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The unusual methanogenic seryl-tRNA synthetase recognizes tRNA^{Ser} species from all three kingdoms of life

Silvija Bilokapic[1,2], Dragana Korencic[2,3], Dieter Söll[3] and Ivana Weygand-Durasevic[1,2]

[1]*Department of Chemistry, Faculty of Science, University of Zagreb, Croatia;* [2]*Rudjer Bošković Institute, Zagreb, Croatia;* [3]*Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, USA*

The methanogenic archaea *Methanococcus jannaschii* and *M. maripaludis* contain an atypical seryl-tRNA synthetase (SerRS), which recognizes eukaryotic and bacterial tRNAs^{Ser}, in addition to the homologous tRNA^{Ser} and tRNA^{Sec} species. The relative flexibility in tRNA recognition displayed by methanogenic SerRSs, shown by aminoacylation and gel mobility shift assays, indicates the conservation of some serine determinants in all three domains. The complex of *M. maripaludis* SerRS with the homologues tRNA^{Ser} was isolated by gel filtration chromatography. Complex formation strongly depends on the conformation of tRNA. Therefore, the renaturation conditions for *in vitro* transcribed tRNA$^{Ser}_{GCU}$ isoacceptor were studied carefully. This tRNA, unlike many other tRNAs, is prone to dimerization, possibly due to several stretches of complementary oligonucleotides within its sequence. Dimerization is facilitated by increased tRNA concentration and can be diminished by fast renaturation in the presence of 5 mM magnesium chloride.

Keywords: methanogenic archaea; seryl-tRNA synthetase; tRNA dimerization; tRNA^{Ser} recognition.

Fidelity of translation depends on accurate charging by aminoacyl-tRNA synthetases. Investigations carried out in recent years on prokaryotic and eukaryotic aminoacylation systems have shown that the specificity of the aminoacylation reaction is correlated with the presence of a set of recognition elements, which is largely conserved among species [1]. Great effort has been undertaken recently to unravel tRNA identities in archaeal organisms [2,3] and to determine to what extent they follow the rules accounting for identities in prokaryotes and eukaryotes [1,4,5]. In spite of the universality of the genetic code, there are often barriers to aminoacylation across taxonomic domains [6], as the recognition manner of tRNA has undergone evolution coupled with changes in the structure and the number of tRNA molecules in the cell, which carry partially overlapping determinants. The serine system is particularly interesting in this respect because the main recognition element required for specific tRNA:synthetase complex formation is a long variable arm, present in all species except in animal mitochondria. While bacteria and organelles contain three isoacceptor families comprising long variable arms (type 2 tRNAs; tRNA^{Ser}, tRNA^{Leu} and tRNA^{Tyr}), eukaryotic cytoplasm and archaea have only two (tRNA^{Ser} and tRNA^{Leu}) [7–9]. Experimental evidence revealed different mechanisms of type 2 tRNA discrimination in different organisms [10–12]. In general, while the discrimination manner is stringent and dependent on tertiary structure in *Escherichia coli* [10], it is less exclusive and more sequence dependent in yeast [13]. However, despite apparent correlation between the substrate stringency of each aminoacyl-tRNA synthetase and the number of type 2 tRNAs in particular cellular compartment, it was shown that tRNA discrimination by SerRS and LeuRS in the archaeon *Haloferax volcanii* depends on tertiary structure differences, presumably involving the D-loops, similarly to *E. coli* [14]. However, D-loop structure is poorly conserved in tRNAs^{Ser} of methanogenic archaea (http://www.uni-bayreuth.de/departments/biochemie/trna/) [15]. We have recently found that the enlargement of the D-loop did not significantly influence the kinetics of serylation and tRNA discrimination by the two SerRSs that coexist in methanogenic archaeon *Methanosarcina barkeri* [16] (D. Korencic, unpublished data). One of these enzymes is bacteria-like SerRSs, while the other is atypical archaeal SerRS [16], which is only marginally related to the homologues in nonmethanogenic species and outside the archaeal kingdom [17].

We show in this paper that two other methanogenic SerRSs with atypical amino acid sequences, one from thermophile *Methanococcus jannaschii* and the other from mesophile *M. maripaludis*, recognize eukaryotic and bacterial tRNAs^{Ser} in addition to their homologous tRNA^{Ser} and tRNA^{Sec} substrates. The relative flexibility in the tRNA recognition pattern displayed by methanogenic SerRSs was shown by aminoacylation and gel mobility shift assays. This indicates the conservation of some serine determinants in all three domains and gives additional support to the existence of a functional connection between archaeal, bacterial and eukaryotic aminoacylation systems. Since the recognition strongly depends on the conformation of tRNA substrates [18], refolding conditions for unmodified *in vitro* transcribed tRNAs^{Ser} were carefully studied.

Correspondence to I. Weygand-Durasevic, Department of Chemistry, Faculty of Science, University of Zagreb, Strossmayerov trg 14, 10000 Zagreb, Croatia. Fax: +385 1 456 1177, Tel.: +385 1 456 1197, E-mail: weygand@rudjer.irb.hr

Abbreviations: IEF, isoelectric focusing; SerRS, seryl-tRNA synthetase.

Enzyme: seryl-tRNA synthetase (EC 6.1.1.11).

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Materials and methods

Materials

Oligonucleotides were synthesized and DNAs were sequenced by the Keck Foundation Biotechnology Resource Laboratory at Yale University. [^{14}C]serine (100 μCi·mL^{-1}, 160 mCi·mmol^{-1}) was from PerkinElmer Life Sciences Inc. Restriction enzymes were from New England Biolabs. Expand High Fidelity polymerase and inorganic pyrophosphatase were from Roche. Nucleotriphosphates were from Sigma. pET28b vector was from Invitrogen. T7 RNA polymerase was purified from an overproducing strain. NAP-5 columns were from Amersham Biosciences. Genomic DNA of *M. jannaschii* was a gift from D. Tumbula-Hansen, Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, USA. Nickel-nitrilotriacetic acid matrix and HiSpeed Plasmid Maxi Kit were from Qiagen.

tRNA cloning and preparation

Yeast tRNA^{Ser} and tRNA^{Tyr}, purified from total brewer's yeast tRNA as described previously [19], accepted 1.2 nmol of serine and 1.4 nmol of tyrosine per A_{260} unit of tRNA, respectively. *E. coli* tRNA$^{Ser}_{1}$ (VGA anticodon, where V is uridin-5-oxyacetic acid) was previously purchased from Subriden. It accepted 1.1 nmol of serine per A_{260} unit of tRNA. The following archaeal tRNAs were prepared by *in vitro* transcription [20] of their synthetic genes, constructed according to the published sequences [15] (http://www.uni-bayreuth.de/departments/biochemie/trna/): *M. maripaludis* tRNA$^{Ser}_{GCU}$, *M. jannaschii* tRNA$^{Ser}_{GCU}$, *M. maripaludis* tRNA$^{Sec}_{UCA}$ and *M. jannaschii* tRNA$^{Sec}_{UCA}$. tRNA transcripts were purified by electrophoresis on denaturing polyacrylamide gels. Full-length tRNAs were eluted, extensively dialysed and refolded carefully. If not stated otherwise, the transcripts were heated for 5 min at 70 °C in 10 mM Tris/HCl pH 7.0, followed by addition of 5 mM $MgCl_2$ before placing on ice. The amount of active tRNA was determined by measuring aminoacylation plateau with homologous SerRSs (at 37 °C for *M. maripaludis* and 55 °C for *M. jannaschii*). The acceptor activities (nmol of serylated tRNA per A_{260} unit tRNA) were 1.1 for *M. maripaludis* tRNA^{Ser}, 0.9 for *M. maripaludis* tRNA^{Sec}, 1.2 for *M. jannaschii* tRNA^{Ser} and 1.4 for *M. jannaschii* tRNA^{Sec}.

Enzyme cloning and preparation

Yeast SerRS was prepared as described previously [21]. *M. maripaludis* (GenBank AF009822) [22] and *M. jannaschii* SerRS genes (GenBank AAB99075) were PCR amplified and cloned into pET28b plasmids (Invitrogen), which were transformed into *E. coli* BL21(DE3)pLysS strain for expression of N terminally His_6-tagged proteins. Cultures were grown at 37 °C in Luria–Bertani medium, supplemented with 20 μg·mL^{-1} kanamycin. Expression of His_6-tagged proteins was induced for 3–4 h at 30 °C with addition of 0.5 mM isopropyl thio-β-D-galactoside before cell harvesting and disruption. Homogenized cells with expressed *M. jannaschii* SerRS were then centrifuged at 12 000 ***g*** for 15 min and heat treated at 70 °C for 30 min to denature *E. coli* proteins. After centrifugation at 100 000 ***g*** for 1 h, the supernatant was applied on Ni–nitrilotriacetic acid chromatography column equilibrated in 50 mM potassium phosphate buffer pH 7.0, containing 10% glycerol, 0.5 M KCl, 5 mM imidazole, 5 mM 2-mercaptoethanol and 0.1 mM phenylmethanesulfonyl fluoride. Unbound proteins were washed off in the same buffer and His-tagged SerRS was eluted with 200 mM imidazole. A similar procedure was used to separate *M. maripaludis* His-tagged SerRS, except that the flocculation step was omitted. Purification of His-tagged *M. jannaschii* SerRS was continued on FPLC MonoS column equilibrated in 25 mM potassium phosphate buffer pH 7.0, containing 10% glycerol, 10 mM magnesium chloride, 14 mM 2-mercaptoethanol and 0.1 mM phenylmethanesulfonyl fluoride. SerRS was eluted with 300 mM KCl. A FPLC MonoQ column was more suitable for purification of His-tagged *M. maripaludis* enzyme, which has low pI and did not bind to the cationic resin. Binding buffer contained 50 mM Hepes pH 7.0, 10 mM NaCl, 10% glycerol, 10 mM magnesium chloride, 14 mM 2-mercaptoethanol and 0.1 mM phenylmethanesulfonyl fluoride. The synthetase was eluted with 300 mM KCl. The preparations of *M. jannaschii* and *M. maripaludis* His-tagged SerRS proteins were free of endogenous bacterial SerRS, which was confirmed by Western analyses. The antibodies against *E. coli* SerRS did not immunoreact with His-tagged archaeal proteins. Therefore, visualization with His-tagged monoclonal antibodies (Novagen) was performed. In order to obtain bacterial SerRS, crude *E. coli* proteins were applied to a FPLC MonoQ column, and the fraction enriched in endogenous SerRS activity was taken as a source of bacterial enzyme. The specific activity of the pure SerRSs, determined at 37 °C, was 10.37 U·mg^{-1} and 7.83 U·mg^{-1} for the *M. maripaludis* and *M. jannaschii* enzymes, respectively (1 U charges 1 nmol *E. coli* tRNA in 1 min). The specific activity of thermophilic enzyme was fivefold higher at 55 °C.

Gel-retardation assay

SerRS:tRNA complexes were prepared by incubation of the enzyme with freshly renatured tRNA, for 10 min at 37 °C, in buffer containing 10 mM Tris/HCl pH 7.0, 5 mM $MgCl_2$, followed by cooling on ice. Glycerol was added to a final concentration of 5% and the preformed complexes were subjected to electrophoresis on a 6% acrylamide/bisacrylamide (19 : 1) gel containing 5% glycerol in electrophoresis buffer (50 mM Tris, 25 mM boric acid, 10 mM magnesium acetate; pH 7.8). Electrophoresis was at 4 °C for 3 h at 100 V and gels were stained with silver.

Isoelectric focusing

Three successive concentration and reconstitution cycles in deionized water ensured both buffer exchange and removal of salts from protein samples for isoelectric focusing (IEF). The samples were than loaded onto a polyacrylamide gel with ampholyte in the 3–10 or 5–8 pH range. The following protocol was used with a 111 Mini IEF Cell (Bio-Rad) electrofocusing unit: 15 min at 100 V, 15 min at 200 V, 60 min at 450 V at 4 °C. After IEF, the gel was stained with silver.

Aminoacylation assay

Aminoacylation was performed at 37 °C as described previously [21] in reaction mixtures containing 50 mM Tris/HCl pH 7.5, 15 mM $MgCl_2$, 4 mM dithiothreitol, 5 mM ATP and 1 mM ^{14}C-labelled serine. All values represent the average of three independent determinations, which varied by less than 10%.

Results

Seryl-tRNA synthetases from *Methanococcales*: overexpression in *E. coli*, purification and properties

The production of *M. maripaludis* His_6-tagged SerRS was easily induced in an *E. coli* overexpression strain. A two-step purification procedure, which included separation on a Ni–nitrilotriacetic acid affinity column, followed by FPLC on a MonoQ column, resulted in apparently pure protein on a Coomassie blue-stained SDS/polyacrylamide gel. A small amount of aggregates and higher molecular mass impurities were removed by gel filtration using Superdex 200. SerRS was eluted in dimeric form, as determined by careful calibration of the column with protein standards. Dissociation into monomers was not observed. The average yield was ≈ 1.5 mg pure enzyme from 1 L bacterial culture. On the other hand, the expression of *M. jannaschii* SerRS in *E. coli* was very inefficient, resulting in an approximately sixfold lower yield than for the *M. maripaludis* enzyme. Purification was facilitated by the thermophilic character of the enzyme. Therefore, heat denaturation of *E. coli* proteins was performed, and after separation of *M. jannaschi* His-tagged SerRS by Ni–NTA affinity chromatography, basic SerRS protein was additionally purified on a FPLC MonoS column. The use of different ion-exchange chromatographies for purification of two methanogenic SerRSs is based on rather different calculated pI values for these enzymes: 5.8 for *M. maripaludis* and 7.9 for *M. jannaschii* SerRS, which were experimentally verified by IEF (Fig. 1). Determination of molecular mass by gel filtration chromatography revealed that the *M. jannaschii* SerRS is also a dimeric enzyme.

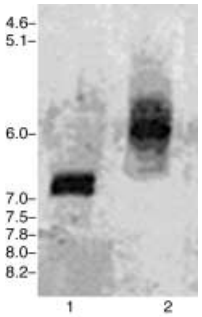

Fig. 1. IEF of two methanogenic SerRSs. Polyacrylamide gel with ampholytes in the pH range 3–10 was visualized by silver staining. Lane 1, *M. jannaschii* (pI ≈ 7.9); lane 2, *M. maripaludis* (pI ≈ 5.8). The pI of IEF protein standards are indicated on the left.

Structural properties of $tRNA^{Ser}$ isoacceptors from methanogenic archaea

The inspection of primary and presumed secondary structures of a number of $tRNA^{Ser}$ isoacceptors from several methanogens available in the databases (*M. jannaschii*, *Methanobacter thermoautotrophicus*, *M. maripaludis*, *Methanopyrus kandleri*, *Methanosarcina mazei* and *M. barkeri*) revealed a strictly conserved G73 nucleotide (with the exception of one *M. mazei* isoacceptor which contains A) and the presence of 16 or 17 nucleotides in the variable arm (positions 44–48, inclusively), which can form five or six base pairs. Thus, the length of the $tRNAs^{Ser}$ variable arms in methanogens exceeds those characteristic for eukaryotic $tRNAs^{Ser}$ (i.e. this identity element in archaea is more bacteria-like), while the number of unpaired nucleotides at the base of variable arm reflects the similarity to eukaryotic tRNAs, due to the presence of at least one unpaired nucleotide between the possible stem of the variable arm and the base at position 48. The most striking feature of $tRNAs^{Ser}$ from methanogens is a variable size of the D-loop. In contrast with other serine-specific tRNAs from bacterial and eukaryotic cells, including those from organelles, many methanogenic $tRNA^{Ser}$ species have D-loops with occupied positions 17 and 17A. Interestingly, the nucleotide at position 20A, which is present in the majority of $tRNA^{Ser}$ isoacceptors from bacteria and eukaryotes, including serine-specific tRNAs from the organelles, is often missing in the sequences of methanogenic $tRNAs^{Ser}$. The same is with base 20B, which is characteristic for bacterial and organellar $tRNA^{Ser}$. The role of D-loop insertion at 20B in orientating the long variable arm in *Thermus thermophilus* $tRNA^{Ser}$ has been clearly observed in the crystal structure of tRNA:synthetase complex [23]. Mixed eukaryotic- and bacteria-like features found in $tRNA^{Ser}$ sequences in methanogenic archaea prompted us to test whether SerRS enzymes from Methanococcales recognize, in addition to their homologous substrates, serine specific tRNAs from other domains of life. The following $tRNAs^{Ser}$ (Fig. 2) were selected for this study: *M. jannaschii* and *M. maripaludis* $tRNA^{Ser}$ transcripts (both with GCU anticodons) and $tRNA^{Sec}$ transcripts which correspond to *selC* genes in the same archaeal species. Fully modified native tRNAs from *E. coli* ($tRNA^{Ser}_1$, anticodon VGA) and *Saccharomyces cerevisiae* (the mixture of serine-specific isoacceptors) represented bacterial and eukaryotic domains in our study, respectively.

$tRNA^{Ser}$ recognition by methanogenic SerRSs

The ability of homologous and heterologous $tRNA^{Ser}$ to be recognized by purified SerRS enzymes from mesophilic and thermophilic methanogenic archaea was tested by aminoacylation (Fig. 3) and tRNA:SerRS complex formation (Fig. 4). Although *M. jannaschii* SerRS is fully active at the temperatures as high as 80 °C (data not shown), serylation capability of different tRNAs was compared at 37 °C due to structural instability of mesophilic tRNA substrates (especially those without post-transcriptional modifications) at elevated temperatures. Both archaeal enzymes efficiently aminoacylated homologous and heterologous archaeal $tRNA^{Ser}$ and $tRNA^{Sec}$ transcripts (Fig. 3A), suggesting

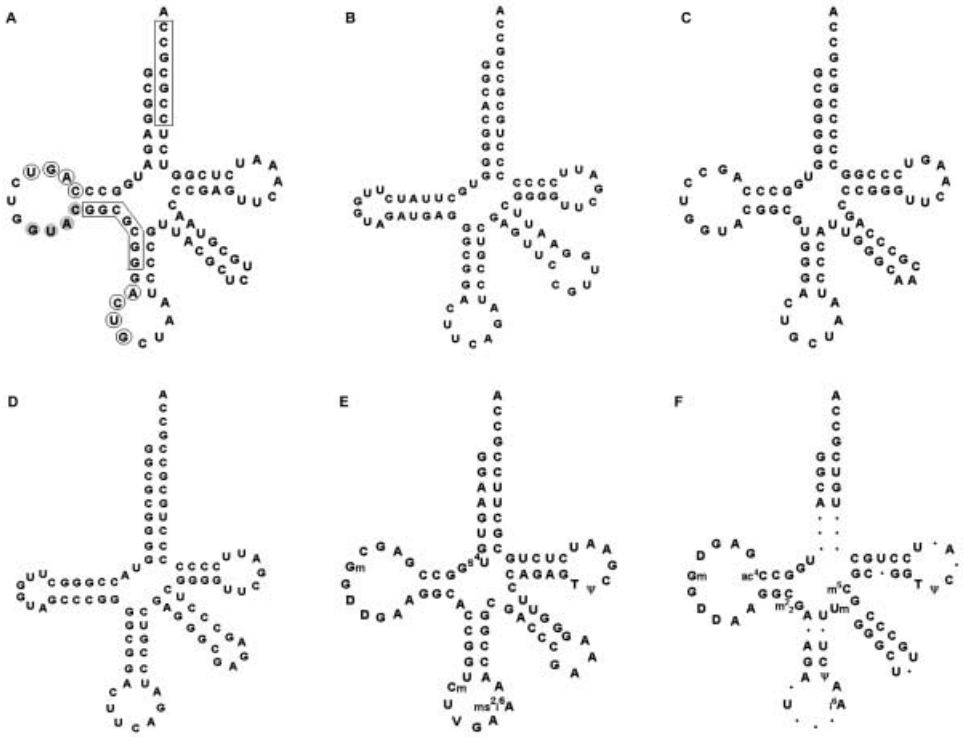

Fig. 2. Sequence and cloverleaf structures of tRNAs^{Ser} and tRNAs^{Sec} used in this study. (A, B) *M. maripaludis in vitro* transcribed tRNAs^{Ser} and tRNAs^{Sec} isoacceptors with anticodons GCU and UCA, respectively. (C, D) *M. jannaschii in vitro* transcribed tRNAs^{Ser} and tRNAs^{Sec} isoacceptors with anticodons GCU and UCA, respectively. (E) *E. coli* tRNA^{Ser}, anticodon VGA (V = uridin-5-oxyacetic acid). (F) *S. cerevisiae* tRNA^{Ser} composite structure. The stretches of oligonucleotides which may participate in intermolecular dimer formation are indicated (and are explained further in the text).

the existence of similar tRNA identity sets in both archaeal organisms. *E. coli* tRNA^{Ser} was charged almost equally well by its homologous enzyme (data not shown) and SerRS from *M. maripaludis*, while about 80% of the charging plateau was reached with SerRS from *M. jannaschii* (at 37 °C). Yeast tRNA^{Ser} was a poorer substrate than *E. coli* tRNA^{Ser} (Fig. 3A and B). We have also noticed that native yeast tRNA^{Ser} was serylated more efficiently than corresponding *in vitro* transcripts (data not shown). Thus, tRNAs^{Ser} from all three domains of life contain the signals required for serylation with SerRSs from both methanogens. However, this flexibility in recognition is unilateral. Archaeal tRNA^{Ser} transcripts were found to be very inefficient substrates for yeast SerRS (I. Gruic-Sovulj, unpublished data), suggesting less constrained recognition in archaea than in yeast. This agrees with previously observed inability of *E. coli* SerRS to serylate *in vitro* transcribed tRNAs^{Ser} from *M. maripaludis* and *T. thermoautotrophicus* [22].

Complex formation among archaeal SerRSs and homologous and heterologous tRNAs^{Ser} was monitored by native gel electrophoresis. As shown in Fig. 4 *M. maripaludis* SerRS forms complexes with homologous and heterologous archaeal tRNAs^{Ser} and tRNAs^{Sec} (lanes 3 and 4, 5 and 6, respectively) as well as with tRNA^{Ser} substrates from other domains of life (*E. coli*, lane 7; yeast, lane 8). The complexes with nonarchaeal serine specific tRNAs are somewhat less stable, as judged by relative amounts of free and SerRS-bound tRNAs. However, noncognate complexes with yeast tRNA^{Tyr} (type 1 tRNA) were not detected (lane 9). The same results were obtained with *M. jannaschii* enzyme (data not shown). This experiment confirms the similarity in the recognition pattern between two archaeal enzymes, which both recognize nonarchaeal tRNAs, observed also in the aminoacylation assay (Fig. 3). tRNA^{Ser}:SerRS complexes were also detected as the bands of altered mobility by IEF (Fig. 4B). Although the two methanogenic SerRSs display very different pI values (pI of 5.8 was estimated for *M. maripaludis* and 7.9 for *M. jannaschii* SerRS) (Fig. 4B, lanes 2 and 3, respectively), both homologous tRNA^{Ser}:SerRS complexes (lanes 1 and 5, respectively) have a pI value of approximately 5.2.

Dimerization of archaeal tRNA transcript

Unlike many other tRNAs, *M. maripaludis* and *M. jannaschii* tRNA$^{Ser}_{GCU}$ transcripts are prone to dimerization, possibly due to several complementary stretches of oligonucleotides present in their primary structures. Consequently, the transcripts, which appeared homogenous on a denaturing gel, did not migrate as single species on a nondenaturing gel, but separate into at least two bands (Fig. 5). One has a mobility consistent with the expected size of monomeric tRNA, while the other corresponds to dimeric species (see also Fig. 6). Dimerization is enhanced

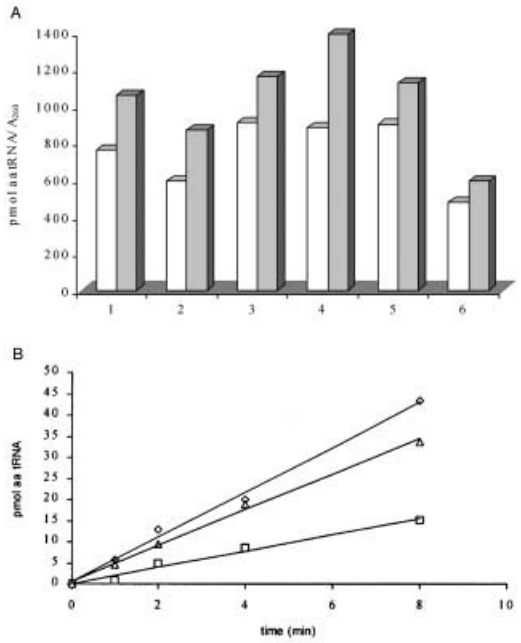

Fig. 3. Serylation of homologous and heterologous tRNAs^{Ser} and tRNAs^{Sec} by SerRSs from methanogenic archaea. (A) Serylation was performed at 37 °C and the charging plateau with *M. maripaludis* (filled bars) and *M. jannaschii* (open bars) SerRS is shown. Histograms compare the aminoacylation of 0.33 μM tRNAs: 1, *M. maripaludis* tRNA^{Ser}; 2, *M. maripaludis* tRNA^{Sec}; 3, *M. jannaschii* tRNA^{Ser}; 4, *M. jannaschii* tRNA^{Sec}; 5, *E. coli* tRNA^{Ser}; 6, *S. cerevisiae* tRNA^{Ser}. The concentration of SerRS was 220 nM. (B) Serylation of tRNAs^{Ser} (5 μM) from three kingdoms of life (△, *M. maripaludis*; ◇, *E. coli*; □, yeast) performed at 37 °C with 90 nM *M. maripaludis* SerRS.

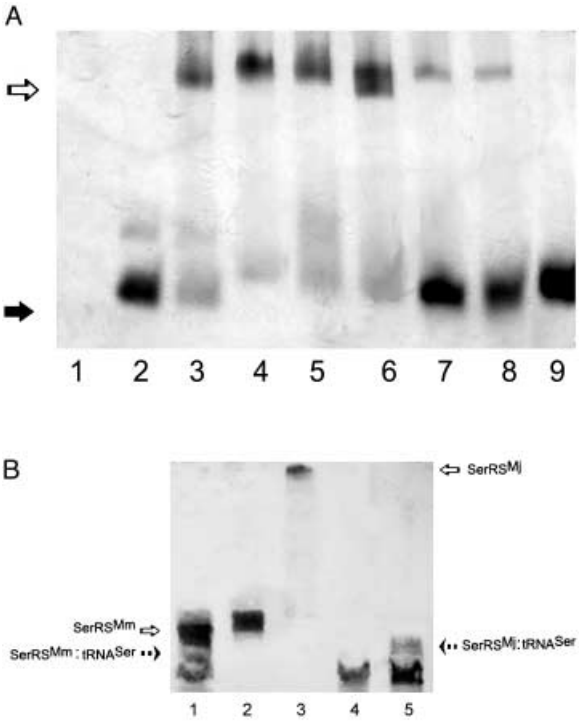

Fig. 4. Detection of homologous and heterologous SerRS complexes. (A) Gel mobility shift assay. *M. maripaludis* SerRS (7 pmol) was incubated with different tRNAs (14 pmol) and subjected to PAGE under native conditions: *M. maripaludis* tRNA^{Ser}, lane 3; *M. maripaludis* tRNA^{Sec}, lane 4; *M. jannaschii* tRNA^{Ser}, lane 5; *M. jannaschii* tRNA^{Sec}, lane 6; *E. coli* tRNA^{Ser}, lane 7; *S. cerevisiae* tRNA^{Ser}, lane 8; *S. cerevisiae* tRNA^{Tyr}, lane 9. Uncomplexed SerRS (7 pmol) and tRNA^{Ser} (14 pmol) were loaded on the gel as electrophoretic mobility markers (lanes 1 and 2, respectively). The black arrow shows uncomplexed tRNA; the white arrow shows SerRS:tRNA complexes. The same pattern was obtained with *M. jannaschii* SerRS (data not shown). (B) IEF detection. SerRS was incubated with the excess of freshly renatured tRNA^{Ser} for 10 min at 37 °C and separated by native PAGE in a gel containing ampholytes in the pI range 5–8. Lanes 1 and 5 show homologous *M. maripaludis* and *M. jannaschii* complexes, respectively. Lanes 2 and 3 contain the proteins (*M. maripaludis* and *M. jannaschii*, respectively) and lane 4 tRNA^{Ser} as electrophoretic markers. The gels showed in both panels were stained with silver.

in the presence of spermine (Fig. 5, lane 1), spermidine or monovalent cations (data not shown). On the other hand, it is diminished by refolding of tRNA in 10 mM Tris/HCl pH 7.0 containing 5–10 mM magnesium. However, the ratio of monomeric and dimeric fractions after refolding depends on the renaturation procedure (as shown in Fig. 5), most importantly on duration of cooling after addition of magnesium (lane 2, 3 and 4). Only fast cooling in the presence of 5–10 mM magnesium leads to a properly folded tertiary structure. The dimerization was significantly enhanced when renaturation was performed at increased tRNA concentrations (Fig. 5B). A similar effect was observed previously by monitoring dimerization of complementary microhelices [24]. As expected, tRNA^{Ser} dimers were not recognized by cognate SerRS, as shown by gel retardation assay (Fig. 5C).

The isolation of *M. maripaludis* tRNA:SerRS complex

In order to explore the stoichiometry of tRNA:SerRS complex in a methanogenic archaeon, gel filtration chromatography, which enabled the size estimation of interacting macromolecules, was performed. To facilitate the complex formation, *M. maripaludis* SerRS was preincubated with excess tRNA and loaded onto a Superdex 200 column, previously calibrated with molecular mass markers. The elution profile consists mainly of three peaks (Fig. 6A), which were analysed further by SDS/PAGE and visualized by ethidium bromide staining (Fig. 6B). The first peak comprised protein and tRNA, while the fractions in the second and the third peaks contained only tRNA. The molecular masses of separated macromolecules or their complexes, which corresponded to elution volumes of peaks I, II and III, were estimated to be 159 kDa, 66 kDa and 32 kDa, respectively. This clearly shows that the separation of dimeric (peak II) and monomeric (peak III) tRNAs can be achieved by gel filtration chromatography. Based on the molecular mass estimation of peak I, the macromolecular complex is composed of dimeric SerRS with one bound tRNA. This finding also supports the results of gel retardation assays (Fig. 5), which revealed that tRNA dimers were not capable of participating in the complex formation with the synthetase.

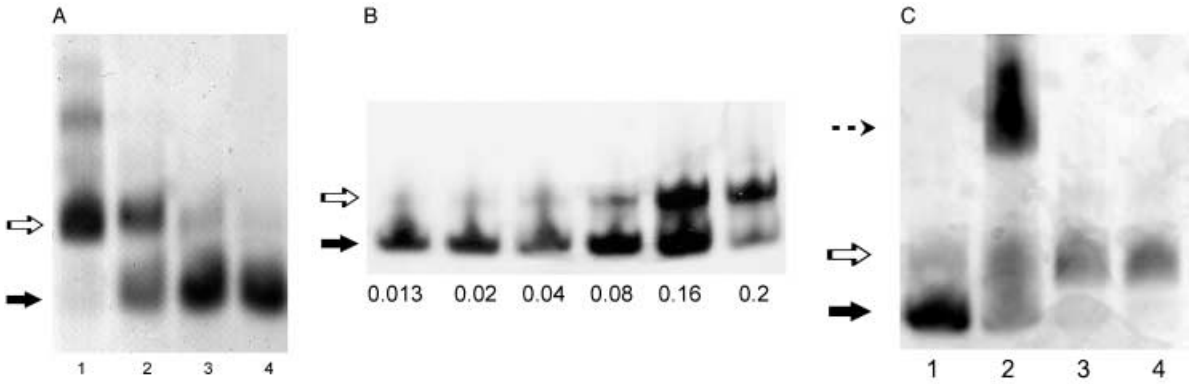

Fig. 5. Formation and properties of *M. maripaludis* tRNA$^{Ser}_{GCU}$ dimers. (A) Sensitivity to refolding conditions: lane 1, tRNA was heated in 10 mM Tris/HCl pH 7.0 for 2 min at 95 °C, then incubated for 5 min at 55 °C prior to addition of 1 mM spermine and slow cooling to room temperature over of 5 h; lane 2, as described for lane 1 but with 5 mM magnesium instead of spermine; lane 3, tRNA was heated in 10 mM Tris/HCl pH 7.0 for 5 min at 70 °C before addition of magnesium and cooling to room temperature over 30 min; lane 4, as described for lane 3 except that the sample was placed on ice immediately after addition of magnesium. Black and white arrows show the positions of monomeric and dimeric tRNA forms, respectively. (B) Renaturation under conditions that favour monomer formation (described in panel A, lane 4) gave a higher proportion of dimers at elevated tRNA concentrations, as shown by the numbers below the lanes (mM). (C) SerRS recognition was monitored by incubation of SerRS and tRNA subjected to a different refolding conditions: properly folded tRNA monomers (conditions indicated at panel A, lane 4), before (lane 1) and after (lane 2) incubation with homologous SerRS. Refolding in the presence of spermine resulted in the formation of tRNA dimers (lane 3), not recognized by the synthetase (lane 4; tRNA + SerRS). tRNA monomers, dimers and tRNA:SerRS complexes are indicated by arrows. The samples were separated by nondenaturing PAGE and visualized by silver staining.

Discussion

Non-stringent tRNA recognition by methanogenic SerRSs

Identity elements required for serylation have been studied in a number of organisms, providing insights into tRNA^{Ser} recognition in different domains of life [9–13,25–35]. While in *E. coli* recognition is rather constrained and depends strongly on the characteristic tertiary structure of tRNA^{Ser}, it is less constrained in yeast and seems to be flexible in archaea as well. We have recently shown that shortening of the extra arm influences recognition by *M. barkeri* SerRSs, while enlargement does not (D. Korencic, unpublished data). This may be also the case for the enzymes from *M. maripaludis* and *M. jannaschii*, that make less stable complexes with yeast tRNA^{Ser} isoacceptors comprising extra arms that are two nucleotides shorter (Fig. 4). On the other hand, both enzymes recognize and efficiently serylate methanogenic tRNAs^{Sec}, whose variable arm lengths exceed those of cognate tRNAs^{Ser} (Figs 3 and 4). In addition to its length, the orientation of the variable arm was shown to be important for proper recognition by SerRS enzymes [8,32]. It is maintained in *E. coli* through tertiary interactions with the D-arm. As this latter region is structurally not conserved in methanogens, it can be speculated that the orientation of the extra arm may be maintained in different manner. In agreement with sequence analyses which revealed that serine specific tRNAs from methanogenic archaea comprise mixed bacterial and eukaryotic features, we show in this paper that two atypical archaeal SerRSs exhibit relatively low stringency in heterologous tRNAs^{Ser} recognition. However, in agreement with our previous results, SerRS does not have a generally relaxed specificity, since the barriers in cross-domain recognition of cognate tRNA^{Ser} are unilateral [22,31] and noncogante tRNA does not form complexes with SerRS enzymes ([36] and Fig. 4).

Alternative conformations of tRNA transcripts and SerRS recognition

Numerous experiments have shown that careful refolding of tRNA transcripts is crucial for biological activity [18,37–41]. Because the refolding path is sequence dependent, each transcript may require substantially different renaturation conditions. Parallel pathways of tRNA folding may produce a variety of stable misfolded conformations besides the native-like molecule [42]. In our hands, special care had to be taken to obtain a uniform population of properly folded *in vitro* transcribed *M. maripaludis* and *M. jannaschii* tRNA$^{Ser}_{GCU}$, as the formation of alternative conformations was prominent. These altered structures were identified as stable tRNA dimers, easily separable by gel filtration from the fraction of active monomeric tRNA (Fig. 6). We assume that dimerization is the consequence of intermolecular hybridization occurring during the renaturation process. Several short complementary sequences within *M. maripaludis* tRNA$^{Ser}_{GCU}$ sequences (Fig. 2) can participate in the tRNA:tRNA association. There is the tetranucleotide GUAC in the D-arm of *M. maripaludis* tRNA^{Ser}, which may anneal with the same stretch in another tRNA^{Ser} molecule in a self-complementary manner. The dimerization can also be caused by the intermolecular association, maintained through the hybridization between CAGU (positions 13–16, D-arm) and ACUG (positions 31–34, anticodon arm) stretches. Similarly, the heptanucleotide GGCGCGG (D-stem/anticodon stem) can potentially pair with the complementary stretch CCGCGCC (corresponding to positions 68–75 in the acceptor stem). The hybrid-

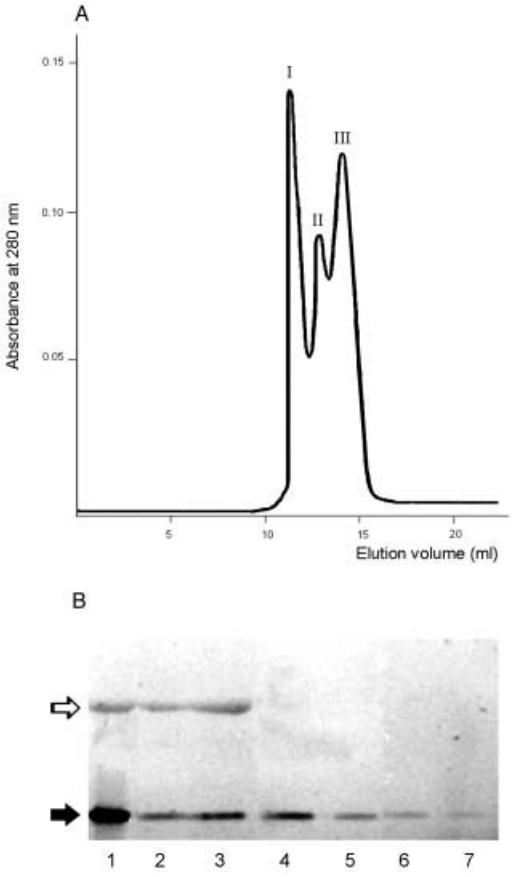

Fig. 6. Separation of macromolecular complexes by gel filtration. *M. maripaludis* SerRS was incubated with an excess of $tRNA^{Ser}$ and loaded on Superdex 200 column HR 10/30 (1 × 30 cm). Elution was performed with 10 mM Hepes pH 7.0, containing 50 mM KCl, 10 mM $MgCl_2$ and 5 mM dithiothreitol, at flow rate of 0.5 $mL \cdot min^{-1}$, and the absorbance at 280 nm was monitored. The fractions of the three peaks appearing in the elution profile (A) were subjected to SDS/PAGE. The gel was stained with ethidium bromide (B): lane 1, the sample before gel filtration chromatography; lanes 2 and 3, peak I fractions; lanes 4 and 5, peak II fractions; lanes 6 and 7, peak III fractions. Molecular masses of 159 kDa, 66 kDa and 32 kDa, corresponding to peaks 1, II and III, respectively, were estimated from the elution volume. Black and white arrows show the positions of tRNA and protein, respectively.

ization which could lead to dimer formation, especially if it involves the oligonucleotides from the stem regions, probably starts at elevated temperature, before monomeric tertiary tRNA structure is stabilized. On the other hand, we imagine that structural fragility in the absence of post-transcriptional modifications might facilitate disruption of contacts stabilizing the $tRNA^{Ser}$ tertiary structure and cause functional deactivation, either by formation of misfolded tRNA monomers or by dimerization. The latter event seems to be preferred in the case of *M. maripaludis* and *M. jannaschii* $tRNA^{Ser}$, a both tRNAs comprise short, although, different, sequences which can produce misfolded tRNA molecules. Several kinds of tRNA dimers, formed by parallel pathways during tRNA folding have been observed previously in other systems [41]. Certainly the most interesting case is dimerization of pathogenic human mitochondrial tRNA, which is facilitated *in vivo* by a single nucleotide mutation in the D-arm, producing a self-complementary hexanucleotide [43].

Potential importance of modified nucleotides for tRNA folding, tRNA:tRNA interactions and SerRS recognition

tRNA sequences and post-transcriptional modifications vary considerably across the three phylogenetic domains of life. In addition to the conserved core of modifications observed in tRNAs of almost all organisms [44] archaea, bacteria and eukarya each make phylogenetically characteristic modifications to their tRNAs following transcription, which exert clear effects on tRNA stability [37,45]. Although the influence of tRNA stabilization by nucleotide modifications is substantial in archaea growing at temperatures that would otherwise denature unmodified tRNAs [45], it was observed that in the two relatively closely related species *M. jannaschii* and *M. maripaludis*, that grow optimally at very different temperatures (85 °C and 37 °C, respectively), the modification profile is relatively similar [46]. Therefore, post-transcriptional modifications can be important for proper folding of mesophilic *M. maripaludis* $tRNA^{Ser}$ as well [46] and can also modulate or prevent tRNA:tRNA interactions [47]. Thus, observed dimerization of $tRNA^{Ser}$ transcript, which occurs *in vitro* probably as a consequence of several stretches of complementary oligonucleotides in tRNA primary sequence, may not occur *in vivo*, where many nucleotides carry modifications, and the cellular concentration of tRNA is far below those used in the experiments described. Efficient charging of *M. jannaschii* and *M. maripaludis* unmodified $tRNA^{Ser}$ and $tRNA^{Sec}$ isoacceptors by their homologous SerRSs indicate that the modifications do not affect the recognition directly, although they may be important for maintaining the structure of tRNA.

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