Core flexibility of a truncated metazoan mitochondrial tRNA

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ABSTRACT

Secondary and tertiary structures of tRNAs are remarkably preserved from bacteria to humans, the notable exception being the mitochondrial (m) tRNAs of metazoans, which often deviate substantially from the canonical cloverleaf (secondary) or ‘L’-shaped (tertiary) structure. Many metazoan mtRNAs lack either the TψC (T) or dihydrouridine (D) loops of the canonical cloverleaf, which are known to confer structural rigidity to the folded structure. Thus, the absence of canonical TψC–D interactions likely results in greater dispersion of anticodon-acceptor interstem angle than for canonical tRNAs. To test this hypothesis, we have assessed the dispersion of the anticodon-acceptor angle for bovine mtRNASer(AGY), which lacks the canonical D arm and is thus incapable of forming stabilizing interarm interactions. Using the method of transient electric birefringence (TEB), and by changing the helical torsion angle between a core mtRNA bend and a second bend of known angle/rigidity, we have demonstrated that the noncanonical bovine mtRNA Ser(AGY) (Figure 1) possesses substantially more flexibility than its canonical tRNA counterparts. These results suggest that increased flexibility, in addition to a more open interstem angle, would allow both noncanonical and canonical mtRNAs to utilize the same protein synthetic apparatus.

INTRODUCTION

The cloverleaf secondary structure and concomitant ‘L’ tertiary structure of tRNAs is remarkably conserved from bacteria to humans, but is not universal. The exceptions are found in the mitochondria of metazoans. Some of these tRNAs exhibit minor additions or alterations while others lack an entire arm of the cloverleaf. Measurements in our laboratory have demonstrated that these noncanonical variations in secondary structure lead to substantial variations in tertiary structure (1–3). In particular, the variant mitochondrial tRNAs (mttRNAs) exhibit apparent angles between their anticodon and acceptor stems (~120–140°) that are substantially larger than the ~80–90° exhibited by canonical yeast tRNA^Phe (1–5).

What remains unknown is the influence that these structural variations have on the overall flexibility of the mtRNA core. Previous measurements of the flexibility of the canonical yeast tRNA^Phe revealed that in the presence of millimolar magnesium concentrations, the tRNA core, as reflected in the anticodon-acceptor interstem angle, remains quite rigid (6).

To address the issue of core flexibility for a variant tRNA, we have used the method of transient electric birefringence (TEB), in combination with a series of phased tRNA–A5 bulge constructs (5), to demonstrate that the noncanonical bovine mtRNA^Ser(AGY) (Figure 1) possesses substantially more flexibility than its canonical tRNA counterparts. The current work should lead to a better understanding of the interplay between tertiary RNA structure and dynamic flexibility, and suggests that tRNA flexibility, in addition to flexibility within the ribosome, may play a role in the transit of noncanonical tRNAs during the translation cycle. Moreover, the presence of flexibility may broaden the scope of the study of the pathogenic mechanisms of point mutations within these noncanonical tRNAs (7).

MATERIALS AND METHODS

Preparation of extended tRNA–bulge heteroduplex molecules

Plasmid construction is largely as described earlier (1,4), with modifications for measuring flexibility as described in Friederich et al. (6). Duplex deoxyoligonucleotides corresponding to the dihydrouridine (dhU or D) arm (5’) and ribothymidine–pseudouridine–cytidine (TψC or T) arm (3’) halves of the tRNA molecule (Table 1) were synthesized and cloned into plasmids pGJ122A and pGJ122B, which code for the 70 bp extensions with a central ‘Hind III’ insertion site (4). Deoxyoligonucleotides containing the A5 bulge, and varying numbers of base pairs for the

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probing with dimethylsulphate (DMS), which modifies exposed adenosine residues. Specifically, 4 μg of gel purified rRNA–A₅ bulge construct was incubated in 200 μl of DS buffer (50 mM NaP, pH 7.2, 1 mM Na₂EDTA, 3 mM MgCl₂, 10% glycerol) with 1.2 μl DMS, for 1.5 h at 4°C. After ethanol precipitation, the DMS reaction products were resuspended in DS buffer and further desalted by passage over 1 ml G-25 spin columns equilibrated in the same buffer without glycerol. The reactions were then phenol extracted, reprecipitated and finally resuspended in H₂O. The probing reactions were then annealed to 5'-fluorescein label primers (fluorescein phosphoramidite, Glen Research, Sterling VA), which were designed to anneal to the extensions specifically on the D or T half as described in Frazer-Abel and Hagerman (1). In all other respects, reactions and primer extension analyses are as described previously (1,4,6).

**Measurement of relative electrophoretic mobilities**

Analytical acrylamide gels (8% acrylamide; 29:1 monomer/bis-acrylamide) were used to examine the relative mobility of the RNA heteroduplex constructs. The gel/running buffer contained 10 mM NaP, pH 7.2, with either 1 mM Na₂EDTA or 1 mM Na₂EDTA plus MgCl₂ to yield the effective MgCl₂ concentration designated in the text (5). The running buffer was recirculated during electrophoresis, and gels were run at room temperature overnight at 30 mA in 17 cm vertical gels.

**TEB methodology and measurements**

**Principle of the TEB method.** The method measures the rotational decay times for a linear or heteroduplex DNA or RNA molecule by monitoring the loss of solution birefringence (optical anisotropy) following removal of an orienting electric field pulse. The method exploits the fact that rotational reorientation is extremely sensitive to changes in length of extended molecules in solution (9–11). In a typical experiment, a transient (~1 μs) electric pulse is applied to the sample cell, which aligns the nucleic acid construct. Once the electric pulse is removed, the solution becomes isotropic as the previously oriented molecules randomize through Brownian motion. For heteroduplex species, the sensitivity of the TEB method can be dramatically increased by extending the helices that flank the central nonhelical structure (Figure 2 of ref. 9). The rate of birefringence decay for a given heteroduplex construct is compared to the decay of a fully duplex species of equivalent axial length. The ratio of the decay times is the outcome of the birefringence experiment, and is related to the apparent angle of the central bend. If the bend in the experimental construct possesses additional flexibility (i.e. more flexibility than an equivalent length of pure helix), this will also lead to a reduction in decay time. To remove this ambiguity, a second nonhelical element is added to the construct; in the current instance, A₅ bulge whose angle has been reported elsewhere (12).

The specific methods employed for the measurement and analysis of the birefringence decay curves have been described (4,11,13,14). For the current measurements, the pulse width was 1.0 μs; pulse repetition frequency, 1 Hz;

**Figure 1.** Secondary and tertiary structure of one canonical and one non-canonical tRNA. Ribbons represent phosphate backbone of: yeast cytoplasmic (c) tRNA Phe from Holbrook et al. (16), bovine mtRNA Ser(AGY) from Steinberg et al. (24), and S. Steinberg (personal communication).
orienting field strength, 10 kV/cm; the instrument response time, \( \leq 20 \text{ ns} \) (buffer birefringence decay). Measurements of the single-bend construct were performed at 3.8 \( /C_6 \) 0.1, 20.8 \( /C_6 \) 0.1 and 37.4 \( /C_6 \) 8 \( ^}\text{C} \) as indicated. For the two-bend constructs, measurements were performed at 3.5 \( /C_6 \) 0.1 \( ^}\text{8} \^\text{C} \), in 5 mM NaPi pH 7.2 with 1 mM Na2EDTA or with 1 mM Na 2EDTA and with the necessary MgCl 2 to yield the indicated effective free concentrations of MgCl 2. Each birefringence decay curve represents the average of 128 transients, which were digitized and averaged on a LeCroy 9310 oscilloscope. Sets of five decay curves were obtained for each construct for each experimental run, and a minimum of two experiments were run for each condition on two independent RNA preparations for a total of \( \geq 10 \) decay curves per sample. Decay curves were analyzed as double-exponential

| n | dhU half | TwC half | Plasmid designation |
|---|----------|----------|---------------------|
| 0 | CTAGCAAAAAAGCG | CTAGCGCG | pGJ122FB0 |
| 2 | GCTGTTTTTTCCGATC | GCGCGATC | pGJ122FT0 |
| 4 | CTAGCGACAAAAAGCG | CTAGCGACGGCG | pGJ122FB2 |
| 6 | GCTGCGTTTCCCGATC | GCGCGATC | pGJ122FT2 |
| 8 | CTAGCGCTGCGAAAAAGCG | CTAGCGCTGCGCG | pGJ122FB4 |
| 10 | GCTGCGACTGGGTTTTTGCGATC | GCGCGATC | pGJ122FT4 |
| 12 | CTAGCGACCAGCAGAGACCCGCG | CTAGCGACCAGACCCGCG | pGJ122FB6 |
| 14 | GCTGCGACTGGGTTTTTGCGATC | GCGCGATC | pGJ122FT6 |
| 16 | CTAGCGACCAGCAGAGACCCGCG | CTAGCGACCAGACCCGCG | pGJ122FB8 |

Table 1. Oligonucleotides used in the production of the single-bend and the tRNA–A 5 bulge constructs

*aOligonucleotides used to create the wild-type bovine mtRNA(AGY) used in temperature studies (1).
*bParent oligonucleotides used to create the parent species that was annealed and inserted into Hind III site of pGJ122A or pGJ122B.
*c, the number of additional (phasing) bases. The additional bases are underlined.
dOligonucleotides used to create each species by annealing and insertion into the NheI site of corresponding parent plasmids.
eP GJ122 corresponds to the original plasmid, B, bulge (dhU) side; T, TwC side, numbers correspond to the number of phasing bases added.
functions using the Levenberg–Marquardt method. For additional analysis of error, refer Hagerman and Hagerman (13).

Interpolation of decay times for linear duplex RNA constructs
For the current studies, five linear constructs (dplx) were utilized to accompany the 10 heteroduplex constructs (htx) containing the tRNA core and the A₅ bulge. To allow the proper determination of the \( t_{htx}/t_{dplx} \) ratios, it was necessary to determine \( t_{dplx} \) values for linear controls of the same contour length for each construct. The estimation of the effective contour length of the tRNA core for bovine mtRNA\(^{Ser}(AGY)\) has been described in detail (1) and determined to be 163 bp. To determine the \( t_{dplx} \) value for linear species of 163 bp, the \( t_{dplx} \) values for the four linear species actually measured were interpolated (Figure 2A and B). Figure 2A demonstrates the fit for each of the conditions presented in Tables 3 and 4. In Figure 2B, the data points for all temperatures were corrected to 20°C and replotted using the following conversion formula:

\[
\tau_{20,w} = \tau_{\text{meas}} \left( \frac{\eta_0}{\eta_T} \right) \left( \frac{T + 273}{293} \right) (^\circ C)
\]

The tight clustering of the data points indicates that the different temperatures were not causing structural alterations in the duplex construct. Decay times for these equivalent duplexes are obtained by interpolation using a set of four duplex species (Tables 3 and 4).

For the two-bend tRNA–A₅ bulge constructs, seven linear species were measured and the \( t_{htx} \) values for intermediate lengths were determined by interpolation (Figure 2C). The results of this interpolation indicate that there were no unexplained or unexpected factors affecting the TEB measurements, and support the use of interpolation to achieve \( \tau \)-values of intermediate linear length. Additionally, the use of five data points to obtain a single \( \tau \)-value reduced the influence of sequence variation that might have influenced a single data point.

Hydrodynamic modeling
All hydrodynamic modeling presented is as described (6). The initial hydrodynamic computations utilized the angle measurements shown in Table 5. The first bend, the tRNA core, was modelled as a point bend at base 83 and the second bend at base pair 100 + \( n \). The other helical parameters used are as follows: twist 12 bp per turn, rise 2.8 Å per bp, hydrodynamic radius 13 Å, helix persistence length (P) 700 Å and helix torsional elastic constant (C)

| RNA species plasmid designations | Oligomer sequences\(^b\) |
|---------------------------------|---------------------------|
| 158 linear pDL158               | 5’-AGCTGCGGAAAAAGTATGCAAG |
| pTL158                          | CGCGTTTATCATGCTTCGA-5’     |
| 170 linear pDL170               | 5’-AGCTAGCGGCGTATGCTTCATACCTTTTCGCC |
| pTL170                          | TCAGGCGATGAGAAGCTGTAGTACTGCTTCGA-5’ |
| 174 linear\(^b\) pGJ122A38+     | 5’-AGCTGCGGCGGATGCTTCATGCTTCGACATGACGTAGAGCT |
| pGJ122B38-                      | CGCGCTATATCGAGATGCTGTTAGATTACGAGCATG-5’ |
| 182 linear pDL182               | 5’-AGCTAGCTAGCAGGCTTACGTCTTGAGGCTGCTTCATCTTTCGCC |
| pTL182                          | TCGACGCGATGAGAAGCTGTAGTACTGCTTCGA-5’ |
| 188 linear pDL188               | 5’-AGCTAGCGGCGGATGCTTCATGCTTCGACATGACGTAGAGCT |
| pTL188                          | TCGGCGGATGAGAAGCTGTAGTACTGCTTCGA-5’ |
| 192 linear pDL192               | 5’-AGCTAGCGGCGGATGCTTCATGCTTCGACATGACGTAGAGCT |
| pTL192                          | TCGCTCGCGGATGAGAAGCTGTAGTACTGCTTCGA-5’ |
| 194 linear pDL194               | 5’-AGCTAGCGGCGGATGCTTCATGCTTCGACATGACGTAGAGCT |
| pTL194                          | TCGGACTCGCGGATGAGAAGCTGTAGTACTGCTTCGA-5’ |

\(^a\)Pairs of plasmids are created with the same insert, with opposite orientations in the two plasmids.

\(^b\)See Friederich et al. (4).
3.0 × 10⁻¹⁹ erg cm. To model the data, the last two parameters were altered within the region between base pairs 73 and 93, roughly the expanse of the tRNA core.

RESULTS

Formation and verification of the extended mtRNASer(AGY)–A₅ bulge constructs

The construction of the single-bend construct of the bovine mtRNASer(AGY) has been described in detail (1). The sequences of the oligonucleotides used to create the single-bend and the two-bend tRNA–A₅ bulge constructs, with the varied spacing (n), are contained in Table 1. The current approach, depicted in Figure 3, is essentially that used in Friederich et al. (6), except for the inclusion of three additional phasing sizes: n = 14, n = 16 and n = 18.

The secondary structure of the tRNA core and the presence of the A₅ bulge were verified by chemical modification probing. DMS alkylates the free N1 of adenosine (A) and, to a lesser extent, the N3 of cytosine (C), when those residues are not involved in Watson–Crick base pairing. Figure 4 shows a probing experiment on the phasing construct, n = 0, where the mtRNA core and A₅ bulge are in the closest proximity; alkylation of the A and C bases stops reverse transcriptase one base before the modification, as can be seen in the figure. DMS also alkylates N7 of guanosine, but that modification does not stop reverse transcriptase. Similar experiments on the n = 8 and n = 10 constructs gave similar results, verifying the presence of the A₅ bulge and of the stems and loop expected for the tRNA core.

TEB measurements at increased temperature and in the presence of magnesium reveal increased dispersion in the interstem angle

Measurement of the apparent interstem angle of the single-bend wild-type bovine mtRNASer(AGY) were made at both 4°C and 37°C to provide an initial assessment of possible enthalpic contributions to the apparent angle, which would suggest the presence of at least some core and/or interstem stabilizing interactions. Measurements made in the presence of 2 mM magnesium do demonstrate a larger apparent angle and/or greater angle dispersion at the higher temperature (Table 5), suggestive of the loss of some stabilizing interactions that may have been present at the lower temperature in the noncanonical bovine mtRNASer(AGY). Measurements made in the absence of magnesium did not vary with temperature, suggesting that such stabilizing interactions are not present in the absence of divalent cations (Table 5).

Absence of a strong phase-dependence of electrophoretic motilities of the tRNA–A₅ bulge constructs is indicative of substantial flexibility in the tRNA core

Heteroduplex RNA constructs containing the bovine mtRNASer(AGY) core element and an A₅ bulge, separated by varying numbers of base pairs, were run on polyacrylamide gels (Figure 5, right panels). The weak phase...
dependence of the mobilities of the two-bend constructs stands in stark contrast to the large phase dependence observed for the yeast tRNA\(^{Phe}\)–A\(_5\) constructs (Figure 5, left panels), for which previous studies demonstrated substantial structural rigidity of the yeast tRNA\(^{Phe}\) core (6). The flexibility contributing to the weak phasing must reside in the bovine mtRNA\(^{Ser}\) (AGY) core element, since the A\(_5\) bulge is shared for heteroduplex constructs involving both tRNA species. Interestingly, comparison also reveals that while the canonical yeast tRNA\(^{Phe}\) constructs exhibit more pronounced phasing in the presence of magnesium than in its absence, the reverse is true for the noncanonical bovine constructs. This latter observation indicates that magnesium is exerting fundamentally different stabilizing effects for the two tRNAs. It should be noted that these effects of magnesium are only determined whether the structural modulation is specific to magnesium or is a more general effect of divalent ions.

Table 3. Birefringence decay times and fractional amplitudes\(^a\) for the single-bend tRNA species and linear controls; absence of magnesium

| Temperature | Species | Fraction fast decay | Fast decay time (μs) | Fraction slow decay | Slow decay time (μs) |
|-------------|---------|---------------------|----------------------|---------------------|---------------------|
| 4°C         | Bovine mtRNA\(^{Ser}\) (AGY) | 0.69 (0.01)\(^c\) | 0.54 (<0.01) | 0.29 (<0.01) | 2.30 (0.02) |
|             | 158 bp linear | 0.12 (<0.01) | 0.27 (0.01) | 0.87 (<0.01) | 2.87 (0.02) |
|             | 170 bp linear | 0.16 (<0.01) | 0.31 (0.03) | 0.82 (0.01) | 3.39 (<0.01) |
|             | 174 bp linear | 0.15 (<0.01) | 0.34 (0.01) | 0.84 (<0.01) | 3.66 (0.03) |
| 20°C        | Bovine mtRNA\(^{Ser}\) (AGY) | 0.72 (0.01) | 0.34 (0.01) | 0.27 (<0.01) | 1.29 (0.12) |
|             | 158 bp linear | 0.08 (<0.01) | 0.17 (0.01) | 0.91 (<0.01) | 1.69 (<0.01) |
|             | 170 bp linear | 0.12 (<0.01) | 0.20 (<0.01) | 0.87 (<0.01) | 1.98 (<0.01) |
|             | 174 bp linear | 0.12 (<0.01) | 0.23 (0.01) | 0.87 (<0.01) | 2.14 (<0.01) |
| 37°C        | Bovine mtRNA\(^{Ser}\) (AGY) | 0.75 (0.01) | 0.23 (<0.01) | 0.25 (0.01) | 0.83 (0.03) |
|             | 158 bp linear | 0.07 (<0.01) | 0.09 (<0.01) | 0.92 (<0.01) | 1.07 (<0.01) |
|             | 160 bp linear | 0.33 (0.03) | 0.17 (<0.01) | 0.67 (0.03) | 0.98 (0.01) |
|             | 170 bp linear | 0.09 (<0.01) | 0.14 (<0.01) | 0.90 (<0.01) | 1.28 (0.01) |
|             | 174 bp linear | 0.14 (0.01) | 0.17 (0.01) | 0.85 (0.01) | 1.40 (0.01) |

\(^a\)TEB measurements were performed as described in Materials and methods section. For each RNA species, the fractional decay amplitudes and times represent at least 10 TEB measurements on two independent RNA preparations.

\(^c\)Precise temperatures are provided in Materials and methods section.

\(^d\)Numbers in parentheses represent standard error.

Table 4. Birefringence decay times and fractional amplitudes\(^a\) for the extended tRNA species and linear controls; presence of 2 mM MgCl\(_2\)

| Temperature | Species | Fraction fast decay | Fast decay time (μs) | Fraction slow decay | Slow decay time (μs) |
|-------------|---------|---------------------|----------------------|---------------------|---------------------|
| 4°C         | Bovine mtRNA\(^{Ser}\) (AGY) | 0.61 (<0.01) | 0.55 (0.01) | 0.37 (<0.01) | 2.10 (0.10) |
|             | 158 bp linear | 0.19 (<0.01) | 0.49 (0.05) | 0.80 (<0.01) | 2.56 (0.02) |
|             | 170 bp linear | 0.18 (<0.01) | 0.34 (0.02) | 0.81 (<0.01) | 2.66 (0.05) |
|             | 174 bp linear | 0.13 (0.01) | 0.20 (0.03) | 0.86 (0.01) | 2.88 (0.04) |
| 20°C        | Bovine mtRNA\(^{Ser}\) (AGY) | 0.57 (0.01) | 0.29 (<0.01) | 0.41 (0.01) | 1.29 (0.03) |
|             | 158 bp linear | 0.12 (<0.01) | 0.22 (0.02) | 0.86 (<0.01) | 1.44 (0.01) |
|             | 160 bp linear | 0.22 (<0.01) | 0.22 (<0.01) | 0.77 (<0.01) | 1.39 (<0.01) |
|             | 170 bp linear | 0.17 (<0.01) | 0.26 (<0.01) | 0.81 (<0.01) | 1.71 (<0.01) |
| 37°C        | Bovine mtRNA\(^{Ser}\) (AGY) | 0.76 (<0.01) | 0.24 (<0.01) | 0.23 (<0.01) | 0.88 (0.04) |
|             | 158 bp linear | 0.09 (<0.01) | 0.11 (<0.01) | 0.91 (<0.01) | 0.92 (<0.01) |
|             | 160 bp linear | 0.18 (<0.01) | 0.14 (0.02) | 0.81 (0.01) | 0.88 (0.02) |
|             | 170 bp linear | 0.11 (<0.01) | 0.13 (0.01) | 0.90 (<0.01) | 1.04 (0.01) |

\(^a\)TEB measurements were performed as described in Materials and methods section. For each RNA species, the fractional decay amplitudes and times represent at least 10 TEB measurements on two independent RNA preparations.

\(^c\)Precise temperatures are provided in Materials and methods section.

\(^d\)Numbers in parentheses represent standard error.
TEB decay ($\tau$ ratio) measurements for the set of two-bend constructs demonstrate that the bovine mtRNA$^{\text{Ser}}$(AGY) structure possesses substantially greater core flexibility than the canonical yeast tRNAPhe. For the canonical yeast tRNAPhe, similar measurements showed a greater than twofold difference between the minimum and maximum $\tau$ ratio values in the presence of millimolar quantities of magnesium (6), as predicted for a tRNA core that is as rigid as an equivalent span of duplex RNA helix. For the highly truncated bovine mtRNA$^{\text{Ser}}$(AGY), there was only a 0.6-fold difference, consistent with a substantially flexible mtRNA core.

In Figure 6, curves computed assuming no added core flexibility (6,11) are superimposed on the experimental $\tau$ ratio data for the set of two-bend constructs. Although it is difficult to quantify the degree of increased flexibility, since there are likely to be contributions from both reduced torsional and flexural constraints, the dramatic disparity between the theoretical curves and the tRNA is flexible (Figure 6). For the canonical yeast tRNAPhe, similar measurements showed a greater than twofold difference between the minimum and maximum $\tau$ ratio values in the presence of millimolar quantities of magnesium (6), as predicted for a tRNA core that is as rigid as an equivalent span of duplex RNA helix. For the highly truncated bovine mtRNA$^{\text{Ser}}$(AGY), there was only a 0.6-fold difference, consistent with a substantially flexible mtRNA core.

### Table 5. Acceptor-anticodon interstem angles for the extended mtRNA$^{\text{Ser}}$(AGY) constructs

| Extended tRNA species | 4°C$^a$ | 20°C | 37°C |
|-----------------------|--------|------|------|
| No magnesium          |        |      |      |
| Bovine mtRNA$^{\text{Ser}}$(AGY) | 119 (3)$^b$ | 115 (3) | 114 (4) |
| 2 mM MgCl$_2$B$_2$    |        |      |      |
| Bovine mtRNA$^{\text{Ser}}$(AGY) | 134 (4) | 131 (2) | 145 (8) |

$^a$Precise temperatures are provided in the Materials and methods section.

$^b$Parentheses in table refer to standard error.

Figure 4. Verification of the expected of secondary structure of the $n = 0$ construct using chemical modification. (A) The sites of modification are indicated by closed arrows on the extended secondary structure representation. (B) The closed triangles point to sites of modification indicating a single stranded area. The open triangles indicate a site of lesser modification.

Figure 3. Outline of the construction of double-bend tRNA–A$_5$ bulge constructs. (A) Construction of the tRNA core and A$_5$ bulge. The phasing bases are indicated as N. The additional extensions are indicated as (dotted line). (B) Schematic representation of the phase constructs for a rigid tRNA core, and (C) for a flexible tRNA core.
experimental data clearly indicates that the truncated mtRNA possesses substantially more flexibility than does the canonical tRNA\(^{\text{Phe}}\) species. Curves computed using the angles reported in Table 5 in effect incorporate an apparent mean angle for the tRNA core that may itself combine both a larger angle of minimum free energy and increased flexibility. Thus, it is difficult to quantify the nature and degree of increased flexibility, since there are likely to be contributions from both reduced torsional and flexural constraints, which render the computational problem underdetermined.

In the insets to Figure 6, the experimental phasing curves are again displayed, along with representative computed curves that approximate the experimental amplitudes.
No attempt has been made to optimize the fit, since the curves are underdetermined with respect to true minimum free energy angles, $P$-values and $C$. However, the total amplitudes of the curves are less than half of the expected values in the absence of added flexibility. For studies in the absence of magnesium, reductions in the torsional elastic constant ($C$) were also incorporated. The improved agreement with the experimental data, while not uniquely defining the nature of the increased flexibility, confirms its presence. The data points represented by open circles ($n = 0, 2$) in the insets to Figure 6 were excluded for reasons that are addressed in the Discussion section.

**DISCUSSION**

Previous measurements (1,2) of the acceptor-anticodon interstem angles of non-canonical, metazoan mtRNAs, including bovine mtRNA$^\text{Ser(IAG)}$, revealed two important distinctions between their core angles and the ‘L’-shaped form of the archetypal yeast tRNA$^{\text{Phe}}$ (5,6,15–17). First, the apparent interstem angles of the truncated mtRNAs are generally greater than that of the nearly right angle of the corresponding angle for yeast tRNA$^{\text{Phe}}$. Second, the core angles for the noncanonical species generally have little if any dependence on the presence of Mg$^{2+}$ ions, in stark contrast to yeast tRNA$^{\text{Phe}}$ (5). Despite these differences, it was difficult to draw any definite conclusions regarding the intrinsic rigidity/flexibility of the mtRNA core from the single-bend analyses, since both added flexibility and/or a larger rigid interstem angle would yield similar results for the apparent bend angles (5,6).

In the present work, we have utilized the dual-bend approach used previously by Friederich et al. (6), who demonstrated that the yeast tRNA$^{\text{Phe}}$ core is intrinsically rigid in the presence of Mg$^{2+}$ ions, but substantially flexible in the absence of divalent cations. For bovine mtRNA$^\text{Ser(IAGY)}$, the more open apparent interstem angle and its relative lack of Mg$^{2+}$ dependence are suggestive of a more flexible core. Indeed, this is born out by the two-bend analysis. What is less clear is the importance and physical properties by which the tRNA bend appears to become more flexible in the presence of Mg$^{2+}$, than in its absence. Although it would be premature to extend the current conclusions to all truncated tRNAs, the similar patterns of behavior (1,2) for a number of other metazoan mtRNAs suggest a common behavior, perhaps due to a paucity of available interstem interactions.

The current work presents the first evidence of a substantially flexible core for any tRNA species. The presence of additional flexibility is not unexpected, based in part on the lack of available D- to T-loop interactions; however, there has thus far been no direct demonstration of the role of such stabilizing interactions in modulating core rigidity. While a study of tRNA transiting the ribosome has indicated that an abbreviated tRNA would necessarily be flexible in order to accommodate the various stages of transit, measurements have focused on the flexibility of the ribosome (18–22) and not on that of the mtRNA.

The markedly reduced phase amplitudes of the experimental curves in Figure 6 relative to expectations for a rigid core, coupled with the minor phase variation of the relative electrophoretic motilities (Figure 5), indicate that the truncated mtRNA is indeed more flexible than the canonical yeast tRNA$^{\text{Phe}}$. Thus, the process of transit may involve flexibility of both the mitochondrial ribosome and the transiting tRNAs.

The fact that the periodicities of the $\tau$ ratio curves (Figure 6) are not uniform may also indicate that the bovine mtRNA is not behaving as a simple hinge, but instead may have a series of discrete conformations. In a mixed pool of conformers, the decay of birefringence due to rotational reorientation would tend to weigh more heavily than the conformations that are more extended in solution because the $\tau$ values are derived from the terminal portion of the TEB decay curves. In fact, were the decay curve to reflect a population of slowly interchanging conformers, including cis- (fast tumbling, $\tau$ ratio minimum) and a trans- (slow tumbling, $\tau$ ratio maximum) phased species, the resulting apparent $\tau$ ratios would be weighted toward larger angles. The possibility of multiple conformers in slow or intermediate exchange (on the time scale of birefringence decay), precludes a quantitative modeling of the nature of the flexibility. Although there is no unique solution for added flexibility, the fundamental conclusion is unambiguous, namely, that the highly truncated mtRNA clearly possesses more flexibility than its canonical counterparts.

Increased flexibility of the bovine mtRNA$^\text{Ser(IAGY)}$ core is consistent with what is known about its secondary structure. This non-canonical tRNA lacks not only many of the conserved residues of the canonical tRNAs, but is also missing one of the arms (DhU arm) of the canonical species. The DhU arm in canonical tRNA is involved in a number of core-stabilizing interactions, including those reinforced by Mg$^{2+}$ ions, that are absent in mtRNA$^\text{Ser(IAGY)}$. Although models for the tertiary structure of bovine mtRNA$^\text{Ser(IAGY)}$ put forth by de Bruijn and Klug (23), as well as models by Steinberg et al. (24), do include interactions between the D replacement loop and the T arm, those interactions are not equal in number to those found in canonical tRNA. As suggested by the current work, the proposed interactions in those studies are in any case likely to be insufficient to stabilize the bend to the extent present in canonical tRNA.

Previous work with yeast tRNA$^{\text{Phe}}$ has demonstrated the validity of using the two-bend constructs and the $\tau$ ratio method to investigate tRNA flexibility (6). In those experiments, where the minimum separation between the two bends was 21 bp, there was a close agreement between the experimental data and the theoretical data derived from independent single-bend measurements. For that study, there was no apparent structural perturbation due to the helical extensions, even when the two arms were nearest to one another, in the cis-confirmation (5). In the current studies, the $n = 0$ and $n = 2$ constructs put the two bends, and consequently two helical extensions, somewhat closer than the 21 bp minimum used for the yeast tRNA$^{\text{Phe}}$ measurements; thus, there
may be some as yet unidentified structural interaction for the smallest separations between bend centers. From the structural probing data (Figure 4), the closer proximity of the two bends does not appear to affect the secondary structure within the core region. Because of this remaining question for the most proximal bend centers, the first two data points are depicted as open circles in all the figures.

Bovine mtRNA_{Ser}^{AGY} and other metazoan mtRNAs challenge the notion of a universal structure of tRNA. Such differences are clear at the secondary structure level, since the mitochondrial species lacks many of the conserved bases of the canonical tRNA, and lacks one of the arms of the canonical cloverleaf secondary structure. Previous studies demonstrated that the global conformation of the tertiary structure was also quite distinct from that of the canonical tRNA, particularly in the more open interstem angle for the mtRNA. In the current work, we have established for the first time that the core of the non-canonical tRNA possesses substantial flexibility. Thus, at the level of tertiary structure, non-canonical tRNAs are likely to deviate from their canonical counterparts not only in net conformation, but also with respect to the conformational freedom of the core itself.

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