Dual Mode Recognition of Two Isoacceptor tRNAs by Mammalian Mitochondrial Seryl-tRNA Synthetase*  

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Animal mitochondrial translation systems contain two serine tRNAs, corresponding to the codons AGY (Y = U and C) and UCN (N = U, C, A, and G), each possessing an unusual secondary structure; tRNA_{Ser}^{UGA} (for AGY) lacks the entire D arm, whereas tRNA_{Ser}^{UCG} (for UCN) has an unusual cloverleaf configuration. We previously demonstrated that a single bovine mitochondrial seryl-tRNA synthetase (mt SerRS) recognizes these topologically distinct isoacceptors having no common sequence or structure. Recombinant mt SerRS clearly footprinted at the TΨC loop of each isoacceptor, and kinetic studies revealed that mt SerRS specifically recognized the TΨC loop sequence in each isoacceptor. However, in the case of tRNA_{Ser}^{UGA}, TΨC loop-D loop interaction was further required for recognition, suggesting that mt SerRS recognizes the two substrates by distinct mechanisms. mt SerRS could slightly but significantly misacylate mitochondrial tRNA_{Ser} also has a long variable arm, and biochemical studies of Saccharomyces cerevisiae and human tRNA_{Ser} have revealed that it contains the major identity element of tRNA_{Ser} (11–14). The recognition mechanism of SerRS thus appears to be evolutionarily conserved in both prokaryote and eukaryotic cytoplasm.  

The fidelity of protein synthesis relies on the specific attachment of amino acids to their cognate tRNA species. This process is catalyzed by aminoacyl-tRNA synthetase (ARS), each species of which discriminates with high selectivity among the many structurally similar tRNAs and amino acids (1, 2). To avoid misacylation of tRNAs from any of the 19 non-cognate groups, tRNAs possess identity elements within their sequence or tertiary structure that are strictly recognized only by the cognate synthetase. These identity elements are most commonly located in the anticodon and in the acceptor stem, particularly the discriminator base at position 73 (2, 3). However, in the case of the serine tRNA of Escherichia coli, several biochemical experiments have revealed that neither the anticodon stem/loop nor the discriminator base is involved in recognition (4, 5); instead, the E. coli tRNA_{Ser} identity elements are located in the characteristic long extra arm (4–7). These findings conform well with analyses of the crystallographic structures of seryl-tRNA synthetase (SerRS)-tRNA_{Ser} complexes from E. coli and Thermus thermophilus (8–10), which indicate that the N-terminal long helical domain of SerRS plays an important role in recognizing the long extra arm and the TΨC loop of tRNA_{Ser}. In eukaryotic systems, cytoplasmic tRNA_{Ser} also has a long variable arm, and biochemical studies of Saccharomyces cerevisiae and human tRNA_{Ser} have revealed that it contains the major identity element of tRNA_{Ser} (11–14). The recognition mechanism of SerRS thus appears to be evolutionarily conserved in both prokaryote and eukaryotic cytoplasm.  

The mammalian mitochondrial (mt) translation system utilizes two tRNA_{Ser} species, one specific for codon AGY and the other for UCN. Neither of these tRNAs has a long extra arm as a recognition site for cytoplasmic SerRS (15). In addition, each possesses an unusual secondary structure; tRNA_{Ser}^{UCG} (for AGY) lacks the entire D arm (16), whereas tRNA_{Ser}^{UGA} (for codon UCN) has an unusual cloverleaf configuration with an extended anticodon stem (17). We previously demonstrated that the single mt SerRS recognizes these distinct isoacceptors with almost the same activity (18). Additionally, inspection of the primary sequences of several mt SerRSs revealed differences between mammalian mt SerRS and its prokaryotic counterpart in the N-terminal domain responsible for tRNA recognition, which are in line with structural and recognition differences between the extra arms of mammalian mt and prokaryotic tRNAs. Because no other tRNA investigated to date recognizes structurally different tRNA isoacceptors, it is supposed that the recognition mechanism of mammalian mt SerRS differs considerably from that of any other ARS.  

Elucidating the mystery of how mammalian mt SerRS recognizes and discriminates two isoacceptors with no common sequence and structure from non-cognate tRNAs will not only extend our knowledge of the recognition mechanism of ARS but also shed light on hidden aspects of the mammalian mt translation system.  

To investigate the recognition mechanism of mammalian mt SerRS, we recombinantly expressed bovine mt SerRS in E. coli and performed a series of biochemical experiments using this enzyme and several tRNA variants. On the basis of the results, we report here the unique recognition mechanism of mammalian mt SerRS.

EXPERIMENTAL PROCEDURES

Materials—Phenylmethanesulfonyl fluoride was purchased from Sigma; [1^14]Cl-serine (5.59 GBq/mmol), [32P]pCp, and a HiTrap chelating column were from Amersham Pharmacia Biotech; the vector pET-
19b was from Novagen; nucleotide-specific RNases T1 and U1 were from Amersham Pharmacia Biotech and Seikagaku Kogyo (Tokyo), respectively; vectors pUC18 and pUC19 were from Takara; an anion-exchange tip was from Qiagen; and a QuikChange site-directed mutagenesis kit was from Stratagene. Recombinant mt LeuRS was overproduced from an E. coli expression vector kindly provided by Dr. L. L. Spremulli (University of North Carolina, Chapel Hill, NC). Native bovine mt tRNAs were purified from bovine mitochondria by selective hybridization using a solid phase DNA probe as described by Wakahisa et al. (19).

Construction of Expression Plasmid—cDNA for bovine mt SerRS without an N-terminal peptide for mitochondrial importation was amplified by PCR using synthetic primers (underlined forward primer and reverse primer) annealing to the N-terminal end of mature mt SerRS (atacatctggggtcatcatcactcactcatgctcgagggagcgacggagggagagatt) and a reverse primer for the C-terminal end (caggcgacctcagggaggggctgg) carrying a BamHI site. The PCR product was cloned into pET-19b to construct an expression vector for mature bovine mt SerRS with a hexahistidine tag in the N-terminal region.

Expression and Purification of mt SerRS—E. coli BL21 (DE3) was used as a host for expression of the recombinant mt SerRS. The culture conditions for overproducing cells were optimized to maximize the expression of soluble enzyme. The transformant was cultured in LB broth (100 μg/ml ampicillin) at 37 °C to an A600 value of 0.6, and then induced by 10 μM isopropyl-β-D-thiogalactopyranoside for 20 h at 28 °C. Cells grown in 3 l of LB broth were resuspended in 60 ml of HT buffer (50 mM HEPES-KOH (pH 7.6), 100 mM KCl, 10 mM MgCl2, and 7 mM β-mercaptoethanol) containing 0.2 mM phenylmethylsulfonyl fluoride, 0.03% (v/v) egg white lysozyme, and 0.1% Triton X-100 and disrupted by 12-min sonication (repeated 1-s bursts after 4-s cooling periods) at 100 watts and 0 °C. The homogenate was cleared by centrifugation at 100,000 × g for 60 min. The supernatant fraction (S100) was loaded onto a nickel-charged HiTrap chelating column (5 ml). After washing out nonbound proteins, the recombinant protein was eluted with a 60-ml linear gradient from 0 to 350 mM imidazole in HT buffer. mt SerRS was eluted in a fraction containing ~200 mM imidazole. Protein concentrations were determined with a Bio-Rad protein assay kit using bovine serum albumin as a standard. Glycerol was added to pooled mt SerRS fractions at a final concentration of 30%, frozen quickly with liquid nitrogen, and stored at −70 °C.

Native PAGE and Gel Retardation Assay—The mt tRNA51-ser—mt SerRS complex was formed as described by Yokogawa et al. (18). Native PAGE was performed as described by Hornung et al. (20); the gel was stained with Coomassie Brilliant Blue and toluidine blue to analyze the footprint site of tRNAser in the presence of mt SerRS. The band position was assigned by comparison with partial NcoI site, and a reverse primer for the C-terminal end (caggcgacctcagggaggggctgg) carrying a BamHI site. The PCR product was cloned into pET-19b to construct an expression vector for mature bovine mt SerRS with a hexahistidine tag in the N-terminal region. Gel retardation assay was performed to analyze the footprint site of tRNAser in the presence of mt SerRS. The band position was assigned by comparison with partial NcoI site, and a reverse primer for the C-terminal end (caggcgacctcagggaggggctgg) carrying a BamHI site. The PCR product was cloned into pET-19b to construct an expression vector for mature bovine mt SerRS with a hexahistidine tag in the N-terminal region.
homodimeric form, as is the case with prokaryotic SerRS (9, 27, 28, 30).

To establish whether the recombinant mt SerRS could specifically recognize the two mt tRNAs, the seryl-lysine activity kinetic parameters were determined using the native mt tRNA\textsubscript{GCU} and mt tRNA\textsubscript{UAG} as substrates. As shown in Table I, the parameters of the recombinant mt SerRS were almost identical to those of the native mt SerRS. A gel retardation assay was performed to ascertain the complex formation of mt SerRS with each isoacceptor. The assay showed that mt SerRS specifically formed binary complexes with the two serine isoacceptors, whereas no complex was formed with non-cognate mt tRNA\textsubscript{UAA} (Fig. 2A). The dissociation constant (K\textsubscript{d}) for each tRNA substrate was determined by means of Scatchard plots (Fig. 2B). The larger K\textsubscript{d} value of tRNA\textsubscript{GCU} (1 μM) is indicative of a lower affinity toward mt SerRS. The difference in the dissociation constants seems to reflect the different K\textsubscript{m} values of the two substrates (Table I). In addition, it appears that the x-intercept of each Scatchard plot approaches 1.8, suggesting that the two tRNAs bind into one dimeric form of mt SerRS, although further structural analysis is necessary to clarify the stoichiometry of the mt SerRS-tRNA\textsubscript{Ser} complex.

Because the foregoing observations strongly suggested that the recombinant mt SerRS retained the original characteristics of the native enzyme, the subsequent experiments were performed with this recombinant enzyme.

mt SerRS Contact Sites on the Two Serine tRNAs—tRNA footprinting was carried out with ethylthiosourea for both mt tRNAs in the presence of mt SerRS, which strongly protected mt tRNA\textsubscript{GCU} against alkylation by ethylthiosourea at two specific regions, i.e., phosphate positions 57–58 and 64–67 (Fig. 3A). mt tRNA\textsubscript{UAG} was also protected at similar phosphate positions (55–59 and 65–67). No protection was observed in the presence of human mt leucyl-tRNA synthetase (mt LeuRS), which was used as a negative control for each mt tRNA (lanes 5 and 6 in Fig. 3A and B, respectively). Moreover, no strong protection was found in the 5′ region of mt tRNA\textsubscript{Ser} (data not shown). These results indicate that the probable contact sites on both tRNAs are on the T\textsuperscript{V}C loop and at the bottom of the acceptor stem. As shown in Fig. 3C, mammalian mt SerRS contacts both tRNAs at similar positions though they have different topologies.

The crystal structure of \textit{T. thermophilus} SerRS reveals that Arg\textsuperscript{195} contacts positions 66–67 in the acceptor helix of tRNA\textsubscript{Ser}, which is the same location as one of the mt SerRS contact sites. Because Arg\textsuperscript{195} is conserved in mammalian mt SerRS (18), it can be speculated that the contact site at phosphate positions 64–65 may not be required for specific recognition but would be involved in the essential interaction needed to arrange the CCA terminus at the catalytic center of the enzyme.

| Enzyme          | Substrate   | K\textsubscript{m} (μM) | k\textsubscript{cat} (s\textsuperscript{-1}) | k\textsubscript{cat}/K\textsubscript{m} (s\textsuperscript{-1}μM\textsuperscript{-1}) |
|-----------------|-------------|--------------------------|---------------------------------------------|-----------------------------------------------------------------|
| Wild-type       | tRNA\textsubscript{GCU} | 0.37 ± 0.10              | 0.35 ± 0.10                                  | 0.95                                                            |
|                 | tRNA\textsubscript{UAG} | 0.22 ± 0.03              | 0.63 ± 0.06                                  | 2.86                                                            |
| Recombinant     | tRNA\textsubscript{GCU} | 0.35 ± 0.08              | 0.64 ± 0.11                                  | 1.84                                                            |
|                 | tRNA\textsubscript{UAG} | 0.29 ± 0.08              | 0.67 ± 0.17                                  | 2.31                                                            |

**Table I**

Kinetic parameters in aminoacylation of bovine mitochondrial serine tRNAs.

Experimental conditions for aminoacylation are described under "Experimental Procedures."
with the canonical sequence was shown to have a $K_m$ value 4 times higher than that of the native tRNA from bovine liver. According to Hayashi et al. (32), replacement of the two terminal base pairs should have no effect on serylation activity, in which case the relatively high $K_m$ value is apparently attributable to the lack of modified bases.

We first examined the variants with mutations in the anticodon at positions 34–36 and at the discriminator base (position 73), which are recognition elements in most tRNAs. Replacement of the anticodon sequence or the discriminator base caused no significant reduction in serylation activity. As it was revealed that the A73G mutation could be recognized more efficiently by the enzyme, mt SerRS apparently prefers G73 as the discriminator base, which is in agreement with a previous observation that a G73A mutation in tRNA$^{\text{Ser}}_{\text{GCU}}$ caused an $-3$-fold reduction in activity (33). It can thus be postulated that tRNA$^{\text{Ser}}_{\text{UGA}}$ possesses the less efficient A73 as a discriminator base to balance the serine-accepting activities of the two isoacceptors in vivo (Table I).

Next, to evaluate the effects of tertiary interactions in mt tRNA$^{\text{Ser}}_{\text{UGA}}$ on serylation activity, point mutations were introduced at bases involved in four possible interactions (31): A15-U59, G18-U55, G19-C56, and U54-A58 (Fig. 4A). The A15-U59 interaction was shown not to be involved in the activity because the individual mutations A15U and U59A as well as the double mutation A15U59A all had relatively little effect in reducing the serylation activity (Table II). The variants G18U and U55G had $k_{\text{cat}}/K_m$ values that were respectively reduced to 15 and 38% that of the wild type, which probably resulted from destabilization of D loop-T WC loop interaction induced by the mutations. Severely reduced serylation activity was induced by the mutations G19C and C56G, the relative
**Recognition Manner of Mammalian Mitochondrial SerRS**

### TABLE II

| Substrate | $K_m$ (μM) | $k_{cat}$ (1/s) | $k_{cat}/K_m$ (1/μM × s) | Position of mutation |
|-----------|------------|-----------------|--------------------------|---------------------|
| Native tRNA$^\text{Ser}_{\text{UGA}}$ | 0.29 | 0.67 | 2.31 | 11 |
| Transcripts | | | | |
| tRNA$^\text{Ser}_{\text{UGA}}$ A15U | 1.2 | 0.25 | 0.21 | 1.0 |
| tRNA$^\text{Ser}_{\text{UGA}}$ U59A | 0.95 | 0.13 | 0.14 | 0.67 |
| tRNA$^\text{Ser}_{\text{UGA}}$ A15U,U59A | 1.1 | 0.13 | 0.12 | 0.57 |
| tRNA$^\text{Ser}_{\text{UGA}}$ G18U | 1.3 | 0.16 | 0.12 | 0.57 |
| tRNA$^\text{Ser}_{\text{UGA}}$ U55G | 0.74 | 0.024 | 0.032 | 0.15 |
| tRNA$^\text{Ser}_{\text{UGA}}$ G18U,U55G | 1.3 | 0.11 | 0.080 | 0.38 |
| tRNA$^\text{Ser}_{\text{UGA}}$ G19C | 5.9 | 0.022 | 0.0037 | 0.018 |
| tRNA$^\text{Ser}_{\text{UGA}}$ C56G | 6.0 | 0.017 | 0.0028 | 0.013 |
| tRNA$^\text{Ser}_{\text{UGA}}$ G19C,C56G | 0.79 | 0.15 | 0.19 | 0.90 |
| tRNA$^\text{Ser}_{\text{UGA}}$ U54A | 4.2 | 0.0099 | 0.0024 | 0.011 |
| tRNA$^\text{Ser}_{\text{UGA}}$ A58U | 3.0 | 0.044 | 0.015 | 0.071 |
| tRNA$^\text{Ser}_{\text{UGA}}$ U54A,A58U | 4.9 | 0.059 | 0.012 | 0.057 |
| tRNA$^\text{Ser}_{\text{UGA}}$ C56U,U59C | 1.1 | 0.0012 | 0.0011 | 0.0052 |
| tRNA$^\text{Ser}_{\text{UGA}}$ C56A | 2.5 | 0.0085 | 0.0034 | 0.016 |
| tRNA$^\text{Ser}_{\text{UGA}}$ U34G,G35U,A36C | 1.0 | 0.22 | 0.22 | 1.0 |
| tRNA$^\text{Ser}_{\text{UGA}}$ A73G | 0.30 | 0.59 | 2.0 | 9.5 |
| tRNA$^\text{Ser}_{\text{UGA}}$ G46.1 insertion | 0.23 | 0.047 | 0.20 | 0.95 |
| tRNA$^\text{Ser}_{\text{UGA}}$ (cloverleaf type) | 2.9 | 0.21 | 0.073 | 0.35 |
| tRNA$^\text{Ser}_{\text{UGA}}$ (D arm → tRNA$^\text{Ser}_{\text{UGA}}$) | 2.1 | 0.0017 | 0.00082 | 0.0039 |
| tRNA$^\text{Ser}_{\text{UGA}}$ (TΨC loop → tRNA$^\text{Ser}_{\text{UGA}}$) | 0.84 | 0.52 | 0.63 | 3.0 |
| tRNA$^\text{Ser}_{\text{UGA}}$ (D arm, TΨC loop → tRNA$^\text{Ser}_{\text{UGA}}$) | 0.84 | 0.25 | 0.30 | 1.4 |
| tRNA$^\text{Ser}_{\text{UGA}}$ (minihelix) | ND* | ND | ND | ND |

* ND, no activity was detected.

### TABLE III

| Substrate | $K_m$ (μM) | $k_{cat}$ (1/s) | $k_{cat}/K_m$ (1/μM × s) | Relative |
|-----------|------------|-----------------|--------------------------|---------|
| Native tRNA$^\text{Ser}_{\text{UGA}}$ | 0.35 | 0.64 | 1.8 | 6.7 |
| tRNA$^\text{Ser}_{\text{UGA}}$ derivatives | | | | |
| tRNA$^\text{Ser}_{\text{UGA}}$ A(A43) | 2.0 | 0.53 | 0.32 | 0.27 |
| tRNA$^\text{Ser}_{\text{UGA}}$ U8A,A9U | 1.6 | 0.33 | 0.21 | 0.78 |
| tRNA$^\text{Ser}_{\text{UGA}}$ U59A,A60,U | 3.1 | 0.29 | 0.094 | 0.35 |
| tRNA$^\text{Ser}_{\text{UGA}}$ U59A,A60,U | 4.4 | 0.094 | 0.021 | 0.078 |
| tRNA$^\text{Ser}_{\text{UGA}}$ U59A,A60,U | 3.3 | 0.54 | 0.16 | 0.59 |
| tRNA$^\text{Ser}_{\text{UGA}}$ G18U | 3.2 | 0.19 | 0.061 | 0.23 |
| tRNA$^\text{Ser}_{\text{UGA}}$ A58U | 2.3 | 0.011 | 0.0047 | 0.017 |
| tRNA$^\text{Ser}_{\text{UGA}}$ U54A,A58U | 2.0 | 0.0032 | 0.0016 | 0.0059 |
| tRNA$^\text{Ser}_{\text{UGA}}$ A57U | 3.4 | 0.070 | 0.021 | 0.078 |
| tRNA$^\text{Ser}_{\text{UGA}}$ (minihelix) | 7.1 | 0.028 | 0.0039 | 0.014 |

$k_{cat}/K_m$ values being 2 orders of magnitude lower (Table II). However, the variant carrying both mutations (G19C and C56G) completely recovered its activity, suggesting that the Watson-Crick base pair G19-C56 supporting D loop-TΨC loop interaction is indispensable for recognition.

U54-A58 interaction in the TΨC loop was also revealed to be a strong recognition element for seryltransferase. The U54A and A58U mutants exhibited significantly reduced setylation activity. Unlike the case of the G19-C56 tertiary base pair, no restoration in the activity was observed in the double mutant U54A,A58U, indicating that mt SerRS probably recognizes the U54-A58 interaction not only structurally but also sequence-specifically in the TΨC loop.

When compared with the canonical cloverleaf structure, mammalian mt tRNA$^\text{Ser}_{\text{UGA}}$ lacks six conserved residues, at positions 8, 16, 17, 21, 47, and 48, but contains one extra base pair (Ψ26a-A43a) in the anticodon stem so as to form a characteristic pseudo-cloverleaf structure (31). On the other hand, chicken mt tRNA$^\text{Ser}_{\text{UGA}}$ possesses the canonical cloverleaf structure (34). Further, it has already been demonstrated that the chicken tRNA$^\text{Ser}_{\text{UGA}}$ can be seryltransferated by the native bovine mt SerRS. To investigate the effect of the unusual cloverleaf structure of bovine mt tRNA$^\text{Ser}_{\text{UGA}}$ on its serylation activity, we designed a variant mt tRNA$^\text{Ser}_{\text{UGA}}$ possessing a canonical cloverleaf structure based on the chicken mt tRNA$^\text{Ser}_{\text{UGA}}$ sequence. All the residues of the chicken mt tRNA$^\text{Ser}_{\text{UGA}}$ apart from the D loop sequence were substituted for those of bovine mt tRNA$^\text{Ser}_{\text{UGA}}$. As this variant showed no significant change in serylation activity, it seems that the unusual cloverleaf structure of mammalian mt tRNA$^\text{Ser}_{\text{UGA}}$ does not function as a key element for discrimination by mammalian mt SerRS.

Tiranti et al. reported the presence of a heteroplastic insertion at nucleotide position 7472 in human mt DNA (35). The insertion consequently adds one guanine at position 46.1 in the extra arm of human mt tRNA$^\text{Ser}_{\text{UGA}}$, and the authors suggest that

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* N. Nishioka, T. Yokogawa, and K. Watanabe, unpublished data.
this mutation etiologically induces deafness by altering the structure of the T\(\text{V}\)C loop in mt tRN\(\text{A}_{\text{UGA}}\) (35). In light of this, we constructed a variant based on bovine mt tRN\(\text{A}_{\text{UGA}}\) containing the same mutation (Fig. 4A; G46.1 insertion) and examined its serylation activity. The mutation had little effect on the \(k_{\text{cat}}/K_m\) value, indicating that the deafness induced by the C7472 insertion is not caused by a defect in the serine-accepting activity.

From the foregoing, it is concluded that mammalian mt SerRS recognizes the mt tRN\(\text{A}_{\text{UGA}}\) T\(\text{V}\)C loop sequence-specifically and requires the D loop-T\(\text{V}\)C loop interaction sustained by the G19-C56 tertiary base pair for efficient aminoacylation, whereas the characteristic pseudo-cloverleaf structure per se is hardly involved in the serylation reaction.

tRN\(\text{A}_{\text{GCU}}\) Identity Elements for mt SerRS—Ueda et al. (33) previously examined the recognition sites of mt tRN\(\text{A}_{\text{Ser}}\) using partially purified enzyme, but the activity was too weak for the kinetic parameters of several tRNA variants with low serine-accepting activity to be determined. Therefore, we investigated the identity elements for mt tRN\(\text{A}_{\text{GCU}}\) using the recombinant enzyme with full activity. tRNA variants for mt tRN\(\text{A}_{\text{GCU}}\) were prepared by the in \textit{vivo} expression system developed by Hayashi et al. (25), which allows variants to be easily purified in large quantities. Five A-U base pairs in the acceptor stem were replaced with G-C pairs (Fig. 4B) for efficient expression as described (25). tRN\(\text{A}_{\text{Ser}}\) expressed with the canonical sequence had a \(K_m\) value 6 times higher than that of native tRNA (Table III). However, because Ueda et al. (33) previously revealed that the identity elements for mt tRN\(\text{A}_{\text{GCU}}\) are not located in the acceptor stem, we prepared a series of tRN\(\text{A}_{\text{GCU}}\) variants using the same expression system. The variations are shown in Fig. 4B, and the kinetic parameters are summarized in Table III. We first examined the serylation activity of the tRNA variant lacking a bulge (A45) in the anticodon stem, which is a conserved structure among mammalian mt tRN\(\text{A}_{\text{Ser}}\) and is considered to be involved in tertiary interaction with the T\(\text{V}\)C loop (16). This deletion hardly reduced the serylation activity. de Bruijn and Klug (36) proposed a tertiary structural model of human and bovine mt tRN\(\text{A}_{\text{GCU}}\) with four possible tertiary interactions, which are indicated by \textit{broken lines} in Fig. 4B. Point mutations were introduced at the bases involved to evaluate the effects of these postulated tertiary interactions on serylation activity. The interactions U8-A60\(\text{A}\), and A9-U59 were revealed to have no relation to the activity because the double mutation U8A,A9U did not substantially reduce serylation (Table III). On the other hand, the double mutation U59A,A60\(\text{U}\) severely reduced the activity, indicating that mt SerRS recognizes U59 and A60\(\text{A}\) base-specifically. Similarly, the U10-A57 tertiary interaction was not involved in serylation because the U10A variant showed only a slight decrease in activity. To examine the U54A,A58 interaction in the T\(\text{V}\)C loop, point mutations were introduced at positions 54 and 58. The U54A mutation reduced the \(k_{\text{cat}}/K_m\) value by four-fifths, but this was relatively little compared with the drastic loss of activity by 2 orders of magnitude in the A58U variant (Table III). Because no activity was restored by introducing the double mutation U54A,A58U, which actually resulted in an even more severe decrease in the \(k_{\text{cat}}/K_m\) value, it can be assumed that the mt SerRS recognizes A58 base-specifically. With regard to the individual effects of the two point mutations on serylation activity, the theoretical activity derived by multiplying the \(k_{\text{cat}}/K_m\) values of the respective mutations should be in good agreement with the observed value. Because the theoretical value for the double mutation (0.0039) did in fact correspond well with the experimental value (0.0059), U54A and A58U were shown to be mutations that individually reduced the serylation activity. Finally, a point mutation was introduced at position 57, which is one of the strong footprint sites; this A57U mutation markedly reduced the activity.

From the foregoing, it can be concluded that mammalian mt SerRS recognizes the T\(\text{V}\)C loop sequence of mt tRN\(\text{A}_{\text{UGA}}\) but, unlike the case of mt tRN\(\text{A}_{\text{UGA}}\) recognition, it does not recognize its tertiary structure. The major determinants, A57 and A58, were shown to be the sites footprinted by the enzyme (Fig. 3A).

**Dual Mode Recognition of the Two Isoacceptors by mt SerRS—** Our findings on the identity elements of the two structurally dissimilar isoaccepting tRNAs raised the possibility that mammalian mt SerRS recognizes each tRNA by two distinct mechanisms. To examine this notion, we constructed mt tRN\(\text{A}_{\text{UGA}}\) variants in which the D arm and/or T\(\text{V}\)C loop were substituted by the D arm and T\(\text{V}\)C loop configurations of mt tRN\(\text{A}_{\text{Ser}}\) (Fig. 4A). As shown in Table II, the variant with the D arm substitution (i.e. lacking a D arm, as is the case in mt tRN\(\text{A}_{\text{UGA}}\)) had a seriously decreased \(k_{\text{cat}}/K_m\) value, whereas the activity was increased 3-fold by substituting the T\(\text{V}\)C loop. The third variant, carrying both the D arm and T\(\text{V}\)C loop substitutions, showed similar serylation activity to that of the canonical transcript. These findings clearly indicated that the D loop-T\(\text{V}\)C loop tertiary interactions are required for the serylation of mt tRN\(\text{A}_{\text{UGA}}\), whereas only the sequence of the T\(\text{V}\)C loop is important for the activity of mt tRN\(\text{A}_{\text{Ser}}\).

Aminoacylation of \textit{E. coli} tRN\(\text{A}_{\text{Ala}}\) by alanyl-tRNA synthetase (AlaRS) depends on a G3-U70 wobble base pair in the acceptor stem (37). Understanding such a unique identity determinant was greatly advanced by the demonstration that the minibehelix variant, composed only of the amino acid acceptor-T\(\text{V}\)C helix, could be a good substrate for AlaRS (38, 39). The...
fore, to confirm the dual mode recognition of the two tRNA\textsuperscript{Ser} species, we similarly prepared two minihelix tRNAs\textsuperscript{Ser} based on the two isoacceptor tRNAs by connecting the acceptor stem and the T\textsuperscript{ΨC} stem (Fig. 5A). As expected, the tRNA\textsuperscript{Ser,Glu} minihelix exhibited significant serylation activity (Fig. 5B, and Table III), whereas the tRNA\textsuperscript{Ser,UGA} minihelix showed none (Fig. 5B, and Table II).

The above results clearly demonstrated that mammalian mt SerRS recognizes the T\textsuperscript{ΨC} loop of tRNA\textsuperscript{Glu} well in a sequence-specific manner. At the same time, the necessity of dissimilar tertiary interactions for the recognition of the two mt tRNAs\textsuperscript{Ser} by mt SerRS was underscored by this experiment. Thus, there are both common and dissimilar features in the recognition mechanisms of the single enzyme for the two substrates.

**Misacylation of mt tRNA\textsuperscript{Gln} by mt SerRS**—Because the T\textsuperscript{ΨC} loop sequence of each serine tRNA was shown to be important for serylation, we searched mt tRNA sequences to discover whether a T\textsuperscript{ΨC} loop sequence similar to that of either of the two serine tRNAs exists among the 20 non-cognate mt tRNAs. Although no other bovine mt tRNA has a sequence resembling the T\textsuperscript{ΨC} loop of mt tRNA\textsuperscript{Glu} (40), three bovine mt tRNAs (for Gln, Glu, and Tyr) have T\textsuperscript{ΨC} loop sequences similar to or identical to that of mt tRNA\textsuperscript{UGA}. Therefore, using native mt tRNAs from bovine liver, we examined the mt SerRS misacylation activity for each of these tRNAs. The results are shown in Fig. 6 and Table IV. No activity was observed for the two tRNAs with T\textsuperscript{ΨC} loop sequences similar to that of mt tRNA\textsuperscript{UGA} (mt tRNA\textsuperscript{Glu} and mt tRNA\textsuperscript{Tyr}).

To check the negative effects of these small changes in the T\textsuperscript{ΨC} loop sequence on serylation activity, we constructed two tRNA\textsuperscript{UGA} variants, C56A and C56U/U59C, with T\textsuperscript{ΨC} loop sequences respectively corresponding to those of mt tRNA\textsuperscript{Glu} and mt tRNA\textsuperscript{Tyr} (Fig. 4A). These mutants showed 60-fold or more reductions in their $k_{cat}/K_m$ values (Table II), indicating that the variant T\textsuperscript{ΨC} loop nucleotides in the mt tRNAs for Glu and Tyr function as strong negative determinants in preventing misacylation by mt SerRS, thereby maintaining the fidelity of translation, i.e. mt SerRS seems to reject these non-cognate mt tRNAs on the basis of their T\textsuperscript{ΨC} loop sequences. mt tRNA\textsuperscript{Gln}, whose T\textsuperscript{ΨC} loop sequence is identical to that of mt tRNA\textsuperscript{UGA}, was found to be misacylated slightly but significantly by mt SerRS (Fig. 6), although its $k_{cat}/K_m$ value was 4 orders of magnitude smaller compared with that of mt tRNA\textsuperscript{UGA} (Table IV). This finding is a novel instance that appears to exist in the aminoacylation network to maintain a high level of translational fidelity. Further experiments were carried out to elucidate this notion.

**Discrimination of mt tRNA\textsuperscript{Ser,UGA} from Non-cognate mt tRNA\textsuperscript{Gln} by mt SerRS**—As noted above, although mt tRNA\textsuperscript{Gln} can be misacylated by mt SerRS, the activity is kept at a low level even though mt tRNA\textsuperscript{Gln} possesses the same T\textsuperscript{ΨC} loop sequence as mt tRNA\textsuperscript{UGA}. To exclude the possible influence of modified nucleotides, unmodified mt tRNA\textsuperscript{Gln} was prepared by

![Fig. 6. Misacylation of non-cognate bovine mt tRNA\textsuperscript{Gln} by mt SerRS. The serine-accepting activities of mt tRNA\textsuperscript{UGA} (★), mt tRNA\textsuperscript{Gln} (●), mt tRNA\textsuperscript{Glu} (×), and mt tRNA\textsuperscript{Tyr} (□) were determined under the same experimental conditions as those used for the plots in Fig. 5B. The kinetic parameters are shown in Table IV.](http://www.jbc.org/doi/fig/6)
The footprinting experiment with the mt SerRS-mt tRNA
Ser complex revealed that the enzyme contacts both tRNAs at nearly the same position; the TΨC loop and the bottom of the acceptor stem (Fig. 3). The series of mutation studies clearly demonstrated that one of the contact sites, the TΨC loop sequence, is important for recognition in both cases. Herein can be found commonality in the recognition of the two dissimilar tRNAs by mt SerRS. However, we next noted that mt SerRS recognizes the TΨC loop in each tRNA by distinctive mechanisms, because TΨC loop-D loop tertiary interaction is required for the recognition of only mt tRNA
Ser. The different modes of recognition for the two substrates was accentuated by the fact that the minihelix variant of mt tRNA
Ser was a good substrate for mt SerRS, whereas that of mt tRNA
Ser was not (Fig. 5).

Crystallographic studies have revealed that E. coli SerRS has a long helical arm consisting of an antiparallel, two-stranded coiled coil in the N-terminal domain (27, 28). In the crystal structure of T. thermophilus SerRS complexed with tRNA
Ser, the N-terminal helical arm is tightly buried in the bulge between the TΨC arm and the long extra arm of tRNA
Ser with several backbone contacts, whereas the C-terminal region of another subunit is responsible for the catalytic activity (9). However, during the process of mitochondrial evolution in the eukaryotic cell, prokaryotic tRNA
Ser would have lost their characteristic extra arms, whereas the D arm was lost only in the case of mt tRNA
Ser. Accordingly, the N-terminal region of mt SerRS might have been obliged to recognize the TΨC loop of mt tRNA
Ser instead of the missing extra arm, because the catalytic core in the C-terminal region is well conserved in mt SerRS (18). Despite low homology in the N-terminal domain between mt SerRS and prokaryotic enzymes, a single, long helical arm was clearly predicted in the N-terminal region of bovine mt SerRS both by the CD spectrum of the recombinant enzyme and computational analysis of the helical structure using the COILS algorithm. It can be speculated that the predicted N-terminal helical arm of mt SerRS may also be responsible for mt tRNA recognition through interaction with the TΨC loop. However, an investigation of the tertiary structure of the mt SerRS-mt tRNA
Ser complex is required to elucidate how the N-terminal arm recognizes the TΨC loop of each tRNA. The molecular basis of the precise mechanism of the dual mode recognition will be clarified by such a structural study, which is now in progress in our laboratory.

The error rate of translation has been estimated to be 10⁻⁴ to 10⁻⁵ (43), which is of the same order as misacylation (44), indicating that the fidelity of translation was maintained by the accuracy of aminoacylation. In Candida species, the codon CUG is known to be translated as serine instead of leucine by the tRNA
Ser responsible for this non-universal decoding (45, 46). We previously reported that this tRNA
Ser can be aminoacylated not only with serine, but also with leucine to some extent in vivo as well as in vitro (47). This was the first report, and a unique instance, of a single tRNA in a natural organism being acceptable to more than one species of amino acid. In the present study, we unexpectedly found that mt tRNA
Glu can be slightly but significantly misacylated by mt SerRS (Table IV and Fig. 6), because mt tRNA
Glu has a TΨC loop sequence identical to that of mt tRNA
Ser. Although ambiguous specificity of mt aminoaeryl-tRNA synthetase has been reported in unilateral aminoacylation between heterologous tRNAs (48), it was a surprise that mt SerRS cannot discriminate a cognate tRNA strictly even inside the mitochondrion. The fact that the recognition of mt tRNA
Glu by mt SerRS in vitro is 3700 times lower than that of mt tRNA
Ser suggests that kinetic discrimination arising from competition between mt SerRS and mt GlnRS occurs in the mitochondrion to maintain the fidelity of
mt translation. It can be speculated that a reduction in the number of tRNA species might impair the discriminatory ability of mt ARS. Our finding raises the possibility that other mammalian mt aminoacyl-tRNA synthetases may also misacylate non-cognate mt tRNAs, with the fidelity of mt translation being maintained by the kinetic discrimination of mt tRNAs in the network of ARSs. Further studies will certainly clarify this notion and, as a result, deepen our understanding of the mammalian mitochondrial translation system.

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Dual Mode Recognition of Two Isoacceptor tRNAs by Mammalian Mitochondrial Seryl-tRNA Synthetase
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