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Title: An archaeal aminoacyl-tRNA synthetase complex for improved substrate quality control

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ABSTRACT

Aminoacyl-tRNA synthetases (aaRS) decode genetic information by coupling tRNAs with cognate amino acids. In the archaeon *Methanothermobacter thermautotrophicus* arginyl- and seryl-tRNA synthetase (ArgRS and SerRS, respectively) form a complex which enhances serylation and facilitates tRNA^{Ser} recycling through its association with the ribosome. Yet, the way by which complex formation participates in Arg-tRNA^{Arg} synthesis is still unresolved. Here we utilized pull down and surface plasmon resonance experiments with truncated ArgRS variants to demonstrate that ArgRS uses its N-terminal domain to establish analogous interactions with both SerRS and cognate tRNA^{Arg}, providing a rationale for the lack of detectable SerRS•[ArgRS•tRNA^{Arg}] complex. In contrast, stable ternary ArgRS•[SerRS•tRNA^{Ser}] complex was easily detected supporting the model wherein ArgRS operates in serylation by modulating SerRS affinity toward tRNA^{Ser}. We also found that the interaction with SerRS suppresses arginylation of unmodified tRNA^{Arg} by ArgRS, which, by itself, does not discriminate against tRNA^{Arg} substrates lacking posttranscriptional modifications. Hence, there is a fundamentally different participation of the protein partners in Arg-tRNA and Ser-tRNA synthesis. Propensity of the ArgRS•SerRS complex to exclude unmodified tRNAs from translation leads to an attractive hypothesis that SerRS•ArgRS complex might act *in vivo* as a safeguarding switch that improves translation accuracy.

Keywords

Aminoacyl-tRNA synthetase, multisynthetase complexes, tRNA, posttranscriptional modifications, surface plasmon resonance.

Abbreviations

aaRS - aminoacyl-tRNA synthetase, ArgRS - arginyl-tRNA synthetase, SerRS - seryl-tRNA synthetase, MtArgRS - arginyl-tRNA synthetase from *Methanothermobacter thermautotrophicus*, MtSerRS - seryl-tRNA synthetase from *M. thermautotrophicus*, MSC - multisynthetase complex, SPR – surface plasmon resonance.

Highlights

- In *Methanothermobacter thermautotrophicus* arginyl- (ArgRS) and seryl-tRNA synthetases (SerRS) interact to form a transient complex.
- ArgRS uses its N-terminal domain to establish interactions with both SerRS and cognate tRNA^{Arg} in a highly analogous manner.
- While a stable ArgRS•[SerRS•tRNA^{Ser}] complex is easily detected, ArgRS, tRNA^{Arg} and SerRS do not associate into a detectable ternary complex.
- ArgRS *per se* aminoacylates the homologous tRNA^{Arg} transcripts with different posttranscriptional modifications with a comparable efficiency.
- In presence of SerRS, arginylation of hypomodified tRNA^{Arg} transcripts is suppressed.

1. Introduction

Aminoacyl-tRNA synthetases (aaRSs) catalyze the first step in protein biosynthesis, the esterification of their cognate tRNAs with appropriate amino acids [1]. To increase the processivity of translation, some aaRSs form complexes with other components of the translation machinery, i.e. other aaRSs, elongation factors and the ribosome [2-5]. Additionally, to expand beyond this, canonical role in protein synthesis, aaRSs frequently associate with other cellular macromolecules that may not be involved in translation [3, 5].

In methanogenic archaeon *Methanothermobacter thermautotrophicus* arginyl- (ArgRS) and seryl-tRNA synthetase (SerRS) establish a transient non-covalent complex [6, 7]. In eukaryotic organisms both SerRS and ArgRS interact with other cellular proteins to form complexes of varying stability [8]. SerRS is associated with the translasome, a supercomplex composed of aaRSs, ribosomal proteins, elongation factors and the proteasome in fission yeast [9] and transcription factor Yin Yang 1 in human cells [10]. ArgRS has been shown to form complexes with other tRNA synthetases in *Caenorhabditis elegans* [11] and mammalia [4].

A multisynthetase complex (MSC) of *M. thermautotrophicus* is composed of leucyl-, lysyl- and prolyl-tRNA synthetases and its interaction with the elongation factor EF-1 α [12, 13] indicates a potential coupling between tRNA aminoacylation and aminoacyl-tRNA decoding [12]. Likewise, both SerRS and ArgRS make a direct interaction with the large ribosomal subunit in *M. thermautotrophicus* [6]. Both ArgRS and SerRS associate with the ribosomal proteins of the L7/L12 stalk and proteins near the stalk base of the 50S subunit. The proximity of the ribosomal A-site may suggest that these interactions improve the efficiency of Arg-tRNA^{Arg} and Ser-tRNA^{Ser} transfer, from respective synthetases to EF-1 α and, subsequently, to the ribosome. Also, overrepresented consecutive usage of synonymous codons for serine and arginine indicates that ribosomal co-localization of the SerRS•ArgRS complex involves purposeful recharging of tRNA isodecoders within the polysomes [6].

Binary ArgRS•SerRS interaction promotes serylation reaction [7] and enhances SerRS•tRNA^{Ser} complex formation [14]. ArgRS was shown to increase SerRS's affinity for cognate tRNA two-fold, but did not affect cooperative properties or the complex' stoichiometry [14]. Driven by these findings, here we set up to determine the topological determinants of the SerRS•ArgRS interaction and its functional relevance in tRNA arginylation. We produced several ArgRS variants bearing sequential deletions of the structural, N-terminal elements (Fig. 1A) and inspected their capacity to participate in SerRS•ArgRS complex formation by various approaches. Furthermore, we assessed the participation of the cognate substrates in order to reveal existence of stable ternary complexes. Finally, we produced several posttranscriptional variants of tRNA^{Arg} and showed the influence that SerRS exerts on arginylation of these substrates.

2. Materials and methods

2.1. Protein expression and purification

Full length SerRS-His₆ and GST-ArgRS were expressed and purified essentially as described [7]. For production of the tagless SerRS, pET28 vector containing *serS* gene [7] was digested with *NcoI* and *NdeI*, blunted using Klenow polymerase and P1 nuclease and ligated. To generate N-terminally His-tagged wild type ArgRS, *argS* sequence was inserted between *BamHI* and *XhoI* sites of a pET28b vector. Truncated ArgRS variants were generated by PCR using pET28b-*argS* as template and T7 promoter / terminator sequences in combination with the specific primers listed in the [Table S1](#). After digestion with *NdeI* and *XhoI* truncated genes were ligated into vectors pET28 and pET21 to later generate proteins with or without His-tag, respectively. All constructs were verified by DNA sequencing. All genes were expressed in Rosetta BL21(DE3). Expression was started by adding 0.3 mM IPTG to the exponentially growing bacterial culture at 0.6 OD₆₀₀. His-tagged ArgRS variants were purified essentially as before [7]. Routinely, proteins were further purified on a Superdex 200 (or Superdex 75) column equilibrated with 25 mM Hepes–NaOH (pH 7.2), 150 mM NaCl, 1 mM DTT.

Tagless forms of analyzed proteins (SerRS, Δ N21-ArgRS, Δ N39-ArgRS, Δ N43-ArgRS, Δ N89-ArgRS) were purified using HiPrep DEAE-FF 16/10 (GE Healthcare), MonoQ (GE Healthcare) and Superdex 200 or Superdex 75 columns. Briefly, cell pellets were resuspended in the LS buffer (50 mM Tris–HCl (pH 8.0), 50 mM NaCl, 5% (v/v) glycerol, 5 mM β -mercaptoethanol and 0.3 mM phenylmethylsulfonyl fluoride). After lysis and centrifugation, collected supernatant was loaded onto HiPrep DEAE-FF 16/10 column equilibrated with the LS buffer. Proteins were then resolved in a gradient 50 mM–700 mM NaCl (in the LS buffer). Concentrated and desalted fractions were loaded on a MonoQ column and separation was achieved in the same gradient as described above. To confirm the correct oligomerization state, gel-filtration on a Superdex 200 or 75 was executed in the same buffer as for the wild type enzymes.

2.2. Fluorescence measurements

Fluorescence spectra of wild type and mutated ArgRSs were recorded at 20 °C on a luminescence spectrophotometer LS 50 B (PerkinElmer Life Sciences, Boston, MA, USA) using 0.4 cm path quartz cuvette and 5 nm slits in the excitation and emission paths. Tryptophan fluorescence of the proteins was excited at 295 nm, and the emission spectrum was recorded from 300 to 500 nm. Protein concentration was 400 nM.

2.3. Circular dichroism (CD)

Temperature melting curves for full-length and truncated ArgRSs were created by monitoring changes in ellipticity at 220 nm using spectropolarimeter JASCO J-815 spectrometer and a 1 mm quartz cuvette. Purified enzymes were subjected to gel-filtration in a buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1 mM EDTA prior to the experiment and adjusted to the concentration of 6.7 μ M. Unfolding was monitored between 4 and 95 °C under the heating rate of 30 °C h⁻¹.

2.4. tRNA expression and fractionation

Genes for *M. thermotrophicus* tRNA^{Ser}_{GGA}

(GCCGGGATAGCCTAGCCAGGTAAGGCGCAAGACTGGAAATCTTGTGGAGCTTTGCTCCTCCTGGGTTCAAATCCCAGTCCCGGCG) and tRNA^{Arg}_{CCU}

(GGGCCCCGTAGCCTAGCCAGGATAGGGCATCAGACTCCTAATCTGAAGGTCCCGGGTCAAATCCCAGGCGGGTCCG) were cloned into pET3A and placed under the inducible T7 promoter. To ensure proper processing in *E. coli*, CCA sequences for introduced at the 3'-end of the tRNA genes. Genes were transcribed *in vivo*, in BL21(DE3) cells and extracted as described [15]. Plateau aminoacylation of tRNA^{Arg} isolated in this manner was ~ 50 - 60%. To achieve higher quality, both tRNA^{Ser} and tRNA^{Arg} were further resolved on a HiPrep DEAE-FF 16/10 column equilibrated with 50 mM NaOAc (pH 4.5), 100 mM NaCl. Separation was conducted in the gradient 0.1 – 1 M NaCl. Collected samples were then run on a Superdex 75 gel-filtration column. By this procedure, the tRNA sample reached ≥ 85 % plateau aminoacylation.

Posttranscriptional tRNA^{Arg}_{CCU} variants were separated by reversed-phase chromatography (RPC) on a Vydac C4 column [16]. Separation was achieved in the ethanol gradient (0 – 30 % (v/v)) in 40 mM NH₄OAc (pH 5.1) and 20 mM Mg(OAc)₂. For highest purity, fractions of individual tRNA^{Arg} variants were subjected to another round of RPC under same conditions.

Before all kinetic and thermodynamic analyses tRNA was denatured for 3 minutes at 83 °C, followed by addition of MgCl₂ to a final concentration of 10 mM and slow cooling to room temperature to allow renaturation.

2.5. Pull down assay

Ni-NTA resins (Qiagen, 30 µl) were equilibrated in 25 mM Tris (pH 7.6), 150 mM NaCl, 5% (v/v) glycerol, 10 mM imidazole (pH 7.6), 5 mM β-mercaptoethanol. His-tagged SerRS or ArgRS were immobilized and unbound protein washed out with the equilibration buffer. The tagless protein partner was then added and the mixture was incubated at room temperature with occasional shaking for 15-20 minutes. After removing unbound proteins, further washes were conducted with 25 mM Tris (pH 7.6), 150 mM NaCl, 5% (v/v) glycerol, 40 mM imidazole (pH 7.6), 5 mM β-mercaptoethanol. Bound proteins and their complexes were eluted by 150 mM imidazole. For ternary complex co-purification the procedure was identical except that the GST-tagged variant of ArgRS was employed. To appropriate mixtures 150 µM serine or arginine (both pH 7) and/or 9 mM ATP (pH 7.7) were added. Cognate tRNAs were added in stoichiometric 1:1 ratio with respect to the corresponding tRNA synthetase.

2.6. Electrophoretic mobility shift assay (EMSA)

For detection of the non-covalent complexes between aaRSs and their cognate tRNAs, samples from the pull down assay were loaded on a 9% polyacrylamide native gel containing 25 mM Tris and 0.15 % acetic acid (pH 6.4). Electrophoresis was performed at room temperature for 1.5 h at 120 V, and gels were stained with silver [17].

2.7. Isolation of aaRS•tRNA complexes via gel-filtration

For the isolation of ArgRS•tRNA^{Arg} complex 100 µg of ArgRS-His₆ and 250 µg of the enriched DEAE-purified tRNA^{Arg} was mixed and incubated for 10 minutes at 43 °C to allow complex formation. The mixture was then centrifuged and loaded onto Superdex 200 column previously equilibrated with 25 mM Hepes–NaOH (pH 7.2), 120 mM NaCl, 5 mM MgCl₂ and 1 mM DTT.

2.8. Surface plasmon resonance (SPR)

Binding studies were performed at 25 °C using a BIACORE T100 SPR instrument (GE Healthcare, Uppsala, Sweden). Affinity of the truncated ArgRS variants toward SerRS was inspected with SerRS immobilized to a carboxymethyl dextran-coated gold surface (CM5 sensor chip). Activation of the carboxymethyl groups with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride and N-hydroxysuccinimide was followed by SerRS injection whose free amino groups react with the activated surface of the chip. The immobilization level of SerRS was 800 response units (RU). The remaining reactive sites on the chip were blocked by the addition of ethanolamine, and any non-covalently adhered SerRS was removed with 50 mM NaOH. Runs were performed in 20 mM Hepes (pH 7.2), 120 mM NaCl, 2 mM DTT and 0.005% v/v surfactant P20, also used for analyte dilution. Binding step was monitored at a flow rate of 30 µl/min. The interaction between ArgRS•tRNA^{Arg} and SerRS was tested under the same conditions. After the dissociation step, the remaining (bound) analyte was removed by the addition of 3M KCl.

For determination of the affinity of wild type and mutated ArgRS variants toward tRNA^{Arg}, the enzymes were non-covalently adhered via N-terminal His-tag to a Ni-NTA surface (Sensor Chip NTA). The samples were run in 20 mM Hepes (pH 7.2), 120 mM NaCl, 6 mM MgCl₂ and 0.005% v/v Surfactant P20 and the flow rate was 10 µl min⁻¹.

The output data represent the differences in the SPR signal between the flow cell containing immobilized protein and the reference cell. The analyte was titrated over the ligand and one concentration of the analyte was injected two times to test the reproducibility. For K_d determination, the signals obtained at the end of each injection were plotted against the corresponding analyte concentration. Initially, the length of the injection was inspected for each interaction in order to obtain curves that have constant signal (no increment in RU) at the end of the injection which indicates that the amount of association events equals the amount of dissociation events (steady state response). K_d was then determined from the steady state affinity model. For each analyte, triple independent titrations were performed and the data is reported as the mean ± standard error of the mean. The data were analyzed with Biacore T100 evaluation software.

2.9. Aminoacylation assay

Aminoacylation reactions for wild type enzymes were performed at 48 °C using 70 µM [¹⁴C]-amino acid, 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 15 mM MgCl₂, 4 mM DTT, 5 mM ATP and 0.4 µg µl⁻¹ BSA. Truncated ArgRS variants were tested at 42 °C and 50 mM instead of 150 mM NaCl. Aliquots of the reaction were removed at given intervals, spotted onto 3MM

filter papers and immersed in 10 % trichloroacetic acid to precipitate aminoacylated tRNA. For the K_m and k_{cat} determination, wild type ArgRS was present at the concentration of 16 nM, ΔN_{tot} -ArgRS at 50 nM and tRNA^{Arg} was varied over the range of 0.375 - 12 μ M. To determine the plateau of tRNA^{Arg} arginylation in the presence of SerRS, SerRS and ArgRS were pre-incubated at the final concentration of 20 μ M each, at 41 °C for 10-15 minutes. Prior the usage in aminoacylation reaction the enzymes were diluted so that the final concentration of the enzymes in the reaction was 75 nM.

2.10. Modeling and structure analysis

Homology modeling of ArgRS was executed by using EsyPred3D [18] and SWISS-MODEL workspace [19] using *Pyrococcus horikoshii* (PDB ID 2ZUE_A) template. Tertiary structure of tRNA^{Arg} was predicted by ModeRNA [20] using unmodified *P. horikoshii* tRNA^{Arg} (PDB ID 2ZUE_B) crystal structure. Structural properties of the *M. thermautotrophicus* ArgRS•tRNA^{Arg} model were investigated using ENTANGLE [21]. Figures were made with The PyMOL Molecular Graphics System (version 1.2.0.3 Schrödinger, LLC).

3. Results

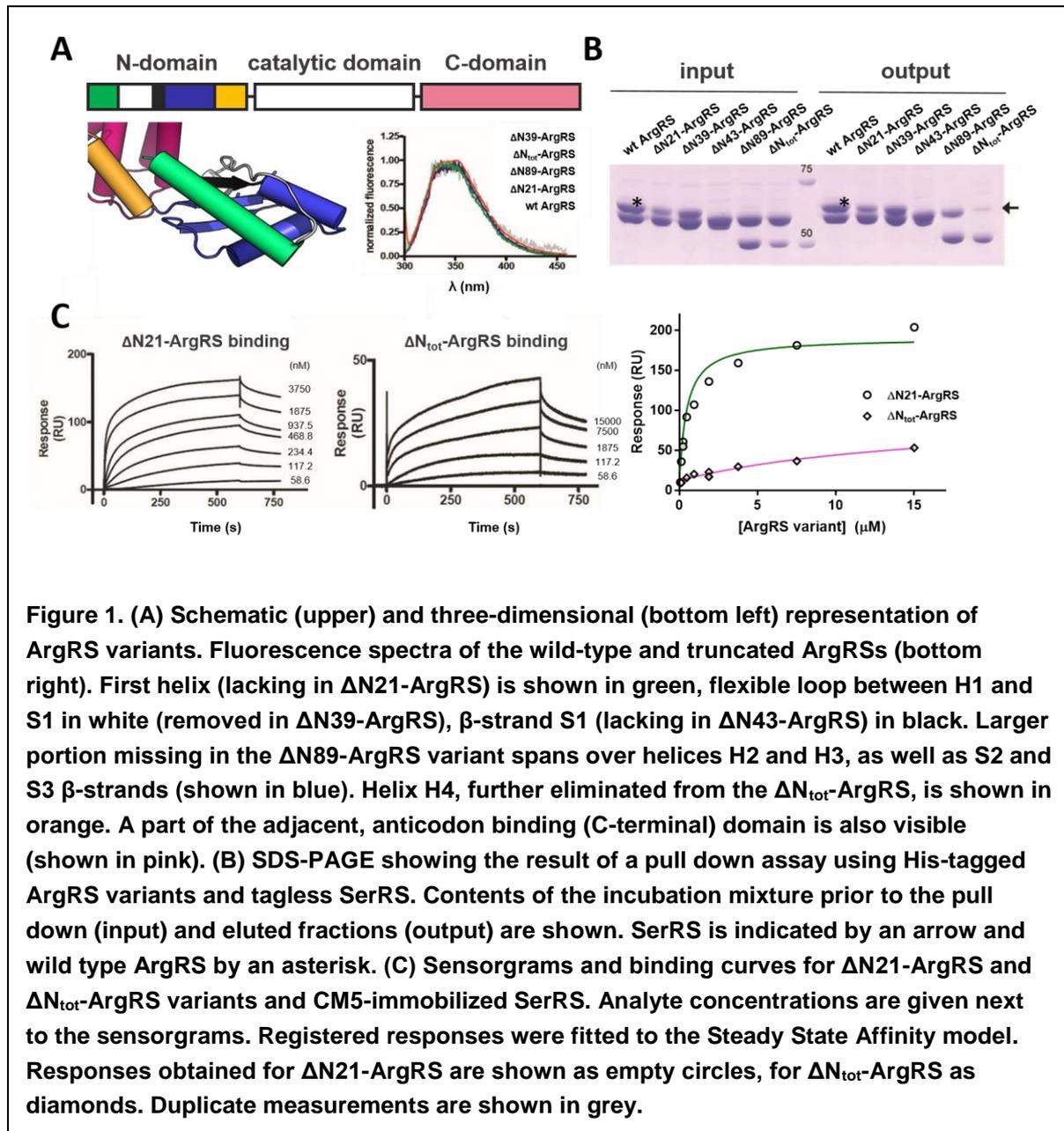
3.1. Determinants that govern ArgRS participation in SerRS•ArgRS assembly

To identify the structural elements of ArgRS that participate in ArgRS•SerRS complex assembly, we generated truncated ArgRS variants and measured their affinity for full length SerRS. Truncations were introduced according to our structural model (Fig. 1A) and their stability was assessed by fluorescence and circular dichroism measurements (Fig. 1A, S2, S3).

The largest truncations eliminated entire N- or C-domains (variants ΔN_{tot} and ΔC_{tot} , Fig. 1A, S3). Removal of the N-terminal domain (fully or in part) had no influence on this variant's stability during expression and purification procedures. However, the removal of the C-domain (residues 399-560) led to protein aggregation during heterologous expression in *E. coli* and formation of inclusion bodies. As efforts to produce a soluble ΔC_{tot} -ArgRS were unsuccessful, production and further use of the ΔC_{tot} variant was omitted from this study. The N-domain was further separated according to the distinct elements of secondary structure which were progressively removed from the ArgRS' N-terminus (Fig. 1A). In doing so, we removed helix H1 ($\Delta N21$ -ArgRS), a loop following the H1 helix ($\Delta N39$ -ArgRS), first β -strand S1 ($\Delta N43$ -ArgRS), and an entire β -sheet of the N-terminal domain along with the intertwining helices ($\Delta N89$ -ArgRS, Fig. S1).

The comparison of the emission spectra taken for the wild type and ArgRS variants indicates that the protein's core structure is largely preserved (Fig. 1A). Because all four tryptophan residues present in the wild type ArgRS (Trp171, Trp256, Trp434, and Trp 473) remain in the shortened variants, comparison of their fluorescence spectra provides a good estimate for core structure preservation (Fig. 1A). The correct folding of the truncated variants was further corroborated by their individual circular dichroism spectra (Fig. S2). Furthermore, denaturation experiments returned T_m values for all the variants near the value

obtained for the wild type enzyme (curves for full length ArgRS and ΔN_{tot} -ArgRS are given Fig. S3). Together, our data show that N-terminally truncated ArgRS variants are correctly folded.



Pull down experiments with truncated ArgRS variants and SerRS reveal that only removal of the entire N-terminal domain appears to compromise ArgRS•SerRS complex assembly (Fig. 1B). The enzymes were produced as both native and His-tagged versions and pull down on Ni-NTA affinity matrix was done in both combinations with the same outcome. Figure 1B shows that the tagless SerRS copurifies with all ArgRS variants except for the variant lacking the entire N-domain (ΔN_{tot} -ArgRS). Compared to wild type, variants ΔN_{21} -ArgRS, ΔN_{39} -ArgRS and ΔN_{43} -ArgRS seem to bind SerRS equally well, while somewhat lessened ability for SerRS binding is observed for ΔN_{89} -ArgRS.

To assess the participation of N-terminal elements of the ArgRS structure in maintaining SerRS•ArgRS interaction in a more quantitative manner, we next employed surface plasmon resonance (SPR). SPR analysis with immobilized SerRS and truncated ArgRS analytes reveals that the progressive removal of the N-terminal region actually has a continuous effect on SerRS binding (Table 1). The only ArgRS variant showing almost full capacity for SerRS binding is Δ N21-ArgRS (2-fold weaker binding, Fig. 1C). After the removal of the first 39 amino acids from N-terminus, ArgRS•SerRS interaction weakens 10-fold. While Δ N39- and Δ N43-ArgRS show comparable affinities, Δ N89-ArgRS shows even weaker binding (20-fold reduction). Finally, variant Δ N_{tot}-ArgRS shows >100-fold higher K_d than the wild type enzyme (Fig. 1C and Table 1). Drastically increased K_d for N_{tot}-ArgRS binding is in good agreement with the pull down analysis (Fig. 1B).

Table 1. Dissociation constants of N-terminally His-tagged ArgRS variants with protein partner SerRS or cognate tRNA^{Arg} determined by SPR^a.

ArgRS variant	Macromolecular partner	
	SerRS	tRNA ^{Arg}
	K_d / (μ M)	K_d / (μ M)
wild type ArgRS	0.28 ± 0.020	0.37 ± 0.080
Δ 21-ArgRS	0.46 ± 0.10	0.56 ± 0.010
Δ 39-ArgRS	2.9 ± 1.8	13 ± 0.57
Δ 43-ArgRS	3.7 ± 0.20	1.8 ± 0.90
Δ 89-ArgRS	5.6 ± 0.11	15 ± 0.29
Δ N _{tot} -ArgRS	30 ± 1.2	21 ± 3.4

^a Data correspond to the average ± S.D. of the three independent titrations.

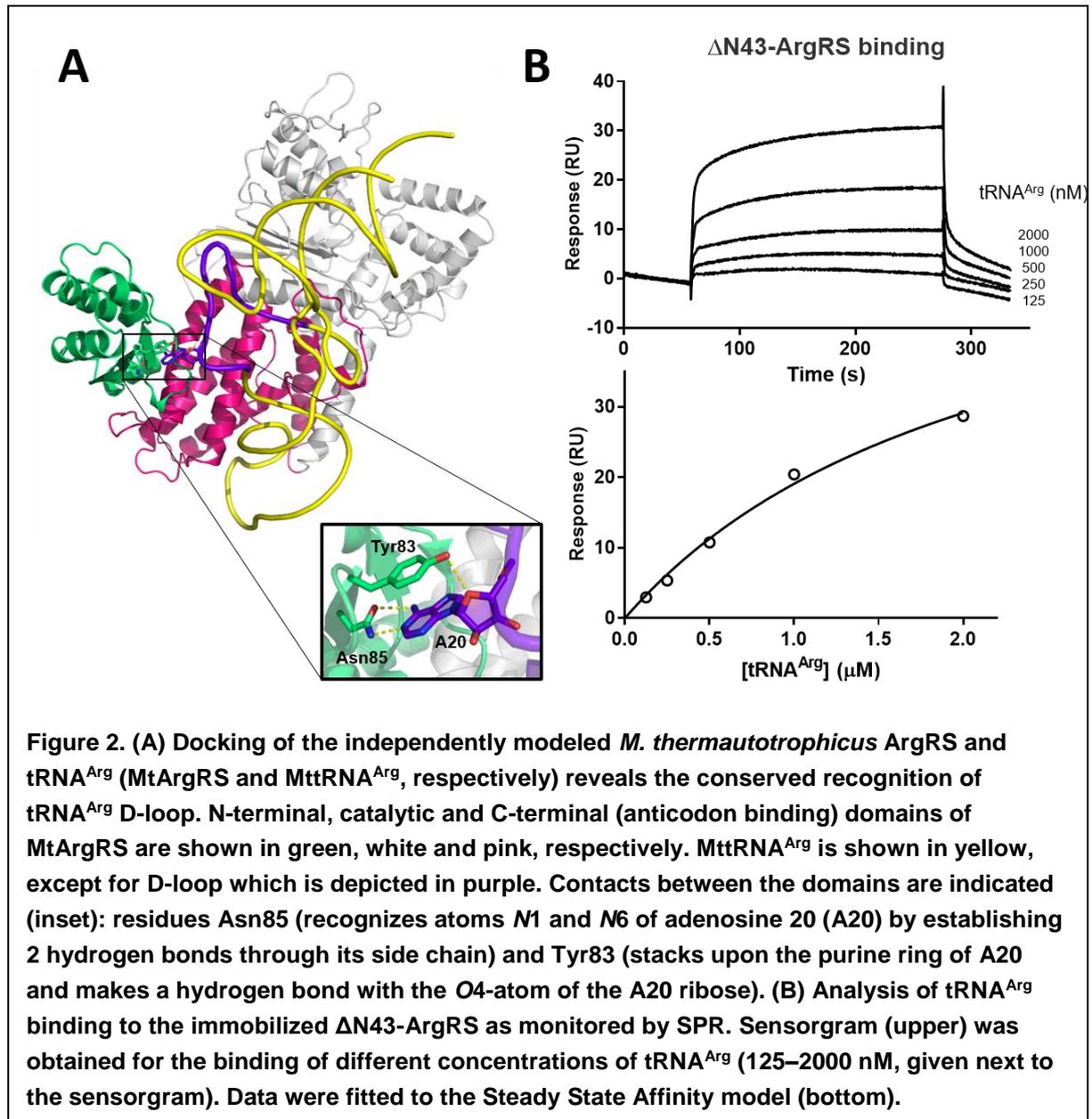
3.2. ArgRS utilizes its N-terminal domain for both tRNA^{Arg} and SerRS binding

Our model of the *M. thermotrophicus* ArgRS•tRNA^{Arg} complex indicates that N-terminal truncations may destroy the D-loop binding platform (Fig. 2A). To test this hypothesis, we measured the affinity of non-covalently immobilized, N-truncated ArgRS variants for their cognate tRNA^{Arg} using SPR.

Variants Δ N89- and Δ N_{tot}-ArgRS exhibit the most significant decrease in affinity for tRNA^{Arg}, as evidenced by 40- and 55-fold higher K_d values relative to the wild type enzyme, respectively (Table 1). In both variants conserved amino acids Tyr83 and Asn85 responsible for A20 determinant recognition have been removed (Fig. 2A, inset). The first 43 residues appear to have a smaller impact on tRNA binding, as, compared to wild type, Δ N21- and Δ N43-ArgRS show only 1.5 and 5-fold weaker tRNA binding (Table 1). Unexpectedly, Δ N39-ArgRS exhibits a 36-fold higher K_d than the wild type. Although a conserved Pro34 is identified as important for tRNA^{Arg} recognition, the high affinity re-established with Δ N43-ArgRS indicates that the observed increase is not likely to be caused by Pro34 removal. Because tertiary structure of that variant was validated by two independent methods and shown to be analogous to the wild type enzyme (section 3.1), it is possible that the high K_d

value for $\Delta N39$ -ArgRS•tRNA^{Arg} interaction reflects an improper orientation of the N-terminally immobilized $\Delta N39$ -ArgRS with respect to the incoming tRNA analyte (section 2.8).

Comparison of the affinities for SerRS and tRNA^{Arg} reveals that the progressive removal of the ArgRS' N-terminal residues leads to a stepwise decrease in binding affinity for both tRNA^{Arg} and SerRS complex (Table 1). The fact that variants with the best ($\Delta N21$ -ArgRS) and least preserved D-loop binding module ($\Delta N89$ - and ΔN_{tot} -ArgRS, Fig. 2, S1) show similar effects in both SerRS and tRNA^{Arg} binding further suggests that ArgRS uses similar elements of its N-terminal domain to interact with both macromolecular partners.



3.3. SerRS•ArgRS complex assembly is sensitive to cognate aaRS substrates

Similar participation of the ArgRS N-terminal domain elements in SerRS and cognate tRNA^{Arg} binding implies that ArgRS recognizes these two macromolecular partners with

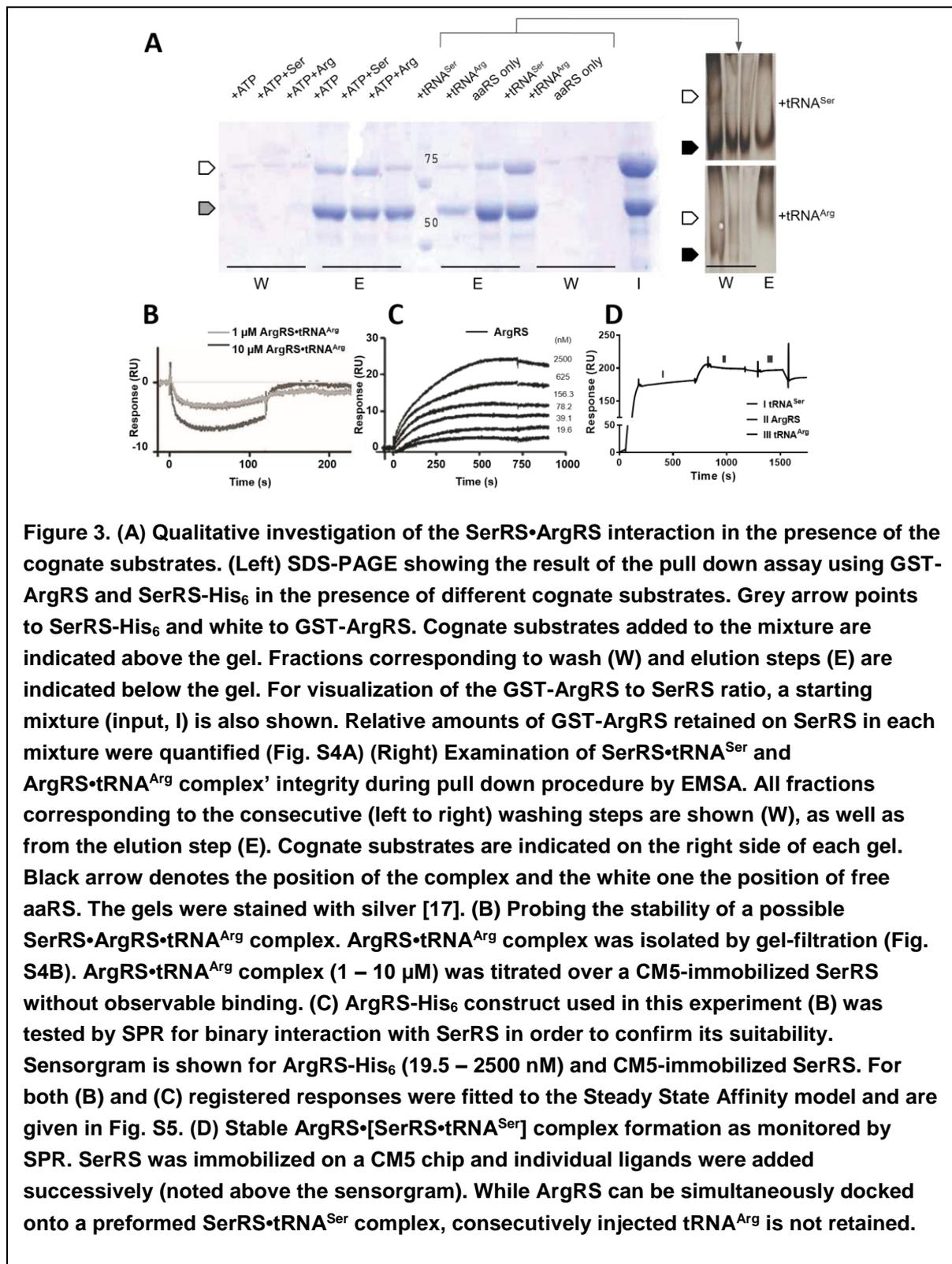
closely placed (or even overlapping) sites on the N-terminal domain's surface. To inspect whether tRNA^{Arg} and SerRS can bind to ArgRS simultaneously, we tested their binding in a pull down- and SPR-based assays (Fig 3A, B, D). Further, because we have previously shown that ArgRS enhances Ser-tRNA^{Ser} synthesis by lowering the K_m for cognate tRNA [7] we wanted to establish whether stable ternary (ArgRS•[SerRS•tRNA^{Ser}], SerRS•[ArgRS•tRNA^{Arg}], ArgRS•[SerRS•Ser-AMP], ArgRS•[SerRS•ATP]) and possibly quaternary ([ArgRS•tRNA^{Arg}]•[SerRS•tRNA^{Ser}] and [ArgRS•Arg]•[SerRS•ATP]) complexes may form under our conditions.

Pull down analysis using SerRS-His₆ and GST-ArgRS executed in the presence of stoichiometric amounts of tRNA^{Arg} reveals that the ArgRS•tRNA^{Arg} complex is not retained on the Ni-NTA immobilized SerRS (Fig. 3A). SDS-PAGE of analyzed fractions shows that a much smaller fraction of GST-ArgRS co-elutes with SerRS once tRNA^{Arg} is added to the incubation mixture. To determine whether retained GST-ArgRS stays bound to SerRS in its free or complexed form, we analyzed collected samples by native PAGE (Fig. 3A, right). During pull down procedure, GST-ArgRS binds its cognate tRNA to form the ArgRS•tRNA^{Arg} complex. However, this complex is not retained by SerRS and is visible in the wash fractions only (Fig. 3A, right). To verify that SerRS, ArgRS and tRNA^{Arg} do not assemble into a high affinity ternary complex we tested the interaction between SerRS and ArgRS•tRNA^{Arg} complex in an SPR-based assay (Fig. 3B). SerRS was immobilized on the CM5 chip and ArgRS was present in its His-tagged form (section 2.8). SPR confirmed that the pre-formed ArgRS•tRNA^{Arg} complex (Fig. S4B) does not bind to immobilized SerRS (Fig. 3B). Because earlier SPR measurements on SerRS•ArgRS interaction used native and GST-tagged variants of ArgRSs [7], here we scrutinized the possibility that the His-tag affects the interaction. This assumption was eliminated by testing the SerRS•ArgRS-His₆ interaction independently (Fig. 3C). Because the dissociation constant of 283 ± 17 nM measured for the ArgRS-His₆•SerRS pair (Table 1 and Fig. S5) matches that of native ArgRS•SerRS pair (253 ± 43 nM [7]), the lack of ArgRS-His₆•tRNA^{Arg} affinity for SerRS cannot be a consequence of a different recombinant tag.

In contrast to tRNA^{Arg}, tRNA^{Ser} forms a detectable ternary complex with ArgRS and SerRS. The same relative amounts of GST-ArgRS retained on the immobilized SerRS in the presence and absence of tRNA^{Ser} suggest that tRNA^{Ser} assembles into a detectable ArgRS•[SerRS•tRNA^{Ser}] complex (Fig. 3A, D). Native gel analysis confirms the SerRS eluted along with GST-ArgRS forms a binary SerRS•tRNA^{Ser} sub-complex (Fig. 3A, right). Utilization of the immobilized SerRS-His₆ in the presence of tRNA^{Ser} in the pull down experiment (Fig. 3A) introduced some uncertainty owing to the fact that tRNA^{Ser} reduces SerRS's affinity for the matrix (Fig. 3A). Therefore, additional pull down experiments were executed using immobilized GST-ArgRS and SerRS-His₆ (in its free or tRNA bound form; Fig. S6). Parallel titration of SerRS and pre-incubated SerRS-tRNA^{Ser} mixture showed that similar amounts of SerRS are being retained by GST-ArgRS, implying that the affinities of GST-ArgRS toward free and tRNA-bound SerRS might be comparable (Fig. S6). In addition, a stable [SerRS•tRNA^{Ser}]•ArgRS complex formation was demonstrated by SPR where ligands were added in a sequential order over immobilized SerRS (Fig. 3D).

Interestingly, pull down analysis executed in the presence of small ligands reveals that the addition of serine and ATP (which allows formation of a SerRS•Ser-AMP complex) does not influence the apparent stability of the SerRS•ArgRS interaction. In contrast, simultaneous addition of arginine and ATP interferes with the SerRS•ArgRS complex formation (Fig. 3A). Although ArgRS does not catalyze the formation of the adenylate intermediate in the absence of tRNA^{Arg} cofactor, it seems that the combination of these two

substrates may obstruct SerRS•ArgRS complex formation.

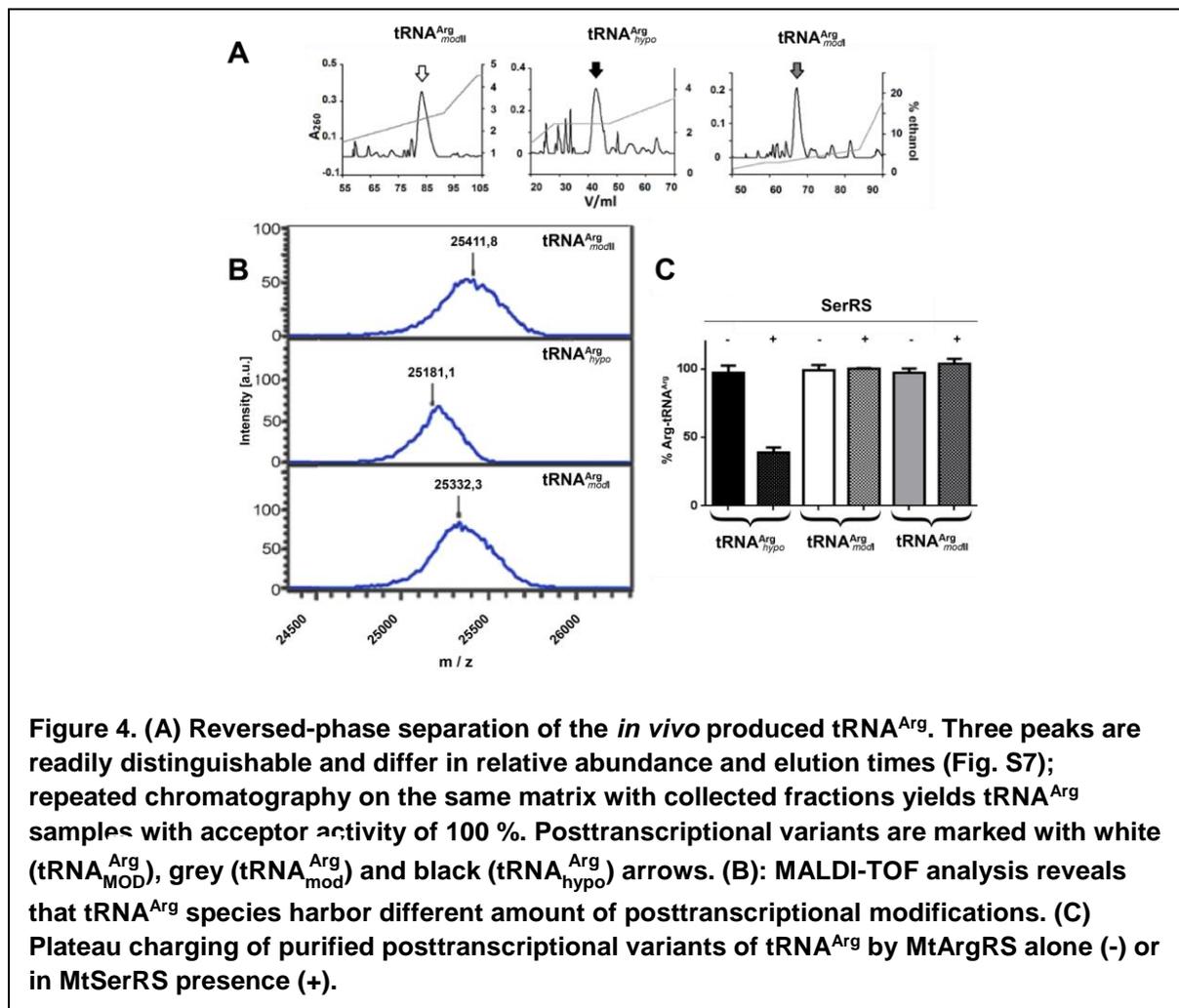


3.4. SerRS helps ArgRS distinguish posttranscriptional variants of cognate tRNA

Bulk *M. thermotrophicus* tRNA^{Arg} overexpressed in *E. coli* separates into three distinct populations that elute at different amounts of organic solvent on a reversed-phase column

(Fig. 4A, S6). It is expected that (i) heterologous tRNA^{Arg} will be modified by *E. coli* enzymes due to its conserved general features [22] and that (ii) tRNA variants with various amounts of posttranscriptional modifications will accumulate when a high rate of transcription is induced [23, 24].

MALDI analysis of isolated variants reveals masses of 25411.8, 25332.3 and 25181.1 Da. The last value equals the theoretical mass of the unmodified *M. thermautotrophicus* tRNA^{Arg}_{CCU} [25], indicating that this population corresponds to either a completely unmodified transcript or the transcript where only pseudouridines have been posttranscriptionally added. Although *M. thermautotrophicus* ArgRS can aminoacylate *E. coli* tRNA^{Arg} [7], none of the collected populations is likely to contain bacterial tRNA, as a fully modified isoacceptor from *E. coli* with the highest molecular weight (tRNA^{Arg}_{UCU}) has a mass >100 Da lower than unmodified *M. thermautotrophicus* tRNA^{Arg}_{CCU} [26]. Further, relative abundance of overexpressed *M. thermautotrophicus* tRNA^{Arg}_{CCU} far exceeds that of *E. coli* tRNA^{Arg}_{UCU} (Fig. S7, [27]). Because all isolated species could be aminoacylated to 100% by MtArgRS, we concluded these are the posttranscriptional variants of *M. thermautotrophicus* tRNA^{Arg} and were designated as tRNA^{Arg}_{hypo}, tRNA^{Arg}_{mod} and tRNA^{Arg}_{MOD} (Fig. 4A, B).



Highly purified tRNA_{MOD}^{Arg} and tRNA_{mod}^{Arg} show mass increments of 230.7 Da and 151.2 Da, respectively, compared to tRNA_{hypo}^{Arg} (Fig. 4B). While modifications introduced by evolutionary conserved enzymes might be expected (e.g. 5-methyluridine (m⁵U) and pseudouridine (Ψ) in the T-arm) [26], others remain elusive. Large mass increments (>100 Da) in *E. coli* arginine isoacceptors result from base 37 (N⁶-threonylcarbamoyladenosine) and base 47 modifications (3-(3-amino-3-carboxypropyl)uridine) [26]. However, these modification are introduced by diverse set of enzymes in bacteria and archaea [28]. Because exogenous tRNA can be modified by *E. coli* enzymes in a unexpected manner [29], it is not possible to determine which modifications have been introduced into tRNA_{hypo}^{Arg}, tRNA_{mod}^{Arg} and tRNA_{MOD}^{Arg} based on the total mass alone.

Steady state aminoacylation with tRNA_{hypo}^{Arg}, tRNA_{mod}^{Arg} and tRNA_{MOD}^{Arg} reveals that these posttranscriptional variants are all suitable substrates for MtArgRS. Similar kinetic properties of these tRNAs indicate that MtArgRS does not distinguish very well between unmodified and modified versions of cognate tRNA (Table 2). The least abundant tRNA, tRNA_{MOD}^{Arg} (Fig. S7), appears to be a slightly better substrate than the most abundant tRNA_{hypo}^{Arg}, as well as tRNA_{mod}^{Arg}.

Table 2. Kinetic parameters for different variants of tRNA^{Arg}_{CCU} from *M. thermotrophicus* and tRNA^{Arg} from *E. coli*.

	k_{cat} / s^{-1}	$K_m / (\mu M)$	$(k_{cat} / K_m) / (s^{-1} \mu M^{-1})$	Susceptibility to SerRS addition
tRNA _{hypo} ^{Arg}	0.51±0.010	1.1±0.20	0.46	yes
tRNA _{mod} ^{Arg}	0.54±0.044	1.6±0.18	0.34	no
tRNA _{MOD} ^{Arg}	0.79±0.041	1.1±0.20	0.73	no
EctRNA _{tot} ^{Arg a}	0.026	4.5	0.0060	no

^a Data for heterologous arginylation with *E. coli* tRNA^{Arg} were taken from [7].

Because our earlier experiments indicated that binding of SerRS and tRNA^{Arg} on ArgRS target closely placed areas in the N-terminal domain (section 3.2), we were interested to determine the impact of SerRS on arginylation reaction *in vitro*. Pre-incubated SerRS•ArgRS complex was used to determine plateau charging of each individual posttranscriptional variant and the values were compared to those obtained by ArgRS alone (Fig. 4B). Interestingly, only tRNA_{hypo}^{Arg} arginylation was affected by SerRS presence. Under standard conditions involving 72 μM [¹⁴C]-arginine only 40% of total tRNA_{hypo}^{Arg} species could be charged (Fig. 4C). Based on the fact that SerRS and tRNA^{Arg} utilize closely placed binding contacts on ArgRS it may be that the subtle differences in tRNA_{hypo}^{Arg} folding, guided by posttranscriptional modification, result in mutual overlap, allowing SerRS to prevent tRNA_{hypo}^{Arg} binding by sterical occlusion.

Another possibility is that SerRS might actively modulate ArgRS activity. Although plateau charging alone cannot reveal details of the mechanism by which SerRS might influence ArgRS activity, it is interesting to note that N-terminal domain has an active, long

range impact on ArgRS catalysis. Steady state aminoacylation with the N-truncated ArgRS variants shows that N-terminal truncations reduce catalytic power of ArgRS predominantly through the effects on k_{cat} . Compared to the wild-type, ΔN_{tot} -ArgRS exhibits 20-fold lower k_{cat} (0.028 s^{-1} vs 0.56 s^{-1}) and 6-fold higher K_m for cognate tRNA^{Arg} ($7.8 \text{ }\mu\text{M}$ vs $1.3 \text{ }\mu\text{M}$, [Table S2](#)). The impact of distal N-domain elements on catalytic activity of ArgRS indicates a possibility of SerRS influencing ArgRS activity via contacts with its N-terminal domain elements. However, to fully unveil the dynamics between ArgRS•SerRS complex formation and arginylation of posttranscriptional tRNA^{Arg} variants *in vivo*, prior knowledge about posttranscriptional status of tRNA^{Arg}_{CCU} in *M. thermautotrophicus* would be needed and “cognate” posttranscriptional variants produced and tested. At this stage, it can only be concluded that the naïve, unmodified tRNA^{Arg} charging can be suppressed by SerRS•ArgRS interaction *in vitro*.

4. Discussion

Here we show that ArgRS uses its N-terminal domain to establish interactions with both SerRS and cognate tRNA^{Arg} in a highly analogous manner. In both cases helix H4 (missing in ΔN_{tot} -ArgRS, but not in ΔN_{89} -ArgRS) exerts the largest influence on the complex formation ([Table 1](#)). Although this element seems to establish direct contacts with SerRS, it does not participate directly in tRNA recognition, as evidenced by our structural model of MtArgRS bound to tRNA^{Arg} [30]. Instead, helix H4 acts as a linker between the globular N-terminal domain and the rest of the enzyme. Communication of this element with the C-terminal anticodon binding domain relies on direct contacts with the long helix devoted to cognate tRNA's body recognition ([Fig. 1A](#)). In that aspect, somewhat lower affinity of ΔN_{tot} -ArgRS toward tRNA^{Arg} relative to ΔN_{89} -ArgRS potentially indicates the effects of mispositioned elements of C-terminal domain involved in tRNA binding. Pronounced effect of N-terminal domain's elements on the turnover number presumably reflects impaired communication between the D-loop binding domain and the distal active site [31, 32]. Likewise, heterologous arginylation of *E. coli* tRNA^{Arg} by MtArgRS shows a ~30 times lower k_{cat} ([Table 2](#)), which might also reflect inadequate recognition of the D-arm [31, 32]

The significance of the N-terminal domain of ArgRS in aminoacylation and other cellular roles is well documented [30, 31, 33-35]. The N-terminal domain of human cytosolic ArgRS binds a nonproteinogenic molecule, hemin [35]. Interestingly, binding of this small molecule is sufficient to inhibit tRNA arginylation. Because hemin also regulates the activity of several critical proteins in the "N-end rule" protein degradation pathway, and ArgRS was assumed to participate in ubiquitin-dependent protein degradation by transmitting Arg-tRNA^{Arg} to arginyl-tRNA transferase (ATE1), it appears that interaction of the N-terminal domain with hemin regulates ArgRS' participation in the N-end rule pathway in human cells [35]. In metazoan organisms, ArgRS forms complexes with other aaRSs via its N-terminal extension which is missing in the archaeal ArgRSs [36]. Both in human cells and *Caenorhabditis elegans* the presence of the N-terminal extension of ArgRS has been proven relevant for maintaining normal translational activity, and, consequently, for cellular growth [37] and homeostasis [38]. Because our data indicate that *M. thermautotrophicus* ArgRS' N-terminal domain

mediates specific interaction with both SerRS and tRNA, it is plausible to assume that a dual role of this domain represents a case where structures devoted solely for protein-protein interaction have not yet arisen.

Posttranscriptional modifications have been shown to modulate both arginylation and decoding. In yeast modifications on tRNA^{Asp} act as negative identity elements and decrease its misarginylation >300-fold [39]. Interestingly, effects on enzyme's specificity originate from the increase in k_{cat} value when tRNA^{Asp} is devoid of modifications [39]. Inspection of the tRNA^{Arg} species from all three kingdoms reveals that the position 37 is universally modified, with the exception of mitochondrial and phage species [26]. The only tRNA^{Arg}_{CCU} isoacceptors with identified modifications (*Bacillus subtilis* and *Bos Taurus*, [26]) bear a N⁶-threonylcarbamoyladenine (t⁶A) at position 37. Although catalyzed by different set of enzymes [40], it is expected that this modification (or its derivative [41]) is present in *M. thermautotrophicus* tRNA^{Arg}_{CCU} *in vivo*. The modification is critical for efficient binding of its cognate codons [22, 42]. Interestingly, inspection of our ArgRS•tRNA^{Arg} model (Fig. S8) shows that a modified anticodon loop with t⁶A at position 37 cannot be recognized in a same manner as unmodified transcript (PDB ID 2ZUE_B, [30]). The bulky modification introduces a sterical clash, thus promoting a conformational change of the anticodon loop. Curiously, if t⁶A is heterologously introduced by *E. coli* enzymes (section 3.4), which might be implied from the mass increase of tRNA^{Arg}_{MOD} and tRNA^{Arg}_{mod} (Fig. 4B), the anticipated conformational changes of the anticodon loop do not affect tRNA^{Arg} aminoacylation by *M. thermautotrophicus* ArgRS severely (Table 2). In contrast, some aspects of ArgRS•tRNA^{Arg}_{hypo} recognition prompt SerRS to act upon its protein partner and suppress charging of the naïve tRNA.

Early reports [40, 43] have shown that in *E. coli* amino acid starvation can be reflected in undermodified tRNA species. More recently, studies in yeast have shown that tRNA modifications can modulate cellular translational capacity in response to the availability of certain amino acids [44]. Furthermore, it has been suggested that posttranscriptional tRNA variants might mediate selective translation of codon-biased mRNAs for stress response proteins [45]. Correlating ArgRS' proximity to the archaeal ribosome, overrepresented consecutive usage of synonymous arginine codons [6] and apparent inability to stringently discriminate between modified and undermodified tRNA decoders, SerRS•ArgRS interaction likely serves to fine-tune release of posttranscriptional tRNA^{Arg} variants or to occlude potentially dangerous, naïve tRNA^{Arg} from translation.

5. Conclusion

Using pull down and SPR analysis, we show that (i) ArgRS uses its N-terminal domain to establish contacts with both SerRS and cognate tRNA^{Arg} and that (ii) only SerRS' substrates participate in the formation of stable ternary complexes (i.e. (SerRS•Ser-AMP)•ArgRS and (SerRS•tRNA^{Ser})•ArgRS). ArgRS does not distinguish between posttranscriptional variants of tRNA^{Arg} and catalyzes arginylation of these substrates with the same efficiency. Aminoacylation of the naïve, unmodified tRNA^{Arg} can be suppressed by SerRS•ArgRS interaction *in vitro*.

CONTRIBUTIONS

AC, GA and IWD conceived and designed the experiments. AC, MČ and VGM performed the experiments. AC, IWD and IGS wrote the manuscript, whereas all authors read, edited and approved the final manuscript.

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