# Crystallization of Bizarre Mitochondrial tRNA$^{Ser}$ using Protein-sandwiching and Symmetry Augmentation

# การตกผลึก tRNA$^{Ser}$โดยวิธีโปรตีนแซนวิชและการเพิ่มสมมาตรโมเลกุล

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## ABSTRACT

Mammalian mitochondrial tRNA$^{Ser}_{GCU}$ is an extraordinary tRNA possessing a three-arm configuration lacking the D-arm in the canonical cloverleaf structure. Despite its aberrant secondary structure, it was shown that the tRNA is fully functional in the translating ribosome. To date, knowledge of the tertiary folding and interactions with translational factors of this '*bizarre*' tRNA are absent because the flexibility and lability of RNA hamper structural determination by X-ray crystallography. To address this difficulty, we here introduce two new approaches to crystallize mitochondrial tRNA$^{Ser}_{GCU}$ by protein-sandwiching and symmetry augmentation. Protein components were expressed in *E. coli* and purified to homogeneity, and tRNAs were prepared using a large-scale *in vitro* run-off transcription. In the end, we observed two crystal forms grown by MPD and PEG MME 5000. These crystals will be further characterized and subjected to X-ray diffraction to solve an enigmatic feature of the translation system.

## บทคัดย่อ

tRNA$^{Ser}_{GCU}$เป็นไมโตคอนเดรียทีอาร์เอ็นเอที่ไม่มีส่วนของ D-arm แต่ยังสามารถแปลรหัสพันธุกรรมได้ปกติ ปัจจุบันความรู้เกี่ยวกับโครงสร้างตติยภูมิ (tertiary structure) และปฏิสัมพันธ์ระหว่างโมเลกุลของทีอาร์เอ็นเอชนิดนี้และโปรตีนที่เกี่ยวข้องยังมีอยู่น้อยมาก เนื่องจากความไม่คงตัวของอาร์เอ็นเอเป็นอุปสรรคต่อการศึกษาโครงสร้าง สามมิติด้วยวิธี X-ray crystallography ดังนั้นงานวิจัยนี้จึงใช้วิธีใหม่ 2 วิธี คือ โปรตีนแซนวิชและการเพิ่มสมมาตรของโมเลกุลเชิงซ้อนระหว่างทีอาร์เอ็นเอและโปรตีน ซึ่งทีอาร์เอ็นเอได้ถูกสร้างขึ้นจาก กระบวนการ *in vitro* run-off transcription ส่วนโปรตีนได้ถูกผลิตขึ้นในเซลล์ *E. coli* และถูกทำให้บริสุทธิ์ จากการศึกษาพบว่าในสภาวะที่มีส่วนประกอบของ เมธิลเพนเทนไดออล (MPD) และ โพลีเอธิลีนไกลคอล (PEG MME 5000) เกิดผลึกขึ้นในรูปแบบที่แตกต่างกัน ซึ่งผลึกดังกล่าวจะถูกนำไปศึกษาต่อไปโดยวิธี X-ray diffraction เพื่อนำไปสู่ความเข้าในในกระบวนการแปลรหัสของ tRNA ดังกล่าว


**Key Words:** mitochondrial tRNA$^{Ser}$, protein-sandwiching, symmetry augmentation

**คำสำคัญ:** ทีอาร์เอ็นเอ โปรตีนแซนวิช การเพิ่มสมมาตรโมเลกุล

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**Introduction**

Mammalian mitochondrial (mt) $tRNA^{Ser}_{GCU}$ (the subscript represents the anticodon letter of tRNA) is an exclusive non-canonical tRNA that has an aberrant secondary structure. This tRNA lacks the entire D-arm, and thus, is folded into a three-arm configuration (Arcari and Brownlee, 1980, Hayashi, 1997 and Helm *et al.*, 2000) or the "truncated cloverleaf" (Bruijin and Klug, 1983). Moreover, it is apparently devoid of the long variable arm, a unique character of $tRNA^{Ser}$, by which the cognate seryl-tRNA synthetase (SerRS) discriminates from other tRNA species. Interestingly, mt $tRNA^{Ser}_{GCU}$ can efficiently function in mt translation system as its isoacceptor $tRNA^{Ser}_{UGA}$, and is recognized by the same SerRS in mitochondria (Ueda *et al.*, 1992). To date, the tertiary folding of mt $tRNA^{Ser}_{GCU}$ remains enigmatic, and the molecular mechanism for discrimination of mt $tRNA^{Ser}$ by SerRS is not fully understood. In addition, it is reported that a single mutation on mt $tRNA^{Ser}$ causes mitochondrial diseases (Lauber *et al.*, 1991). For example, G12207A, a point mutation at the 5′-end of on mt $tRNA^{Ser}_{GCU}$, can raise some features of MELAS/MERRF syndromes (Wong *et al.*, 2006).

To provide new insights into mt $tRNA^{Ser}_{GCU}$'s structure and function, it requires crystallization of mt $tRNA^{Ser}_{GCU}$ for X-ray structural determination. However, RNA crystallization is not always straightforward due to the fact that RNA is highly flexible, heat-unstable, and sensitive to hydrolysis. Moreover, preparation of pure RNA in a milligram scale is laborious and requires skill. Thus, crystallization of mt $tRNA^{Ser}_{GCU}$ has not been so far accomplished. In this study, we introduce two distinct "protein-sandwiching" strategies to stabilize the RNA using protein modules and RNA operation in crystallizing mt $tRNA^{Ser}_{GCU}$ for X-ray crystallographic analysis.

The first "protein-sandwiching" approach utilizes two proteins: bovine mt SerRS and human U1A to concomitantly stabilize mt $tRNA^{Ser}_{GCU}$ (Figure 1A). The use of mt SerRS will also provide understanding of native $tRNA^{Ser}$–SerRS interactions. On the other hand, U1A is a protein member of eukaryotic spliceosomal subunits, which has been previously reported as a useful crystallizing module for structural determination of several ribozymes with scarce effect on the whole RNA structure (Ferré-D'Amaré *et al.*, 2000 and 2010). To create the U1A binding site in mt $tRNA^{Ser}_{GCU}$, the original anticodon loop is replaced by a U1A binding loop motif.

The second "protein-sandwiching" approach includes an RNA operation to introduce the 2-fold symmetry into the tRNA molecule (Figure 1B). Since we hypothesize that "symmetry augmentation" can enhance molecular alignment in the crystal, the introduction of the symmetry into tRNA molecule should stabilize RNA and raise the success rate of crystallization. In this approach, the anticodon loop of mt $tRNA^{Ser}_{GCU}$ is replaced by a 'kissing loop' (Laughrea and Jette, 1994) containing GC-repeat that strongly induces dimerization through G–C base pairs. mtSerRS is employed for sandwiching as the first approach.

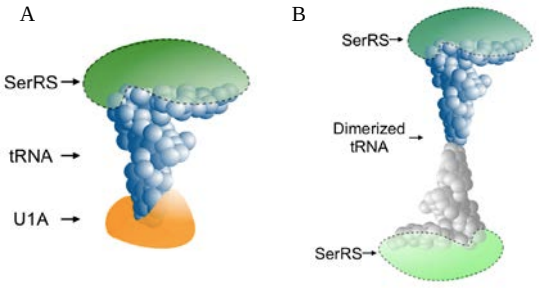


**Figure 1.** Two strategies used in this study for $tRNA^{Ser}_{GCU}$ crystallization: (A) protein sandwiching with mt SerRS and U1A proteins, and (B) SerRS-sandwiching via the RNA kissing loop.

## Methods

*Overexpression and purification of proteins*

The full-length genes of bovine mt SerRS and U1A fused with N-terminal hexahistidine tag were constructed into vectors pET-19b and pET-28a (Novagen), respectively, together with thrombin cleavage signal. The proteins were overexpressed in the Rosetta (DE3) *E. coli* strain under 50 mM isopropyl-β-D-thiogalactopyranoside (IPTG) induction for 18 hours at 25ºC in a 2-liter medium. Cells were lysed by sonication and the soluble fractions were collected after ultracentrifugation at 100,000 × g for 1 hour. Thereafter, all protein purification processes were conducted at 4ºC due to the labile nature of mammalian proteins. Recombinant proteins were purified to homogeneity through a three-step chromatography: nickel affinity, ion exchange, and gel filtration. The hexahistidine tag was completely removed by the treatment with the thrombin protease at 4ºC (1 unit thrombin per mg recombinant protein). Afterwards, purified recombinant proteins were verified and quantitated using SDS-PAGE and Bradford assay, respectively. Purified SerRS and U1A proteins were finally concentrated by ultrafiltration to approximately 10 and 5 mg/ml, respectively, and then divided into small aliquots, flash-frozen with liquid nitrogen, and stored at -80ºC until crystallization.

*Large-scale preparation of mt* $tRNA^{Ser}_{GCU}$

The genes of engineered bovine mt $tRNA^{Ser}_{GCU}$ were constructed and cloned into the pUC19 vector with the upstream T7 RNA promoter sequence. The transcription dsDNA templates were PCR-amplified with two specific primers: 5′-***TAATACGACTCACTATA***G-3′ and 5′-T*Gm*GCGAGAAAGCCATAC-3′ in a 10-ml reaction (The T7 promoter sequence is in bold italic characters, and Gm represents 2′-O-methyl deoxyguanosine, used for manipulating the uniform 3′-end of transcribed products). PCR products were then purified via the gel filtration to remove contaminants including excess primers and nucleotides. Afterwards, the large-scale run-off *in vitro* transcription was carried out at 37ºC for 5-6 hours in a 50-ml reaction containing 40 mM HEPES pH 7.8, 100 mM NaCl, 8 mM $MgCl_2$, 1 mM spermidine, 5 mM DTT, 0.05 mg/ml BSA, 0.01% Triton X-100, 4 μg/ml dsDNA template, 1.3 mM ATP, 1.4 mM UTP, 1.3 mM CTP, 1 mM GTP, 10 mM GMP, and in-house T7 RNA polymerase. Thereafter, transcribed products were ethanol-precipitated and purified via 10% urea denaturing gel (gel excision). The expected band of $tRNA^{Ser}_{GCU}$ was excised, and RNA was twice extracted from gels by 'soak and shake' at 25ºC. At the end, the gel filtration was performed to remove any possible contaminants such as acrylamide. RNAs were stored at -30ºC until use.

*Gel shift assay*

Gel shift assay was conducted to confirm the proper folding and binding activity of proteins and RNA. Protein(s) and RNA were mixed together in the solution containing 50 mM HEPES pH 7.5, 5 mM $Mg(OAc)_2$, 20 mM KCl, 1 mM spermine, 0.5 mM DTT and 1 mM ADPNP at the room temperature (r.t.)

for 20 minutes before loading onto 6% native polyacrylamide gel, and electrophoresed at 120 V constant at 4ºC for 90 minutes. Gels were stained by both ethidium bromide and Coomassie blue to visualize RNA and protein, respectively. Theoretically, when the protein–RNA complex is formed, protein in the complex moves faster into the gel according to the increment of the negative charge by RNA binding. Oppositely, RNA complexed with protein migrates slower due to the bigger size than RNA alone.

*Crystallization screening*

Protein and RNA components were mixed together in the identical condition as gel shift assay and then reacted at r.t. for 20 minutes, and immediately incubated on ice. The molar ratios of the complex were tRNA:SerRS:U1A = 1:1:1 and tRNA:SerRS = 1:1 for approach 1 and 2, respectively. The mixture was centrifuged at 15,000 rpm prior to crystallization to remove denatured protein and aggregation. Matrix screen was conducted at 20ºC using the sitting drop vapor diffusion technique with Crystal Screening Kit 1 and 2 from Hampton Research, yielding 96 different crystallization conditions with various salts, pH and precipitants in MRC 96-well crystallization plate from Molecular Dimensions.

**Results and discussion**

*Pure protein components*

Mitochondrial SerRS and U1A are both mammalian proteins that are sensitive to heat-denaturation and easy to precipitate. We, therefore, performed all purification processes at 4ºC. We note that all solutions and equipment used in the experiment are RNase-free.

His-tagged mt SerRS and U1A were overexpressed as soluble forms showing the sizes of 56.4 kDa and 13.4 kDa, respectively. After nickel-affinity chromatography, since high imidazole concentration can inhibit thrombin's activities, we directly added thrombin protease into protein fraction and used dialysis method to reduce the imidazole concentration. For mt SerRS, thrombin digestion was done for 48 hours resulting in approximately 70% complete. On the other hand, U1A was very sensitive to salt concentration. Rapid reduction of salt caused severe protein precipitation. After removal of His tag, the size of mt SerRS and U1A are 54.78 kDa and 11.6 kDa, respectively. Finally, the gel filtration purification yielded highly pure proteins (Figure 2) that were free from RNase trace as supported in the gel-shift assay (Figure 5).

The final yields of SerRS and U1A from 2-liter culture were 3 mg and 1.6 mg, respectively.

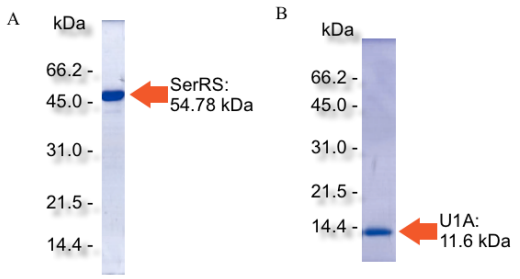


**Figure 2.** High purity of SerRS (A) and U1A (B) verified by SDS-PAGE

*Milligram quantity of modified mt tRNA$^{Ser}_{GCU}$*

We modified the original anticodon loop sequence of mt tRNA$^{Ser}_{GCU}$: 5′-CUGCUAA-3′ to the U1A binding motif: 5′-AUUGCACUCC-3′, and the kissing loop: 5′-AAGCGCGCA-3′ for approach 1 and 2, respectively. In this study, we used PCR to produce DNA template to prevent the addition of extra adenine residues at the 3′-end of transcripts by T7 RNA polymerase, because precise 3′-CCA end is critical for SerRS recognition. Finally, both tRNAs were very

pure (Figure 3). The yield of $tRNA^{Ser}_{GCU}$ with the U1A binding motif was 8.2 mg.

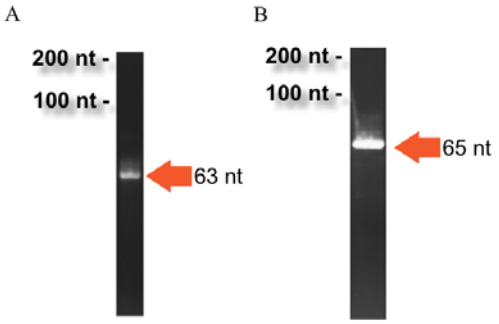


**Figure 3.** Purified $tRNA^{Ser}_{GCU}$ with the U1A binding loop (A) and $tRNA^{Ser}_{GCU}$ with the kissing loop (B) on 10% urea denaturing gel

*Formation of super complexes*

We used the gel shift assay to verify the interactions among components because it is convenient and has high sensitivity. We performed the assay at 4ºC to preserve the native structures of components.

According to lanes 3-5 in Figure 4A, our recombinant SerRS and U1A did not show any bands of RNA contamination from *E. coli*, indicating the quality of purification processes. For approach 1, the tRNA with U1A binding motif can form complexes with both mt SerRS (Figure 4A, lanes 6, complex 1) and U1A (Figure 4A, lane 8, complex 2) as shown by clearly band shift compered to tRNA alone (Figure 4A, lane 2). These results indicate that all components were properly folded and have binding activities. However, tRNA-U1A complex showed a sharper band than that of tRNA-SerRS complex, suggesting that the interaction between tRNA and U1A interaction were stronger and the complex was more stable.

In addition, the mixture of all components showed slower migration than tRNA complexed with a single protein (Figure 4A, lanes 9-10, complex 3). This obviously reveals that the tertiary complex of tRNA, SerRS, and U1A, or the "protein-sandwiching" complex was formed.

For approach 2, modified tRNA with the kissing loop was denatured by urea and heat, resulting in a partially denatured tRNA (Figure 4B, lane 1). This indicates that the tRNA dimer was very stable. In fact, modified tRNA preferred dimeric form in non-denatured condition as shown by a sharp band (Figure 4B, lane 2). The dimeric tRNA and SerRS formed complex as shown by a band shift (Figure 4B, lanes 3-4). Taken together, these results support our ideas of protein sandwiching.

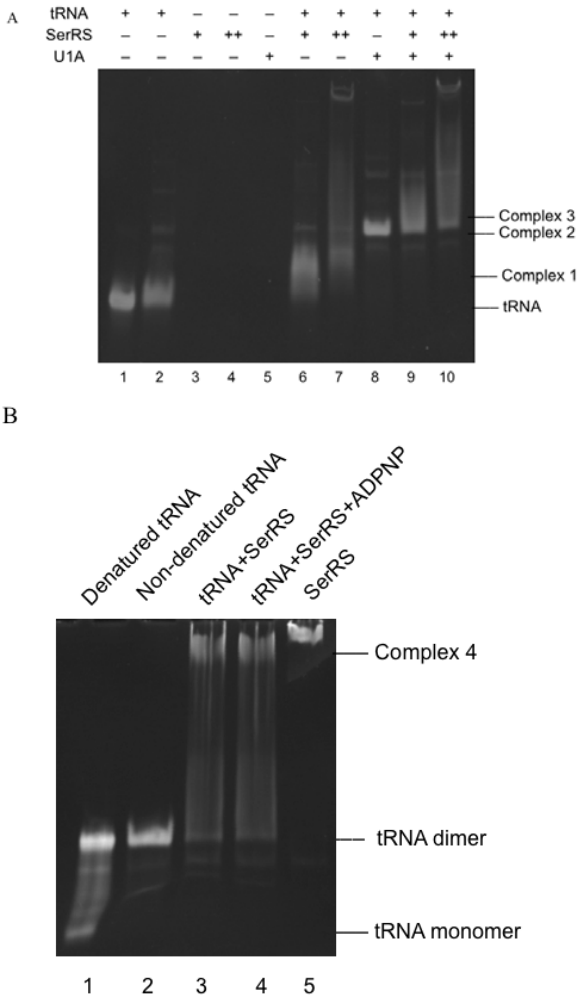


**Figure 4.** Gel shift assays of approach 1 (A) and approach 2 (B). (+) represents 50 pmol and (++) represents 250 pmol of input.

*Two different crystal forms*

After a couple weeks, we observed two crystal forms from each approach. For approach 1, crystals were found in the condition containing 0.2 M ammonium phosphate monobasic, 50% 2-methyl-2,4-pentanediol (MPD), and 0.1 M Tris pH 8.5 (Figure 5A). Cubic-shaped crystal forms of approach 2 were grown in the condition containing 50 mM Tris pH 8.0, 100 mM NaCl, 150 mM $LiSO_4$, 5% Glycerol and 18% PEG MME 5000 (Figure 5B). These two crystal forms will be subjected to X-ray diffraction experiment in the near future to intensively characterize and verify whether the crystals contains all expected components. These results illustrate that two "protein-sandwiching" approaches could be very useful for crystallization of flexible RNA.

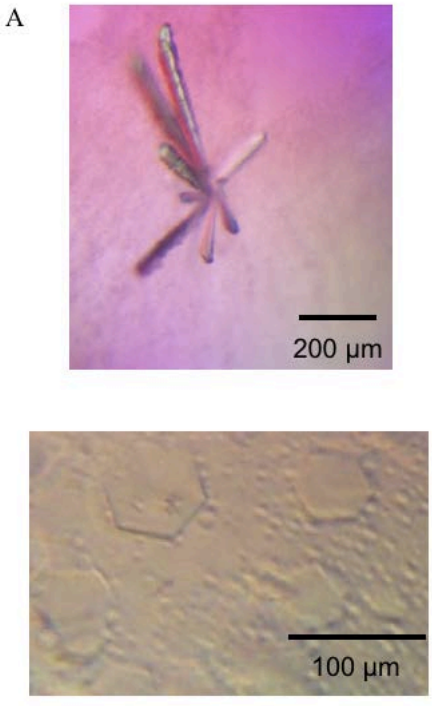


**Figure 5.** Two crystal forms from approach 1 (A) and approach 2 (B)

**Conclusion**

We show here the preliminary results of two new "protein-sandwiching" approaches for successful crystallization of flexible $tRNA^{Ser}_{GCU}$. Our results will lead to the 3D-structural determination of this bizarre tRNA to understand its molecular structure and function in mitochondrial translation system in the near future. Importantly, our strategy should be generally applied for X-ray crystallographic analysis of labile non-coding RNAs in eukaryotes.

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