Communication

A Methyl Group Controls Conformational Equilibrium in Human Mitochondrial tRNA

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A Methyl Group Controls Conformational Equilibrium in Human Mitochondrial tRNA^{Lys}

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Regulation of gene expression, a ubiquitous feature of life, is often achieved by the simultaneous action of several mechanisms on the synthesis, maturation, and degradation of biopolymers, creating a balance to produce a desired expression level. Transfer RNA (tRNA), the key adaptor molecule in the translation of the genetic code from messenger RNA into an amino acid sequence, undergoes a multitude of maturation steps. These include post-transcriptional modification reactions that synthesize more than 70 chemically distinct ribonucleotides in all domains of life. The absence of certain modifications leads to accelerated degradation of tRNA.[1] Nucleotide modifications in the tRNA structural core are believed to stabilize its functional conformation characterized by the cloverleaf secondary structure and the 3D-L-shape. The abundance of modified nucleotides in classical tRNA has rendered a detailed analysis difficult so far. Here we have used single molecule spectroscopy to detect multiple conformations in a tRNA and to show that a post-transcriptional modification consisting of a single methyl group shifts a dynamic equilibrium of an unmatured mitochondrial (mt) tRNA precursor toward the functional cloverleaf structure.

Analysis of the impact of a single nucleotide modification in a tRNA requires the site-specific introduction of a single modified nucleotide into a ∼76-mer RNA. This daunting task is compounded by the presence of additional nucleotide modifications, of which there are typically ∼10 in classical tRNAs. The situation is alleviated in animal mitochondria, which contain only ∼4−5 modifications per tRNA. Of particular interest is human mitochondrial tRNA^{Lys}, whose unmodified transcript Kwt **1** does not exhibit properties expected from a cloverleaf-shaped tRNA. Structural probing in solution suggested an extended hairpin structure for Kwt, whereas a cloverleaf structure was inferred for Km^{1}A **2**, in which a methyl group was introduced on *N1* of adenosine 9 (Figure 1).[2] The m^{1}A9 disrupts a Watson−Crick base pair (A9-U64) in the extended acceptor stem of the unmodified Kwt, which can also be achieved by mutating A9 to C, or U64 to A or C.[2] All these mutants show cloverleaf-type probing patterns like **2** and are aminoacylated by human lysyl-tRNA synthetase (LysRS), while **1** is not.[3] *In vitro* modification assays with proteins from human mitochondria have shown that **1**, despite its apparent non-tRNA structure, is slowly methylated at *N1-A9*.[4] This is intriguing since tRNA modification enzymes would be expected to act best on tRNA structures. Indeed, cloverleaf-type mutants are methylated faster at *N1-A9*. Compared to **1**, several other modifications are also effected faster on cloverleaf-shaped tRNAs, including pseudouridine (Ψ) at position 27, which is synthesized by pseudouridine synthase 1 (Pus1).[4] These data support the hypothesis that at least two alternative conformations of **1** coexist in equilibrium, one of which would be cloverleaf-shaped and thus recognized by modification enzymes. According to this hypothesis, m^{1}A9 would destabilize the extended hairpin of **1** and thus favor the cloverleaf structure, effectively making the methylation event a rate-limiting step in the biogenesis of tRNA^{Lys}.

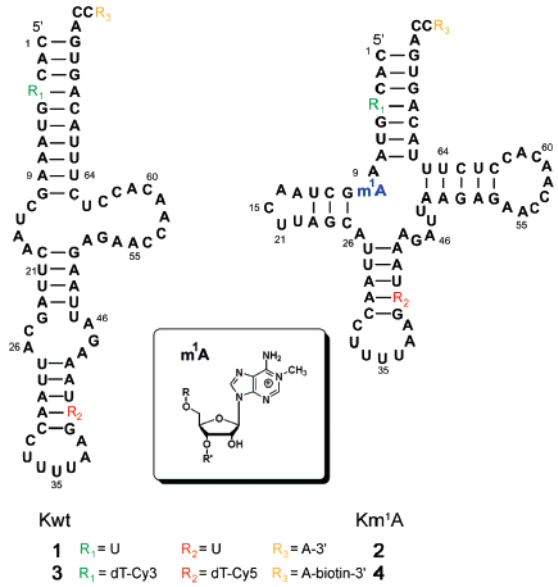

Figure 1. Maturation intermediates and derivatives of human mitochondrial tRNA^{Lys}. **1**: unmodified RNA of genomic sequence (Kwt) in extended hairpin conformation. **2**: RNA containing 1-methyladenosine (m^{1}A, depicted in the context of an RNA chain) as sole nucleotide modification (Km^{1}A). In **3** and **4**, Cy3 and Cy5 dyes are attached via a spacer to the 5-position of U4 and U41, respectively, and a biotin residue is linked to the 3′-end.

To address this hypothesis by single molecule fluorescence resonance energy transfer (smFRET), we have constructed Kwt and Km^{1}A molecules carrying Cy3 and Cy5 dyes by combining phosphoramidite chemistry[5] and ligation.[6] On the basis of the X-ray structure of yeast tRNA^{Phe},[7] nucleotides 4 and 41 were chosen as attachment sites since the C-5 atoms of the respective uracils were about 53 Å apart, which is the Förster radius of the Cy3−Cy5 pair.[8] Because the resulting dye-labeled derivatives of Kwt **3** and Km^{1}A **4** represent typical tRNA maturation intermediates, their substrate properties in representative tRNA maturation events were investigated in vitro. Precursors of **3** and **4** were good substrates in processing of tRNA precursors by RNase P as well as in post-transcriptional modification of nucleotide U27 to Ψ27 (Figure 2). Moreover, their substrate properties were very similar to those of

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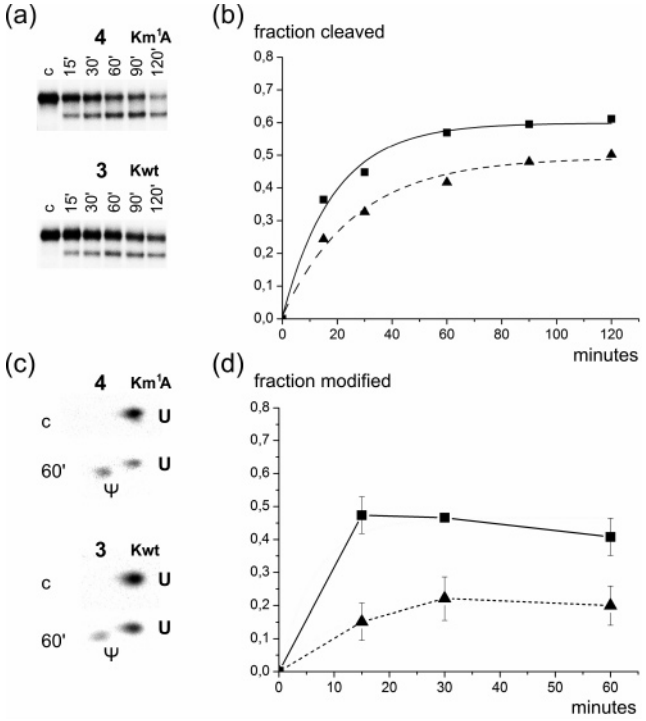

Figure 2. Biochemical characterization of dye-labeled tRNA derivatives. (a) Cleavage products of tRNA precursors after incubation with RNase P. (b) Kinetics of precursor processing of **3** (solid triangles) and **4** (solid squares). (c) TLC analysis of conversion of U27 to Ψ after incubation with recombinant Pus1p. (d) Kinetics of Ψ27 formation.

unlabeled derivatives **1** and **2** (Supporting Information Figure S1). These data suggest that the attachment of the two dyes does not significantly perturb the overall tRNA structure, and that **3** and **4** are suitable models for the investigation of maturation processes by FRET techniques.

The presence of multiple conformations in the FRET-labeled RNA constructs was indeed revealed by smFRET experiments on freely diffusing and surface-immobilized molecules using a confocal microscope.[9,10] FRET efficiency values were obtained by ratiometric analysis of the fluorescence intensities emitted by the Cy3 and Cy5 dye molecules.[11] Histograms of FRET efficiency values measured on freely diffusing molecules (Figure 3a) revealed two subpopulations which can be assigned to the more extended hairpin ($\langle E \rangle \sim 0.4$) and to the highly compact, cloverleaf-based L-shape ($\langle E \rangle \sim 0.8$) structures, respectively. A simultaneous fit of continuous standard Gaussian (low FRET) and log-normal (high FRET) functions to both FRET histograms yielded a ratio of ~2:1 for Kwt and of ~1:2 for Km^{1}A. This change in population corresponds to an overall reduction of the free energy of Km^{1}A by 3.9 kJ/mol due to the m^{1}A modification.

Interconversion between the two subpopulations was examined by measuring time traces of the donor and acceptor emission of individual molecules immobilized on a bovine serum albumin coated glass surface.[10] Switching between two conformational states is clearly observed in the FRET trajectories of **3** and **4** (Figure 3b). Cross-correlation analysis of more than 40 traces for each

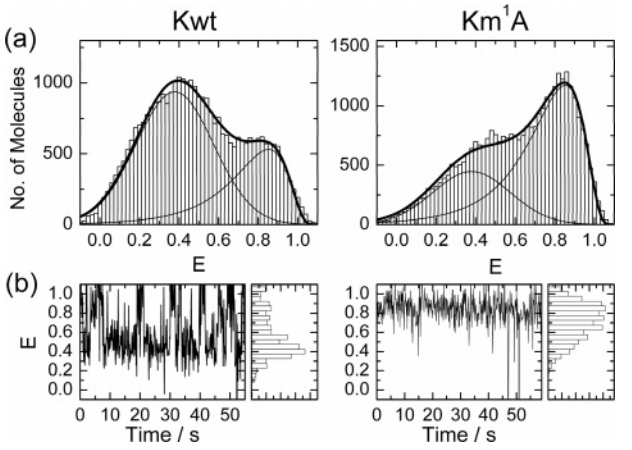

Figure 3. Intramolecular tRNA dynamics of Kwt (left) and Km^{1}A (right) revealed by smFRET. (a) Histograms of FRET efficiency values, *E*, taken from freely diffusing single molecules. (b) FRET trajectories of immobilized single molecules and the corresponding histograms of FRET efficiencies, obtained with 100 ms dwell time binning.

sample revealed average apparent rate coefficients of (4 ± 1) and (2 ± 1) s^{-1} for constructs **3** and **4**, respectively. A simple model involving two conformations interconverting on the ~100 ms time scale appears sufficient to describe the dynamics; there is no evidence of additional metastable conformations. This result underscores that the m^{1}A9 modification induces a thermodynamic preference of the biologically active cloverleaf conformation of tRNA^{Lys} against the inactive conformation.

This work presents insights into the intramolecular dynamics of a tRNA. Such model tRNA systems will allow detailed characterization of the effect of further post-transcriptional modifications in tRNA on its internal structural dynamics.

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Supporting Information Available: Supplemental Figure S1 and experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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