Structure of transfer RNA by carbon NMR: resolution of single carbon resonances from ^{13}C -enriched, purified species

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ABSTRACT

Methyl carbon-13 NMR spectra of purified tRNA species are presented for the first time. In addition, these spectra of tRNA species specific for phenylalanine, tyrosine, and cysteine exhibited the first resolution of single methyl carbon resonances. Carbon-13 enriched methyl groups of ribothymidine (T) and 7-methylguanosine (m⁷G) and the methylthio group of 2-methylthio-N⁶-(Δ^2 -isopentenyl) adenosine (ms²i⁶A) were resolved. The T methyl signal of tRNA^{phe} shifted from 12.3 ppm at 45° in the absence of added Mg²⁺ to 11.1 ppm at 30° in the presence of 10mM MgCl₂. The same change in conditions led to a 0.4 ppm shift of the m⁷G methyl signal in the opposite direction. The relative ease in obtainment of single carbon resonances of purified tRNA species, and display of the sensitivity of their chemical shifts to changes in local structure, are requisite criteria for ^{13}C -NMR to be a useful technique in probing tRNA conformation and its changes during interaction with proteins and other nucleic acids.

INTRODUCTION

The utilization of ^{13}C -NMR spectroscopy in tRNA structure/function studies is desirable for several reasons. Primarily, this magnetically active nucleus exhibits a wide range (200 ppm) of chemical shifts which are markedly sensitive to the chemical environment. The spin-spin relaxation rates are relatively small, even for large molecules, resulting in narrow resonance signals. ^{13}C -NMR spectra for complex molecules tend to be more resolved and relaxation times are easier to interpret in terms of motional parameters than are proton spectra (1). The main difficulty in utilizing ^{13}C -NMR spectroscopy arises from the very low natural isotopic abundance of this nucleus, only 1.1%. With the advent of high sensitivity signal averaging and Fourier transform techniques, as well as the development of technology for large-scale ^{13}C enrichment, this nucleus has become at least as useful as the proton in NMR research (2). Labeling of biological molecules with ^{13}C precursors (2-8) is advantageous over enrichment with other magnetically

active nuclei, such as ^{19}F , because this involves a simple isotopic replacement of a naturally occurring atom. Consequently, the structure of the ^{13}C -labeled biomolecule remains unperturbed.

Komoroski and Allerhand first demonstrated in 1972 (9) the tremendous potential for examining structural properties of tRNA in solution by means of natural-abundance ^{13}C Fourier transform NMR spectroscopy. The development of a probe for 20 mm diameter sample tubes increased the sensitivity of natural-abundance ^{13}C -NMR and facilitated the identification of minor base resonances in unfractionated yeast tRNA made by Komoroski and Allerhand in 1974 (10). Carbon resonances of the less prevalent modified nucleosides, however, were still not visible above background noise, even after fourteen hours of signal accumulation time, due to the very low abundance of the ^{13}C nucleus.

Chang and Lee (11) were able to identify methyl carbon resonances from five methylated nucleosides (m^7G , m^1A , m^3C , m^1G and methyl phosphate) in a natural-abundance ^{13}C -NMR spectrum of unfractionated yeast tRNA methylated in vitro by treatment with an alkylating agent, methyl methanesulfonate. A large improvement in the signal-to-noise ratio was observed with the use of a ^{13}C -enriched methylating agent. Hamill et al. (5) also examined ^{13}C -enriched unfractionated tRNA by incorporating a labeled precursor, ^{13}C -uracil, into tRNA in vivo using a mutant strain of Salmonella typhimurium. This enrichment of ^{13}C into specific residues of tRNA greatly improved the signal-to-noise ratio and decreased the time required for data accumulation. Signals from the labeled C-4 position of dihydrouridine, uridine, ribothymidine, and pseudouridine were quite prominent.

To overcome the weakness of the natural-abundance methyl carbon resonance signals, we initiated studies on the incorporation of ^{13}C -methyl groups into tRNA by a mutant E. coli strain auxotrophic for methionine (4-6). Exogenously provided (^{13}C -methyl) methionine served as the methyl donor for all methylated nucleosides, so use of this labeled metabolite provided a very specific ^{13}C probe into methyl groups (3, 12). Analysis of spectra from unfractionated tRNA enriched to 56.7 atom % ^{13}C at the methyl groups yielded: (a) signal assignments for all methyl carbon resonances; and (b) observation of the release of the T ψ CG loop - hU loop interaction as well as release of anti-codon base stacking during thermal denaturation (3). However, the strength of the enrichment method is realized only with the proven ability to study resonances from individual carbons within purified species of tRNA. The purpose of this communication is to report the resolution of single carbon

resonance signals from methyl groups of three purified *E. coli* tRNAs: phenylalanine, tyrosine and cysteine.

MATERIALS AND METHODS

Preparation of *E. coli* ^{13}C -methyl enriched tRNA--A specifically selected strain (M1) of *E. coli* C6 met⁻cys⁻rel⁻ was grown in a stringently defined medium containing methionine (from Merck, Canada) ^{13}C -enriched in the methyl group by 90+ atom %. Thirty liter cultures were grown, harvested, and tRNA, enriched in ^{13}C solely in methyl groups, was isolated from the cells according to published methods (12). Three 30 l cultures yielded enough cells for the isolation of approximately 300 mg of tRNA.

Purification of tRNA species--Transfer RNA was initially fractionated by benzozylated-DEAE cellulose column chromatography (13). Column fractions were assayed for specific aminoacyl-tRNAs by aminoacylation with radiolabeled amino acids (New England Nuclear). Aminoacylations were catalyzed by enzymes contained in a supernatant fraction from a high speed centrifugation of an extract from *E. coli* B cells (13, 14). Fractions of interest containing tRNA^{phe}, tRNA^{cys}, and tRNA^{tyr} were pooled, the tRNA precipitated with ethanol and the precipitate collected by centrifugation and dissolved in H₂O. After dialysis against buffer (0.01 M sodium acetate, pH 4.5, 0.01 M MgCl₂, 0.001 M disodium ethylenediaminetetracetic acid, 3.0 mM 2-mercaptoethanol 0.5 M NaCl), the tRNA fractions of some 30 mg each were subjected to further purification by high performance liquid chromatography on an RPC-5 column (1.5 x 80 cm). tRNA was eluted with a 500 ml salt gradient of 0.5-0.85 M NaCl in the above buffer, the fractions assayed by aminoacylation and those fractions of interest pooled. The tRNA was precipitated with ethanol, the precipitate dissolved in glass distilled H₂O and extensively dialyzed against H₂O before being concentrated to 0.2 ml by evaporation under vacuum (12). The purity of the tRNA species isolated in this manner was determined by quantitative aminoacylation (13, 14) and polyacrylamide gel electrophoresis (15). tRNA^{phe} (approximately 8 mg), tRNA^{tyr} (3 mg), and tRNA^{cys} (4 mg) accepted greater than 1600 pmoles amino acid per A₂₆₀ unit (50 μg). All three tRNA preparations exhibited one predominant spot upon methylene blue staining of material subjected to polyacrylamide gel electrophoresis.

NMR spectroscopy--The tRNA^{phe} sample was diluted to 1.1 ml with D₂O and placed in a 10 mm NMR tube. tRNA^{tyr} and tRNA^{cys} samples were placed into 0.24 ml cylindrical small sample bulbs (Wilma Glass) and diluted to

this volume with D₂O.

¹³C NMR spectra were obtained at 67.9 MHz, using a quadrature detection Fourier transform spectrometer of hybrid construction. Pulses of 60° (9 μs width) were used with a pulse repetition time of 0.6 sec. Under these instrumental conditions, peak areas might not be proportional to spin concentrations, if T₁ values are significantly different. Our T₁ measurements for unfractionated tRNA give values close to 1 second for all methyl groups, suggesting that peak areas in the present spectra correctly represent relative concentrations. Broadband noise-modulated proton decoupling was centered at a frequency in the methyl region. Transients of 36,000 or 54,000 were averaged in blocks of 2 or 3 to yield the spectra shown here. Digital filtering, equivalent to a line broadening of 10 Hz, was applied to the free induction decays. Temperature control, when required, was maintained by a Bruker Instruments unit.

RESULTS

Carbon NMR spectra of the methyl region of tRNA^{phe}, tRNA^{tyr} and tRNA^{cys} of *E. coli* are shown in Figure 1. Based on previous ¹³C NMR work with modified nucleosides and spectra of unfractionated tRNA (3), the peak assignments were straightforward: 36.8 ppm, CH₃ of m⁷G; 14.7 ppm, methylthio of ms^{2,6}A; 11.9 ppm, CH₃ of T, relative to an internal standard of p-dioxane set at 67.4 ppm. Methyl peaks which appear in each spectrum (Figure 1) are precisely those expected from the published nucleoside sequences (16) and the known positions of methylation derived from methionine. The tRNA^{phe} spectrum is especially interesting in that it shows 2 peaks for the T methyl, one at 12.5 ppm and the other at 11.2 ppm, although the latter is a tentative assignment in view of the limited signal to noise ratio. Proton NMR spectra of tRNA^{phe} (*coli*) (unpublished data, P.G. Schmidt and C.A. Glass) show the T to exist in at least two conformations whose methyl peaks are separated by 0.3 ppm, when the sample has been dialyzed to lower the concentration of Mg²⁺ as is the case here.

Addition of 10 mM MgCl₂ to the ¹³C-enriched tRNA^{phe} sample led to coalescence of the T resonances (top of Figure 2) into a single peak at 11.1 ppm, equal in area to the S-CH₃ of ms^{2,6}A. Raising the temperature of the tRNA^{phe} sample (minus MgCl₂) to 45° (middle spectrum of Figure 2) also had the effect of coalescing the methyl peak, but the chemical shift was 12.3 ppm, 1.2 ppm downfield of the lower temperature spectrum with Mg²⁺ added. A further effect of MgCl₂ addition was seen to involve the m⁷G methyl. While the m⁷G

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Figure 1. Methyl region of ^{13}C NMR spectra of ^{13}C -enriched tRNA^{Phe} , tRNA^{Tyr} and tRNA^{Cys} of *E. coli*. The three tRNA samples had been extensively dialyzed and no Mg^{2+} had been added. Sample temperature was 30°C and 54,000 scans required 9 hrs of data accumulation for each spectrum.

resonance signal is a narrow signal without Mg^{2+} at 30° or 45° , it is broad in the presence of the cation (Figure 2). The most straightforward interpretation is that the m^7G methyl was sampling different environments with an exchange rate on the order of the chemical shift difference.

DISCUSSION

Two key conclusions stem from the results: 1) single methyl carbon resonances of highly purified tRNA isoacceptors can be obtained with relative ease, and 2) these resonances are sensitive to structure in their vicinity. Both of these are requisite conditions if ^{13}C NMR is to be a useful technique in probing tRNA conformation and, more importantly, if the method is to serve in examining tRNA-protein and tRNA-RNA interactions. The advantages of using methyl carbons of modified bases are the ease of making correct assignments and their complete resolution from one another in the spectrum. Furthermore, each group is a probe for a specific locus in the molecule, and the methyls

Figure 2. Spectra of tRNA^{phe} under different solution conditions. Spectra of tRNA^{phe} showing the methyl region and taken at 30° and 45° in the absence of Mg²⁺ are displayed in the lower portion of the figure. MgCl₂ was added to the solution used for the lower two spectra and the sample run at 30° to give the upper trace. Transients numbering 54,000 were averaged for each of the two spectra at 30°; 36,000 scans were sufficient at 45°.

are located in interesting positions with regard to the functions of tRNA.

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