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Fluorine-19 NMR studies of the thermal unfolding of 5-fluorouracil-substituted *Escherichia coli* valine transfer RNA

Wen-Chy Chu and Jack Horowitz

Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011, USA

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^{19}F NMR spectroscopy was used to monitor the thermal unfolding of *E. coli* $tRNA^{Val}$ labeled by incorporation of 5-fluorouracil (FUra). With rising temperatures, resonances in the ^{19}F NMR spectrum of (FUra)$tRNA^{Val}$ gradually shift towards the central region of the spectrum and merge into a single broad peak above 85°C. FU55 and FU12 are the first to shift, beginning at temperatures below 40°C, which suggests that the initial steps of thermal denaturation of $tRNA^{Val}$ involve disruption of the tertiary interactions between the D- and T-arms. The acceptor stem and the FU64-G50 wobble base pair in the T-stem are particularly stable to thermal denaturation. A temperature-dependent splitting of the ^{19}F resonance assigned to FU64, at temperatures above 40°C, suggests that the T-arm of (FUra)$tRNA^{Val}$ exists in two conformations in slow exchange on the NMR time scale.

^{19}F NMR; Fluorouracil; Transfer RNA; Thermal unfolding; *Escherichia coli*

1. INTRODUCTION

The thermal unfolding of tRNA has been investigated by e.g. temperature-jump relaxation kinetic and calorimetric methods (summarized in [1]), ^{31}P and proton NMR spectroscopy [2–6], and chemical modification studies [7]. We have recently shown that ^{19}F NMR provides a powerful tool for probing structural changes in tRNA labeled by incorporation of 5-fluorouracil (FUra) [8–13]. The 14 FUra residues in 5-fluorouracil-substituted *E. coli* $tRNA^{Val}$ ((FUra)$tRNA^{Val}$) are distributed throughout all stems and loops of the molecule (Fig. 1), making it possible to examine conformational changes in many regions of the tRNA. Its ^{19}F NMR spectrum shows a resolved resonance for each of the chemically identical fluorines, which reflects differences in their environments due to the higher order structure of native tRNA [9,12]. Because assignment of the ^{19}F spectrum of (FUra)$tRNA^{Val}$ has recently been completed [13–15], it is now possible to use fluorine-19 NMR spectroscopy to describe in detail the effects of temperature on the unfolding of the tRNA molecule and to compare the results with those obtained with other methods.

2. MATERIALS AND METHODS

5-Fluorouracil-substituted $tRNA^{Val}$ was transcribed in vitro with T7 RNA polymerase from a $tRNA^{Val}$ gene ligated into pUC119 directly adjacent to a T7 promoter [13]. Transfer RNA samples (3 mg) were prepared for ^{19}F NMR study as previously described [13]. For experiments in Mg^{2+}-free buffer, (FUra)$tRNA^{Val}$ was dissolved in 10 mM sodium cacodylate buffer, pH 6.0, containing 15 mM $MgCl_2$, and renatured by heating at 55°C for 20 min followed by slow cooling to room temperature. The renatured tRNA sample was precipitated with ethanol, redissolved in autoclaved water, and dialyzed against three changes of autoclaved water in a flow-dialysis microcell (BRL). After dialysis the sample was dried in a Speed-Vac and dissolved in 330 μl NMR buffer (50 mM sodium cacodylate, pH 6.0, 100 mM NaCl, 1 mM EDTA and 10% D_2O). Transfer RNA prepared in this way retains bound magnesium ions because measures to remove endogenous Mg^{2+}, e.g. EDTA treatment, were not employed.

^{19}F NMR spectra were obtained at 282 MHz on a Bruker WM-300 pulsed FT NMR spectrometer. Data were accumulated by using 8000 data points, no relaxation delay, and an excitation pulse angle sufficient to optimize the Ernst condition. Chemical shifts are reported as ppm downfield from the external standard, 5-fluorouracil.

Abbreviations: NMR, nuclear magnetic resonance; FUra, 5-fluorouracil; (FUra)$tRNA^{Val}$, 5-fluorouracil-substituted *E. coli* $tRNA^{Val}$.

Correspondence address: W.-C. Chu, Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011, USA.

3. RESULTS AND DISCUSSION

3.1. *Fluorine-19 NMR spectra of 5-fluorouracil-substituted $tRNA^{Val}$*

We have completely assigned the ^{19}F NMR spectrum of in vitro transcribed (FUra)$tRNA^{Val}$ recorded in pH 6 buffer containing 15 mM Mg^{2+} and 100 mM NaCl [13–15] (see Fig. 2A). At room temperature, 12 resolved resonances are evident; at 47°C two additional peaks are resolved [9]. Several interesting generalities relating ^{19}F chemical shift to tRNA structure become evident. Signals from FUra residues in unstructured polynucleotides or in loop regions of (FUra)$tRNA^{Val}$ resonate in the central region of the ^{19}F NMR spectrum, 4.7–4.8 ppm downfield of free FUra [9,12]. Resonances assigned to FUra base-paired with A in helical regions, with

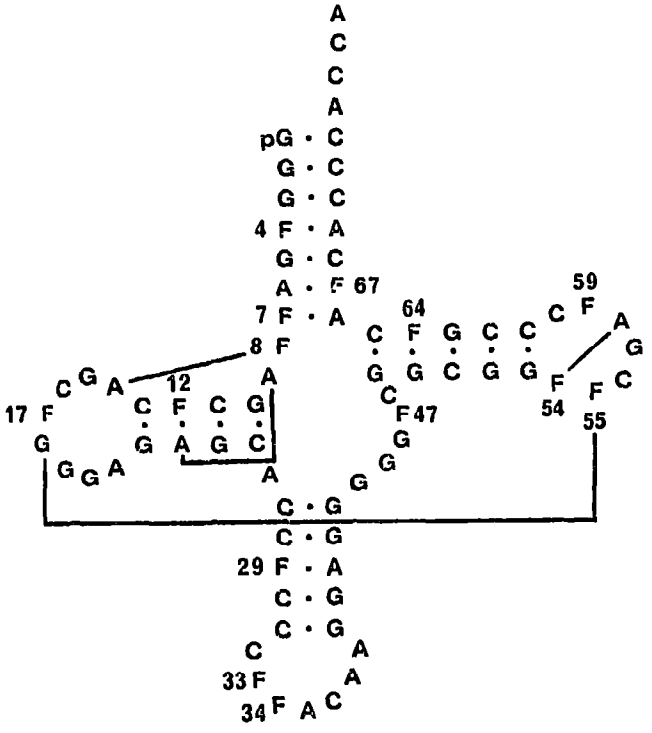


Fig. 1. Cloverleaf structure of 5-fluorouracil-substituted $tRNA^{Val}$. Tertiary base pairs involving 5-fluorouracil (F) are connected by solid lines.

the exception of FU7, are found in the upfield region of the ^{19}F spectrum, presumably due largely to ring current effects of adjacent stacked base pairs (see discussion in [9]). FU7 is base-paired with A66 at the base of the acceptor stem and its signal may be shifted toward the center of the spectrum because it is only partly stacked on G49 in the continuous acceptor stem/T-stem helix. 5-Fluorouracil hydrogen-bonded to G gives a signal shifted 4–5 ppm downfield of resonances from FUra paired with A [13]. These observations indicate that disruption of the secondary and tertiary structural elements of the tRNA by thermal denaturation will cause ^{19}F resonances from the affected FUra residues to move toward the central part of the spectrum.

Thermal unfolding studies were carried out in Mg^{2+}-free buffer to minimize magnesium ion-catalyzed hydrolysis of the tRNA at high temperatures. Removal of exogenous Mg^{2+} produces several spectral changes reflecting alterations in $tRNA^{Val}$ structure [9–12]. By following chemical shift changes as magnesium ion concentration is varied and from the spectra of $tRNA^{Val}$ variants having individual FUra residues replaced by other nucleotides [13–15], we could assign the low-Mg^{2+} spectrum of (FUra)$tRNA^{Val}$ (Fig. 2B). Tightly bound magnesium ions were not removed in these experiments because in the complete absence of Mg^{2+}, the ^{19}F NMR spectrum of in vitro transcripts is poorly defined, especially in the upfield region [13].

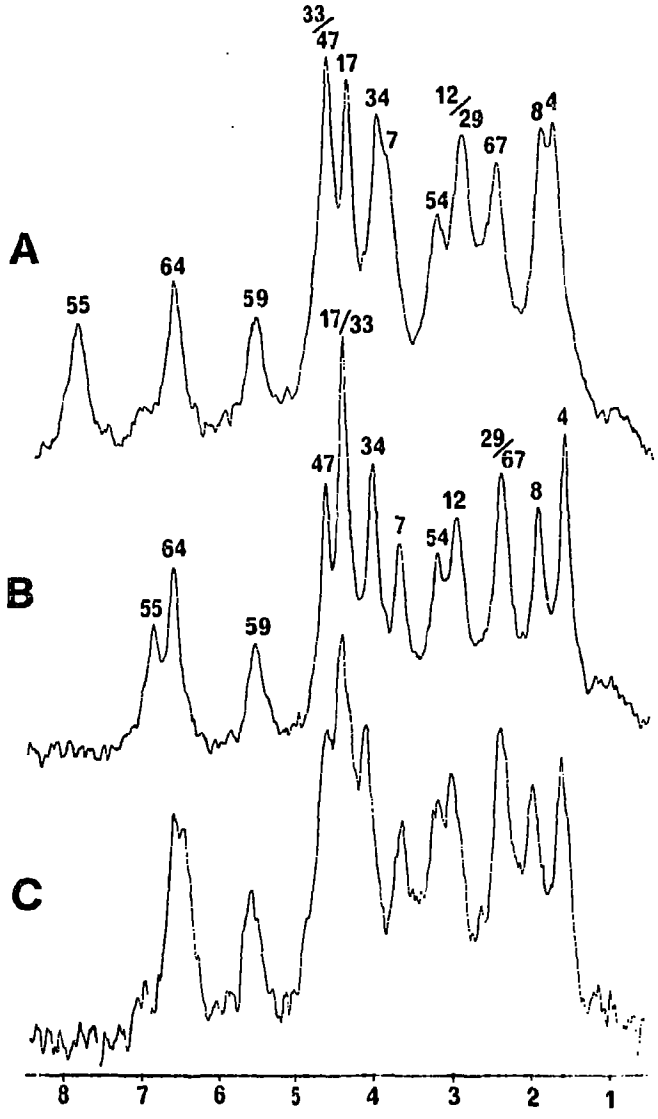
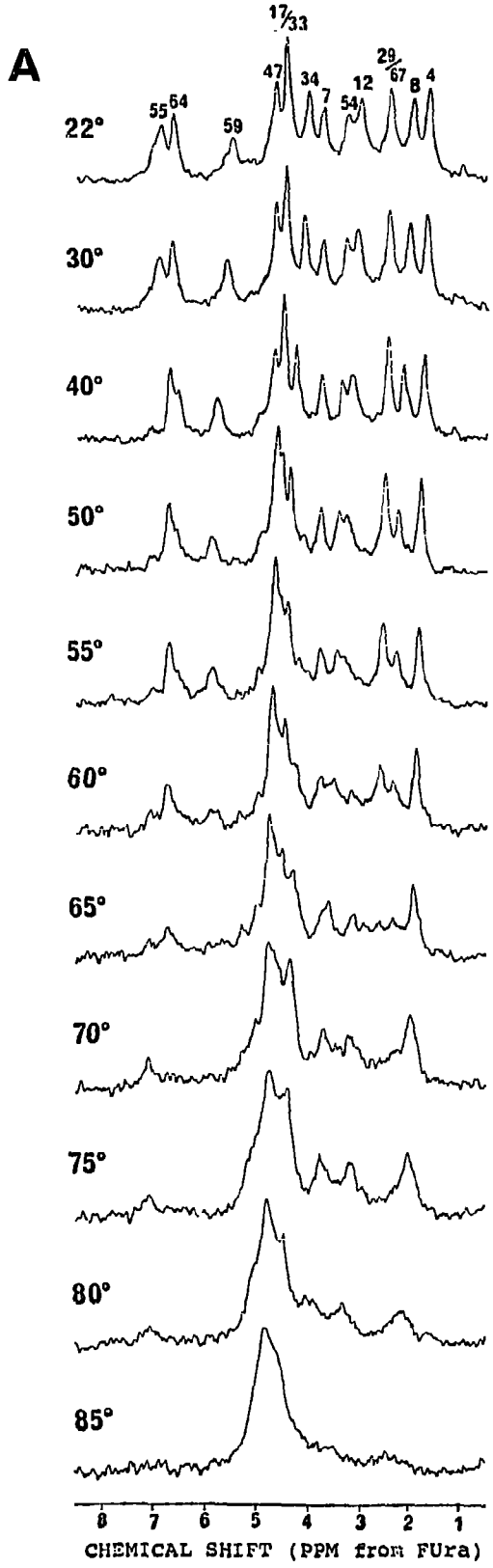


Fig. 2. ^{19}F NMR spectra of (FUra)$tRNA^{Val}$. (A) Spectrum recorded at 25°C in the presence of 15 mM Mg^{2+}. (B) Spectrum recorded at 25°C in the absence of exogenous magnesium ions. (C) Spectrum of (FUra)$tRNA^{Val}$ after thermal denaturation followed by slow cooling to room temperature; recorded at 30°C in the absence of exogenous Mg^{2+}.

3.2. *Thermal denaturation of (FUra)$tRNA^{Val}$*

Changes in the ^{19}F NMR spectrum of *E. coli* (FUra)$tRNA^{Val}$ with increasing temperature are depicted in Fig. 3A. The results, summarized in Fig. 3B, show that, as expected from the known chemical shifts of signals from FUra in nucleotides or polynucleotides devoid of secondary and tertiary structure [9,12], resonances shift towards the center of the spectrum as temperature rises, until at 85°C a single broad peak centered at 4.8 ppm is observed. The thermal denaturation of (FUra)$tRNA^{Val}$ is largely reversible. After slow



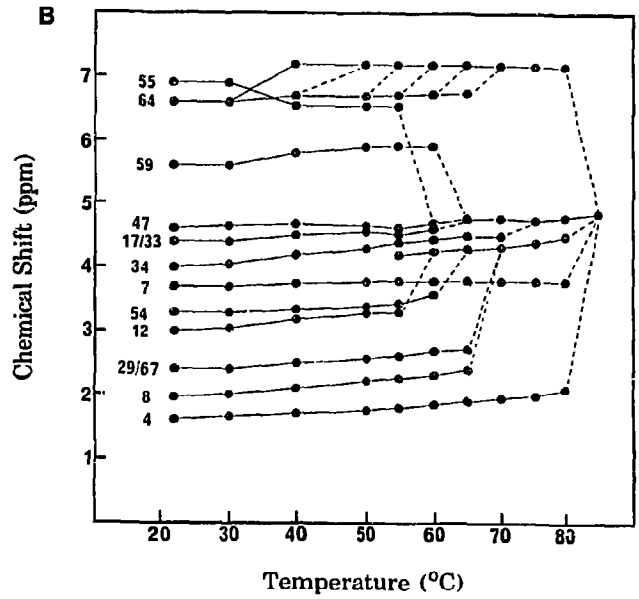


Fig. 3. (A) ^{19}F NMR spectral changes of (FUra)tRNA^{Val} as a function of temperature. The tRNA sample (3 mg), prepared as described in Materials and Methods, was dissolved in Mg^{2+}-free buffer. (B) Temperature dependence of the chemical shifts of resonances in the ^{19}F NMR spectrum of (FUra)tRNA^{Val}.

cooling, the ^{19}F NMR spectrum of (FUra)tRNA^{Val} is almost identical to that recorded at the start of the experiment (Fig. 2C), indicating that little or no degradation occurs at elevated temperatures. Electrophoresis in 15% denaturing (7 M urea) polyacrylamide gels confirms that the tRNA is undamaged after the melting experiment (results not shown).

The first resonances in the ^{19}F NMR spectrum of (FUra)tRNA^{Val} affected by increasing temperature are those assigned to FU55 and FU12. FU55 shifts upfield from 6.9 to 6.6 ppm beginning at temperatures below 40°C. In the same temperature range, FU12 shifts downfield from 3.0 to 3.65 ppm. Although it is difficult to trace the line width and chemical shift position of these resonances at temperatures above 50–55°C as they merge into the large central peak, the gradual shift or broadening of FU55 and FU12 suggest they are in fast or intermediate exchange between the folded and unfolded state. FU55, located in the T-loop, is tertiary hydrogen bonded to G18 in the D-loop, helping to stabilize the interaction between D- and T-loops. FU12 is situated in the D-stem and involved in a semiconserved base triple, A9-A23-FU12. These results suggest that the first stages of thermal denaturation of *E. coli* tRNA^{Val} involve disruption of tertiary interactions between the D- and T-loops.

Another indication of structural changes in the T-arm is the splitting of the peak of FU64 at temperatures greater than 40°C (Fig. 3). The peak assigned to FU64 resonates at 6.6 ppm, in the downfield region of the ^{19}F NMR spectrum of (FUra)tRNA^{Val}, as expected for

FUra residues base-paired with G [13]. Beginning at 40°C, an increasing fraction of peak FU64 intensity shifts from 6.6 ppm downfield to 7.2 ppm (Fig. 3). Observation of two distinct peaks indicates that FU64 exists in two different environments that are in slow exchange on the NMR time scale. The base pair between FU64 and G50 is particularly stable. Its ^{19}F signal persists as a well-defined downfield peak at 80°C (Fig. 3).

With increasing temperature, signals from FUra residues located in single-stranded loops of (FUra) $tRNA^{Val}$, FU47, FU17, FU33, FU34 and FU59, gradually shift towards the middle of the spectrum and merge into the broad central resonance (Fig. 3). These changes can be followed most clearly for the resonance corresponding to FU34, which remains clearly resolved to temperatures above 65°C (Fig. 3).

Most of the remaining resonances in the ^{19}F NMR spectrum of (FUra)$tRNA^{Val}$ disappear as individual peaks and coalesce into the large central peak at temperatures between 60–65°C (Fig. 3). Resonances from several 5-fluorouracil residues base-paired with A in the acceptor stem persist to higher temperatures, however. These include FU7, FU67, and FU4; the FU4:A69 base pair is particularly stable. Its signal, at 1.7 ppm, remains sharp to temperatures above 70°C, with little broadening or loss of intensity as the tRNA unfolds; it remains clearly visible even at 80°C (Fig. 3).

3.3. *Comparison of fluorine-19 NMR results with earlier studies*

Previous ^{19}F NMR thermal denaturation studies of native (FUra)$tRNA^{Val}$ (isolated from 5-fluorouracil-treated *E. coli* cells), conducted before the spectrum had been assigned, gave results comparable to those reported here [12]: the acceptor stem and the FU64-G50 wobble base pair are especially stable to thermal melting and a splitting of the resonance now assigned to FU64 is observed. However, with the native tRNA, increasing temperature causes this resonance to shift upfield from 6.6 to 6.4 ppm [12]. This difference suggests that the stacking geometry of the FU64-G50 base pair in the T-stem of native (FUra)$tRNA^{Val}$ differs from that of the in vitro transcript. The two tRNAs differ only in that native (FUra)$tRNA^{Val}$ contains two modified nucleotides, m^6A37 and m^7G46, whereas the in vitro transcript is completely unmodified.

The molecular mechanism of the thermal denaturation of several tRNA species has been monitored by ^1H NMR spectroscopy [2–6]. By following methyl and methylene proton resonances of modified bases as a function of temperature, Kastrup and Schmidt observed at least 3 conformational transitions in *E. coli* $tRNA^{Val}$ in the complete absence of Mg^{2+} [2,3]. The earliest involves disruption of the native tertiary structure and changes in the vicinity of the rT54 and the m^7G46 methyl protons. This is followed by a conformational change about m^6A37 in the anticodon loop and a melting of the T-stem.

The order of thermal unfolding of common structural elements depends upon tRNA species. In *E. coli* $tRNA^{Phe}$, the tertiary base pairs G15–C48 and G46–G22, and the secondary base pair A31–ψ39 at the bottom of the acceptor stem were reported to be the most labile, followed by base pairs in the D- and T-stems [5]. In *E. coli* $tRNA_f^{Met}$, the first step in heat denaturation involves transient opening of the dihydrouridine helix, followed by a simultaneous disruption of the D-helix and a tertiary interaction; sequential melting of the T-stem, the anticodon stem, and finally the acceptor stem helix, is observed at higher temperatures [6]. In *E. coli* $tRNA_3^{Gly}$, the secondary base pair A7–U66 melts earlier than several tertiary hydrogen bonds [4]. Results with $tRNA^{Phe}$ and $tRNA_3^{Gly}$ demonstrate that the acceptor stem is the most stable structure in the molecule [4,5]. This stability was attributed to the 5–6 consecutive G–C base pairs in the acceptor stem. *E. coli* $tRNA^{Val}$ has 3 consecutive G–C base pairs at the 3'-end of acceptor stem, and this fact may account for the stability of the secondary structure in this region of the tRNA.

These studies of the thermal denaturation of 5-fluorouracil-substituted *E. coli* $tRNA^{Val}$ demonstrate the value of ^{19}F NMR for monitoring conformational changes of tRNA in solution.

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