Spermine Binds in Solution to the TψC Loop of tRNA^{Phe}: Evidence from a 750 MHz ^{1}H-NMR Analysis

Benjamin Frydman*

U. W. Medical School and School of Pharmacy, University of Wisconsin−Madison, 425 North Charter Street, Madison, Wisconsin 53706

William M. Westler

National Magnetic Resonance Facility, University of Wisconsin−Madison, Madison, Wisconsin 53706

Keijiro Samejima

Faculty of Pharmaceutical Sciences, Josai University, Saitama, Japan

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Spermine and spermidine are natural polyamines widely distributed in biological systems and have been shown to be critical for cell growth and carcinogenesis.[1,2] The crystallographic X-ray analysis of yeast tRNA^{Phe} reported the presence of one spermine wrapped at the beginning of the D stem, with another spermine located in the major groove between the anticodon and the D stems.[3,4] Nevertheless, members of that group more recently have stated that the precise location of spermine in tRNA^{Phe} requires a higher degree of resolution.[5] Using ^{13}C-NMR and ^{15}N-NMR, we previously studied the interactions of spermidine and spermine with tRNA.[6−8] Those results showed (1) that spermine binds to tRNA more strongly than spermidine, (2) that the binding takes place mainly through the formation of hydrogen bonds, and (3) that the NH_3^+ groups bind much more weakly than the NH_2^+ groups (see Figure 1).

To identify the binding site of spermine on tRNA we have adopted a strategy based on ^{1}H−^{1}H NOE interactions, which result from the spatial proximity of the spermine and the tRNA protons. We have chosen the well-characterized tRNA^{Phe} from yeast for which a large number of resonances have been previously assigned.[9−11] The hydrogen-bonded imino protons of the paired nucleotide bases resonate in the 9.5−15.0 ppm region of the tRNA ^{1}H-NMR spectrum.[9,10] Labeling the NH_2^+ and NH_3^+ resonances, groups directly involved in the binding process,[7,8] would be the ideal solution. Fast exchange of these protons with the aqueous solvent precludes, however, their observation. In this study, therefore, we use the carbon-bound methylene protons next to the amino groups as the "spies" to follow the interaction with tRNA. These protons resonate between 2.8−3.1 ppm where they overlap with the tRNA sugar resonances. To alleviate this problem of overlap, we used spermines that are enriched with ^{13}C in the carbons bonded to the $-NH_2^+-$ and NH_3^+ groups (Figure 1). We acquired 1D ^{13}C-half-filtered NOE ^{1}H-NMR spectra, which limits the observed intermolecular NOE's to those tRNA protons that are within ca. 5 Å from the ^{13}C bonded spermine protons. The sequence used to obtain these spectra is summarized in (1):

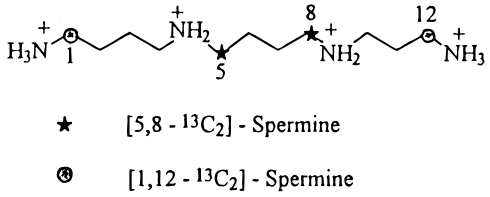

Figure 1. Structures of the [^{13}C]-enriched spermines at pH 7.2.

$$D1 - 90^H - \Delta - \pm 90^C - 180^H - 90^C - \Delta - 90^H - T_{mix} - 90^H - \delta - (-)90^H \pm \text{acquisition} \quad (1)$$

A ^{13}C half-filter selects the protons bound to the ^{13}C-nuclei. The selected protons then cross-relax during T_{mix} followed by a jump return read pulse used for water suppression.

The proton ^{13}C-half-filtered NOE spectra of both [1,12-$^{13}C_2$]spermine and [5,8-$^{13}C_2$]spermine showed a strong peak at 3.1 ppm corresponding to the frequency of the ^{13}C-bound protons. The imino proton region of tRNA (9.0−15.0 ppm), shown in Figure 2D, was recorded in cacodylate buffer in the absence of Mg^{2+}. It is known[12] that the tRNA structure in the absence of Mg^{2+} is basically unchanged from that in the presence of Mg^{2+} and that the 9.0−15.0 ppm imino proton region is essentially the same in both cases. In the 1D ^{13}C half-filtered NOE spectra of the imino region of the 1:2 and 1:8 tRNA:spermine complexes, a NOE signal at 12.46 ppm was measured for both ^{13}C-enriched spermines (Figure 2A−C). A second NOE was observed at 1.0 ppm for both labeled spermines (Figure 2A−C). The resonance at 1.0 ppm has been unambiguously assigned to the 5-methyl group of T54.[10] The NOE signal at 12.46 ppm was assigned to the N(3)H of the T54A58 base pair, based on previous determinations.[10] To further correlate both signals we preirradiated the T54 methyl resonance (1.0 ppm), and a NOE difference spectrum with a peak at 12.46 ppm was obtained. Hence, the signal at 12.46 ppm is resonance M as assigned by Hilbers.[10] Resonance M is a strong peak, and there are several imino protons other than the N(3)H signal of T54-m^{1}A58 whose chemical shifts overlap under this peak. They are the imino protons of the vicinal G53C61, G51C63, and m^{5}C49G65 base pairs of the T-stem, all Watson−Crick-type hydrogen-bonds. Also resonating at this frequency is the imino proton of the G19C56 base pair which holds together the TψC and D loops. Even when it is impossible to unequivocally correlate each one of these resonances with the protons of the spermine ^{13}C-methylenes as in the case

* To whom correspondence should be addressed. Phone: (608) 263-0648. Fax: (608) 262-3397. E-mail: Benjamin.Frydman@mail.admin.wisc.edu.

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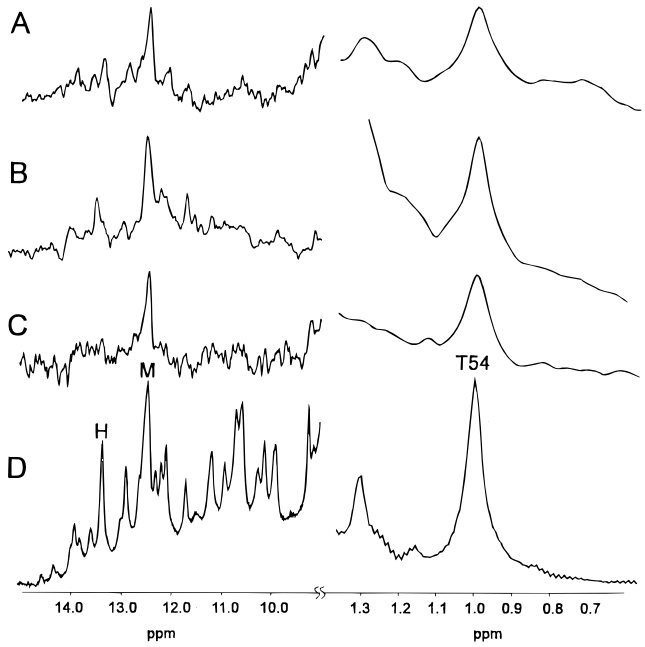

Figure 2. (A−C) 1D ^{13}C half-filtered $^{1}H-^{1}H$ NOE spectra of complexes of labeled spermine and $tRNA^{Phe}$. (D) Control 1D ^{1}H spectrum of the complex. (Left) Imino region of the $tRNA^{Phe}$. (Right) Methyl peak assigned to T54. Spectrum A is from the 1:2 complex of $tRNA^{Phe}$:[1,12-$^{13}C_2$] spermine; spectrum B is from the 1:8 complex of $tRNA^{Phe}$:[5,8-$^{13}C_2$]spermine; and spectrum C is from the 1:2 complex of $tRNA^{Phe}$:[5,8-$^{13}C_2$] spermine. The $tRNA^{Phe}$ from brewers yeast was obtained from Sigma. The sample was dissolved in 0.5 mL of a buffer containing 50 mM sodium cacodylate, 2 mM EDTA, 300 mM NaCl (pH 7.2), and dialyzed for 20 h against the same buffer in double-sided biodialysers (Sialomed). The resulting $tRNA^{Phe}$ solution was polyamine-free as assayed by HPLC. The isotopically enriched spermines (Figure 1) were prepared by synthesis[14] and were added to the dialyzed tRNA solution to yield final 1 mM $tRNA^{Phe}$, 2 mM spermine mixtures (A and C); or 1 mM $tRNA^{Phe}$, 8 mM spermine mixtures (B). ^{1}H-NMR spectra were recorded at 750 mHz at 5 °C. The water signal was suppressed by a jump-return read pulse. The Δ period was set to 2.85 ms, the mixing period, T_{mix} (τ), was 300 ms (75 and 150 ms spectra were also collected but are not shown) and the δ period was 45.3 μs; which placed the maximum of excitation at 12.1 ppm. The relaxation delay D1 was 1 s, and the acquisition time was 0.182 s. The ^{1}H transmitter frequency was on-resonance for H_2O, and the ^{13}C transmitter frequency was at 41 ppm. The ^{1}H spectral width was 22 522 Hz (30 ppm), and for each acquisition 2048 complex data points were collected. The number of acquisitions for these spectra was 120 000 for the sample with [1,12-^{13}C]spermine (49 h collection time) and 100 000 for the [5,8-^{13}C]spermine samples (41 h collection time). The solvent composition was 95% $^{1}H_2O$/5% $^{2}H_2O$. All chemical shifts are referenced to internal DSS. (D) Imino region of $tRNA^{Phe}$.

of the T54m^{1}A58 base pair, it is obvious that the NOE between the M signal and the spermine protons lend support to the binding of spermine to the TψC loop. Further evidence that the TψC loop is the binding site for spermine is provided by the NOE signal at 13.30 ppm (Figure 2A−C) which corresponds to the imino proton of U50A64, a base pair of the T stem. We confirmed Hilbers assignment of this resonance (resonance H)[10] to the U50A64 base pair using 2D-NOE correlations (not shown).

The NOE signals between the protons of the C_1 (C_{12}) and C_5 (C_8) methylenes of spermine and the resonances of the T54m^{1}A58 base pair suggest interactions between the $-NH_3^+$ and the NH_2^+- groups of the polyamine and the TψC loop. The weaker amplitude of the NOE peaks of resonance H point to interactions of the NH_3^+ and NH_2^+ groups in the vicinity of the U50A64 base pair of the TC stem. A space-filling model of tRNA-bound spermine, which was obtained from the $^{1}H-^{1}H$ NOE cross peak constraints, is shown in Figure 3.

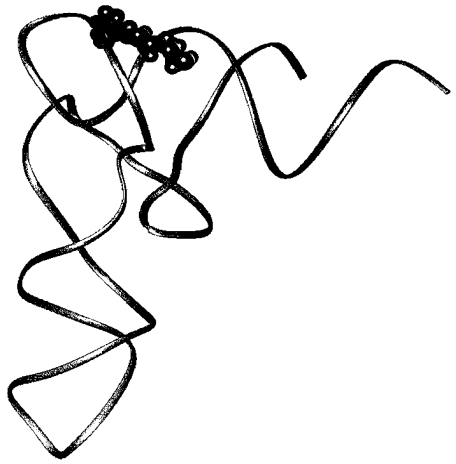

Figure 3. Model of the regioselective binding to $tRNA^{Phe}$ in solution showing a CPK model of spermine bound near the corner of the L shaped tRNA molecule where the TΨC and D loops meet. This structure was obtained using energy minimization (Amber force field) of the observed NOE contraints between spermine and tRNA. The 2.5Å structure of $tRNA^{Phe}$ (PBD entity 4TNA) was used. The calculations were performed using the program Discover from Biosym Technologies Inc.

The NOE mixing time of 300 ms used in these experiments gives rise to significant spin diffusion in complexes of this molecular weight. These effects underestimate the distances measured through NOEs. However, since the intermolecular NOE interaction is averaged between the bound ligand and the noninteracting, unbound ligand, the effective time during which cross-relaxation occurs is governed by the fraction of bound polyamine. No NOE cross-peaks were detected between spermine and the anticodon or D stems of $tRNA^{Phe}$. This suggests that the reported X-ray results[3,4] on the location of spermine in $tRNA^{Phe}$ are either only valid for the structure in the crystal but not in solution or that higher resolution is required to resolve the bound spermine in the crystal.[5]

Our studies show that spermine interacts at the corner of the L-shaped tRNA molecule where the TψC and D loops meet. The importance of this part of the tRNA molecule for correct *in vivo* function has been probed by random mutagenesis.[13] The localization of a specific binding site of polyamines to tRNA yields a target for the further understanding of the function of these ubiquitous molecules in cell growth and carcinogenesis.

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Supporting Information Available: Supplemental Figure 1 shows the $^{1}H-^{1}H$ NOE spectrum at a mixing time of 75 ms. Supplemental Figure 2 shows that NOE effects of the $^{12}CH_2$ neighboring the ^{13}C label can be observed but are rather weak (3 pages).

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