A carbon-13 nuclear magnetic resonance study of the 3'-terminus of 16S ribosomal RNA of *Escherichia coli* specifically labeled with carbon-13 in the methylgroups of the $m_2^6Am_2^6A$ sequence

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ABSTRACT

30S ribosomes were isolated from a kasugamycin resistant mutant of *E. coli* that lacks methylgroups on two adjacent adenines in 16S ribosomal RNA. These ribosomes were methylated *in vitro* with a purified methylating enzyme and 5-S-adenosyl-(^{13}C-methyl)-L-methionine chloride ((^{13}C-methyl)-SAM) as methyldonor. After *in situ* cleavage of the 16S ribosomal RNA by the bacteriocin cloacin DF_{13}, the 49 nucleotide fragment from the 3'-end of the RNA was isolated. The carbon-13 nuclear magnetic resonance spectra of the fragment at various temperatures were compared with those of 6-N-dimethyladenosine (m_2^6A) and 6-N-dimethyladenylyl-(3'→5')-6-N-dimethyladenosine ($m_2^6Am_2^6A$). The data show that the two methylated adenines, which are part of a four membered hairpin $loop_4$ show a strong tendency to be stacked in analogy to the dinucleotide $m_2^6Am_2^6A$.

INTRODUCTION

The 3'-end of 16S ribosomal RNA is part of a site on the 30S ribosomal subunit that is important for initiation of protein synthesis (1). This region of the 16S RNA is very conserved during evolution and is characterized by a nine basepairs long hairpin with a loop of four nucleotides containing the characteristic sequence $m_2^6Am_2^6A$ (2). In previous work (3-7) we have studied the influence of the methylgroups on the function of the ribosome and on the structure of the 16S RNA. Recent UV melting experiments have been carried out on the RNA fragment that results from cleavage by the bacteriocin cloacin DF_{13} and comprises 49 nucleotides from the 3'-end of the 16S RNA (8) (cloacin-fragment), part of which forms the above mentioned hairpin. Comparison of the results obtained for the fragment obtained from wild type *E. coli* with those obtained for the fragment from a mutant strain of *E. coli* that specifically lacks the methylgroups on the two A residues shows that the methylgroups have a subtle but definite influence on the thermodynamic properties of the hairpin. It was found that the helix in the wild type fragment was somewhat less stable compared to the corresponding helix in the mutant fragment. This was

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interpreted (8) in terms of the increased stacking properties of $m_2^6Am_2^6A$ versus A-A segments.

Ribosomes from the mutant strain can be methylated *in vitro* using a purified methylase and S-adenosyl-L-methionine. In principle this would enable one to label this site on the ribosome with a chemical or physical discernable probe using analogs of SAM altered in the methyl moiety provided the enzyme can use the analog. In the study reported here we have synthesized 90% enriched (^{13}C-methyl)-SAM and used this as a donor in the methylation of mutant 30S ribosomes. From these ^{13}C-labeled ribosomes we have isolated the cloacin fragment. In order to obtain a better insight into the stacking properties of the $m_2^6Am_2^6A$ in the fragment its ^{13}C-spectra were recorded at various temperatures and compared with those of the nucleoside m_2^6A and the dinucleotide $m_2^6Am_2^6A$ containing ^{13}C at its natural abundance.

MATERIALS AND METHODS

Thin layer chromatography (TLC). The reaction described below was constantly followed by TLC. Schleicher & Schüll D.C. Fertigfoliën F1500, LS254 (7 x 7 cm) were developed in n-butanol-acetic acid-pyridin-water (15 : 3 : 10 : 12, v/v/v/v). After elution, the products were visualized by (*i*) ninhydrin staining (10% ninhydrin in aceton, 5 min 100-120°C), (*ii*) H_2SO_4-methanol (20 : 80, v/v) at 120°C, and (*iii*) UV light (254 nm). The ninhydrin stains the primary aminogroup of SAM yellow while it stains the primary aminogroup of S-adenosyl-L-homocysteine (SAH) pink. The dye does not stain the primary aminogroups of adenine and adenosine. With these methods adenine, adenosine, SAH and SAM can be distinguished.

Column chromatography. Chromatography was performed on a column (1.5 x 13.5 cm) packed with Cellex-P (Biorad Richmond, California, U.S.A.). The material was activated by three times washing with alternate 100 ml 1 mM HCl and 100 ml demineralized water.

^{13}C NMR. ^{13}C-NMR of SAH, SAM and (^{13}C-methyl)-SAM, all dissolved in D_2O, were measured at 25.1 MHz on a JEOL PFT-100 spectrometer equipped with an EC-100 computer. Probe temperature was kept at 25°C. Dioxane (67.4 ppm from tetramethylsilane (TMS)) was used as an internal standard for chemical shifts.

The ^{13}C-NMR spectra of m_2^6A, $m_2^6Am_2^6A$ and the ^{13}C-labeled 49 nucleotide fragment (100 OD units dissolved in 1.2 ml) were recorded on a Bruker WM-200 WB spectrometer (operating at 50.3 MHz) interfaced with an ASPECT-2000 com-

puter for data accumulation (3,000-10,000 transients). The spectrometer was field/frequency locked on the deuterium resonance of D_2O used as solvent. Heteronuclear proton-noise decoupling was used throughout. Delays of ca. 3.2 seconds, during which the proton-noise decouples was switched to a low power level (∿ 0.3 Watt), were implemented between scans in order to minimize heating of the sample. Chemical shifts were measured relative to the center peak of the tetramethylammoniumchloride (TMA) triplet used as an internal reference and converted to the normal TMS chemical shift δ-scale (56.1 ppm).

Synthesis of 5-S-adenosyl-(^{35}C-methyl)-L-methionine chloride. A mixture of SAH (190 mg, 0.49 mmole; Sigma), 95% formic acid (2.14 ml, 62 mmole), 96% acetic acid (0.5 ml, 9.8 mmole) and methyliodide (0.25 ml, 3.92 mmole) (90% ^{13}C enriched in the methylgroup; Merck, Sharp & Dohme) was stirred with a magnetic bar at room temperature in the dark. After 72 h a second lot of ^{13}C-methyliodide (0.06 ml, 0.98 mmole) was added. TLC after 96 h indicated that the reaction had gone to completion.

The reaction was concentrated under reduced pressure at room temperature to give an oil. Final traces of formic acid and acetic acid were removed by co-evaporating the residue three times with 3 ml toluene. Finally, the residue was evaporated three times from 5 ml ethanol p.a. The brown residue was taken up in 5 ml demineralized water and lyophilized, yielding a redbrown residue which was redissolved in 20 ml demineralized water and four times extracted with 20 ml ether. The water layer was again lyophilized and the resulting product was taken up in 2 ml 1 mM HCl and purified by passing it through a Cellex-P column. The column was first equilibrated with 100 ml 1 mM HCl and then with 100 mM HCl. The product was eluted with a 150 ml linear HCl gradient (50-400 mM). The fractions (2.5 ml) were analyzed by measuring the absorbance at 260 nm and by TLC. Fractions 1-8 contained SAH and 9-35 SAM. These fractions were collected and concentrated using a rotovaporizer. The final yield of (^{13}C-methyl)-SAM was 175 mg (82%).

Activity assay. To test the biological activity of the (^{13}C-methyl)-SAM, it was used as a substrate for a specific methylase that transfers the methylgroups to two adjacent adenines in 16S RNA of *E. coli* (5, compare Results and Table 2).

RESULTS

Characterization and biological activity of (^{13}C-methyl)-SAM

The 25.1 MHz ^{13}C-spectra of the commercial compounds SAH and SAM were

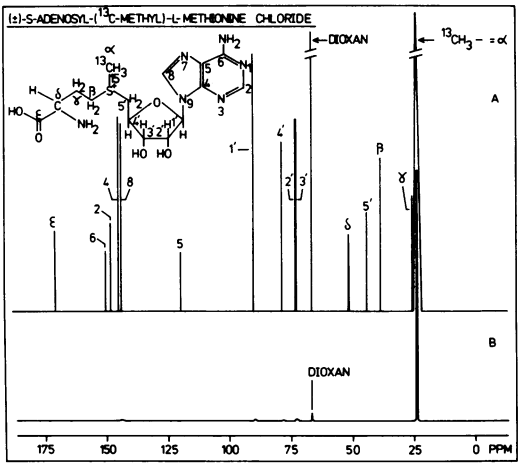

Figure 1. Stick diagram representation of the 25.1 MHz ^{13}C-spectrum of (^{13}C-methyl)-SAM recorded at two different gains. (A): The spectrum recorded at a higher gain, showing the details of the natural abundance of this isotope in the molecule. (B): The spectrum recorded at lower gain.

compared with the synthesized (^{13}C-methyl)-SAM. Chemical shifts and tentative assignments (based on comparison with the chemical shift data of adenosine (9)) are indicated in Fig. 1. The signal at about 23 ppm is obviously caused by the enriched ^{13}C-methylgroups.

The overall yield of SAM obtained by our procedure is 82% as compared to 55% in another study (10) using the same approach.

The ^{13}C-labeled product was tested in a biological system as described before (5). In this *in vitro* system methylase transfers methylgroups from SAM to two adjacent adenines at the 3'-end of 16S RNA of a mutant of *E. coli* (strain TPR_{201}) after which a "pseudo" wild type 30S subunit is obtained. The results shown in Table 1 indicate that the synthetic (^{13}C-methyl)-SAM is completely active in this assay.

^{13}C-NMR of isolated cloacin fragment

30S ribosomes of the mutant strain, labeled with ^{13}C-methylgroups by *in vitro* methylation of these subunits with (^{13}C-methyl)-SAM were treated with the bacteriocin cloacin DF_{13}. This treatment cleaves the RNA at 49 nucleotides from the 3'-end (11). The 49 nucleotide fragment was isolated according to Baan *et al.* (12) and used for ^{13}C-NMR studies. The 50 MHz ^{13}C-NMR spectra of the ^{13}C-methyl enriched wild type cloacin fragment dissolved in D_2O were

Table 1. Dilution of radioactive isotope of (^{3}H-methyl)-SAM by (^{13}C-methyl)-SAM in the methylation of mutant 30S ribosomal subunits.

(^{13}C-methyl)-SAM added (pmoles)	(^{3}H-methyl)-SAM incorporated (cpm)	(percentage)	Relative specific activity of SAM
0	18.336	100	1
500	8.818	48	0.53
1.000	6.752	37	0.36
2.000	3.884	21	0.22
5.000	1.806	10	0.11

A system consisting of 30S subunits (7 pmoles), methylase and (^{3}H-methyl)-SAM (567 pmoles, 780 cpm/pmole) was used according to (5) to measure the effect of (^{13}C-methyl)-SAM on methyltransfer from SAM to 30S.

recorded at 31°C, 12°C and 1°C (Fig. 2, lowest trace). The spectra show that at 31°C all four methylgroups are isochronous (i.e. resonate at identical positions), but upon lowering the temperature the ^{13}C resonance considerably broadens. The line broadening does not result from the increase of the overall rotational correlation time, τ_c, of the molecule at lower temperatures; the estimated increase of τ_c (based on the increase of viscosity of the solvent) at 0°C only marginally contributes (about 6 Hz) to the line width. Indeed other signals than those from the methylgroups do not show the extreme

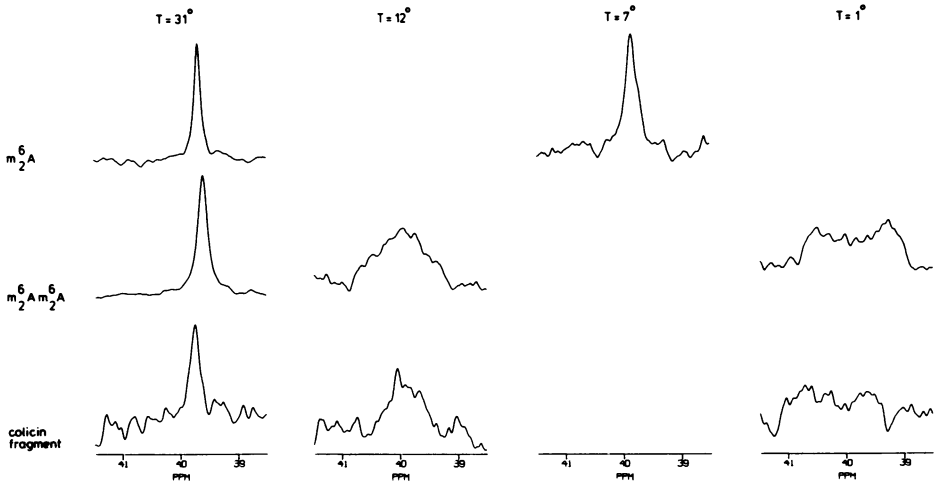

Figure 2. 50 MHz ^{13}C-NMR spectra of the methylgroups of m_2^6A (upper trace), $m_2^6Am_2^6A$ (middle trace) and the ^{13}C-enriched "49 fragment" (lower trace) recorded at various temperatures (°C).

line broadening observed for the latter. If line broadening were due to τ_c alone all signals would be similarly affected. Therefore another mechanism must be held responsible for the observed line broadening, and from the temperature profile of the methyl resonances (Fig. 2) it may be deduced that chemical exchange is a likely candidate.

At this point two reference compounds are introduced: 6-N-dimethyladenosine (m_2^6A) and 6-N-dimethyladenylyl-(3',5')-6-N-dimethyladenosine ($m_2^6Am_2^6A$). The relevant part of the ^{13}C-NMR spectra of m_2^6A (upper trace) and $m_2^6Am_2^6A$ (middle trace) recorded at various temperatures are shown in Fig. 2. Interestingly, the spectra of m_2^6A at 31°C and 7°C display *one* resonance for the *two* 6-N-methylgroups, notwithstanding the fact that these methylgroups are in chemically distinct surroundings (site A and B, respectively, see Fig. 3A). This could be due to an accidental chemical shift equivalence of both methylgroups. More likely the methylgroups give rise to a single time averaged resonance as a result of fast rotation about the C_6-N_6 bond. It can be estimated from proton NMR experiments that the dimethyl aminogroup in m_2^6A rotates

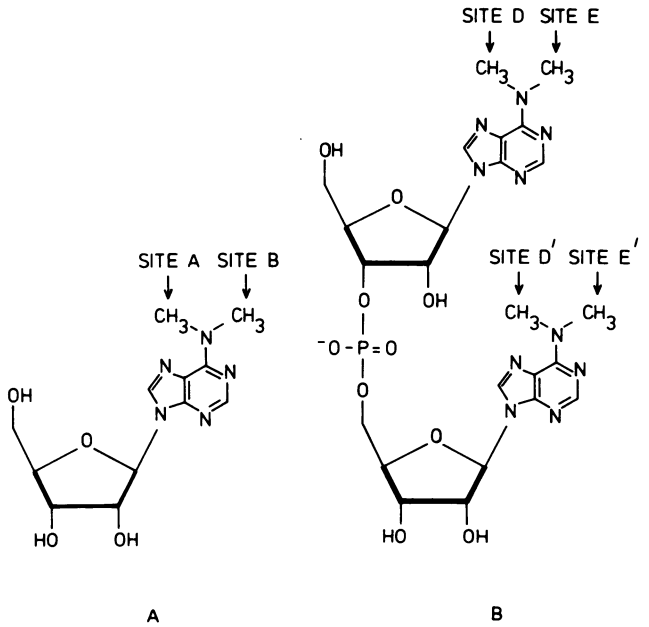

Figure 3. Structure of (A) 6-N-dimethyladenosine (m_2^6A) and (B) 6-N-dimethyladenylyl-(3',5')-6-N-dimethyladenosine ($m_2^6Am_2^6A$). The (in principle) different sites for the 6-N-methylgroups are denoted site A and B in m_2^6A and D/D' and E/E' in $m_2^6Am_2^6A$.

around the C_6-N_6 bond with a rate of $\sim$ 1.000 s^{-1} at 0°C (13).

A similar but even more extreme case is presented by $m^6_2Am^6_2A$ at 31°C: although all four methylgroups involved are, at least in principle, non-equivalent (cf. Fig. 3B, sites, D, D', E and E', respectively), they nevertheless give rise to one single resonance at approximately 40 ppm (Fig. 2). However, upon lowering the temperature the behaviour of $m^6_2Am^6_2A$ diverges from that of the parent compound m^6_2A as the aforementioned methyl signal of $m^6_2Am^6_2A$ extensively broadens (up to about 50 Hz at 12°C) and eventually (at 1°C) splits up in two distinct, albeit broad resonances. The most conspicuous difference between monomer (m^6_2A) and dimer ($m^6_2Am^6_2A$) is found in the excellent intramolecular stacking properties of the latter compound (> 85% stacked at 0°C, see ref. 14) and it seems reasonable to explain the line broadening observed for the ^{13}C-methyl resonances in the dinucleotide at lower temperatures as a result of the increase in population of the single helical conformation of $m^6_2Am^6_2A$. As a result of the stacking, the energy barrier (ΔG) for rotation around the C_6-N_6 bond is raised with respect to the monomer so that at low temperature the rotation slows down giving rise to the line splitting. It is known that the stacking-destacking process is extremely rapid (15). This is also demonstrated by the observation that all resonances but the methylcarbon resonances display line widths less than 4 Hz at 1°C. This means that the lifetime of a particular state of the dimethyl aminogroup must be much longer than that of the destacked state which is reasonable in view of the known rate constants (13,15).

One could reason that because of the stacking process one of the resonances of the dimethyl aminogroups is shifted as a result of anisotropic shielding (ring current effects) caused by the neighbouring adenine base instead of by a slowing down of the rotational motion. We observe, however, an upfield as well as a downfield shift of about 0.5 ppm relative to the $m^6_2Am^6_2A$ methyl resonance at 12°C, while only an upfield shift is expected in such a situation. Moreover, the chemical shift difference between the two methyl signals at 1°C (> 1 ppm), is by far too large to be accounted for by the aforementioned "ring current" effects. A study of molecular models demonstrates that all methylgroups are located well outside the center of the shielding cone of the neighbouring adenine base (where the shielding effect is at maximum (16)).

We now turn to examination of the temperature profile of the ^{13}C-methyl resonance of the cloacin fragment displayed in Fig. 2 shows that the temperature dependence of its methyl resonance closely parallels that of the methyl

resonances of $m_2^6Am_2^6A$. Therefore, by analogy, the strong line broadening of the carbon resonances of the cloacin fragment is interpreted to be caused by stacking of the adenine residues in the loop of the central helix.

DISCUSSION

In previous studies we have found that dimethylation of the adenines in the hairpin loop of the 49 nucleotide fragment of 16S RNA destabilizes the hairpin (8). One of the possibilities that was envisaged in that study in order to explain this effect, was based on the strong tendency for base stacking of the dimethylated adenines (14,18). The measurements reported here indeed show that the base stacking tendency, as reported for the dinucleotide $m_2^6Am_2^6A$ is preserved in the isolated RNA fragment. Probably stacking of the bases imposes a stereochemical strain in the four-membered loop (composed of the sequence $m^2GGm_2^6Am_2^6A$, ref. 17) thereby destabilizing the overal structure.

The final aim of our studies is to correlate the details of ribosomal structure with function. In the case at hand, the question is whether the methylgroups exert their effects on initiation of protein synthesis (4) and on ribosomal subunit interaction (6) through destabilization of the local secondary structure or by some other mode of action.

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Abbreviations: SAM: S-adenosyl-L-methionine;
SAH: S-adenosyl-L-homocysteine.

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