. thermophilus* ArgRS and *T. thermophilus* GluRS, suggesting that their binding to ATP may be similar, although the two ArgRSs have no KMSK but the GluRS and GlnRS do have this motif [15,30]. The ATP-binding mode of *B. stearothermophilus* ArgRS should be similar to that of *E. coli* GlnRS, because they both have the KMSK signature sequence. In the case of *B. stearothermophilus* ArgRS, Lys³⁸⁵ or Lys¹³⁶ forms a putative contact with the ATP α -phosphate oxygen. However, the ATP-binding site can only contain one Lys residue at these positions. Mutants lacking Lys (ArgRS-K385A) or possessing Lys at both sites (ArgRS-G136K) were completely inactive. In contrast, mutant ArgRS-G136K/K385A, which possessed only one Lys residue, was active, though at a lower level. Schmitt et al. [31] suggested that the flexibility of the KMSK loop in the structure of methionyl-tRNA synthetase was required to reach the transition state during the formation of methionyl-adenylate [31]. Therefore the reason why mutant ArgRS-G136K/K385A cannot restore the activity fully may be due to the subtle local structural changes in the active site around these positions.

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