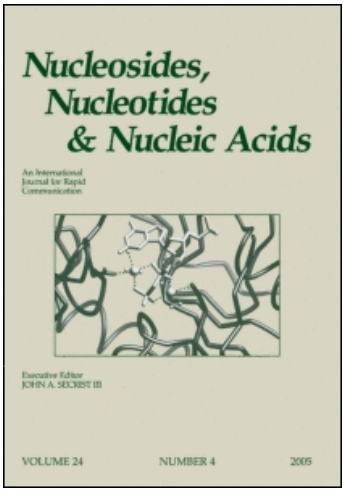


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# Conformational Rigidity of $N^4$-Acetyl-2'-O-methylcytidine Found in tRNA of Extremely Thermophilic Archaebacteria (Archaea)

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NUCLEOSIDES AND NUCLEOTIDES, 11(2-4), 759-771 (1992)

# CONFORMATIONAL RIGIDITY OF $N^4$-ACETYL-2'-*O*-METHYLCYTIDINE FOUND IN tRNA of EXTREMELY THERMOPHILIC ARCHAEBACTERIA (Archaea)*

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Abstract: The conformational characteristics of $N^4$-acetyl-2'-*O*-methylcytidine ($ac^4Cm$), which is one of the modified cytidines unique to the tRNA of extremely thermophilic archaebacteria, and related nucleosides, $N^4$-acetylcytidine ($ac^4C$), 2'-*O*-methylcytidine (Cm) and cytidine, were analyzed by proton nuclear magnetic resonance spectroscopy. Ribose methylation and $N^4$-acylation were found to confer high conformational rigidity to the ribose moiety, suggesting that these post-transcriptional modifications play a role in structural stabilization of tRNA, which is of particular importance at high temperature.

## INTRODUCTION

Earlier studies have indicated that the post-transcriptional modifications represented by 5-methyl-2-thiouridine, 5-methoxyuridine, 2'-*O*-

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* The authors wish to dedicate this paper to the memory of Dr. Tohru Ueda.

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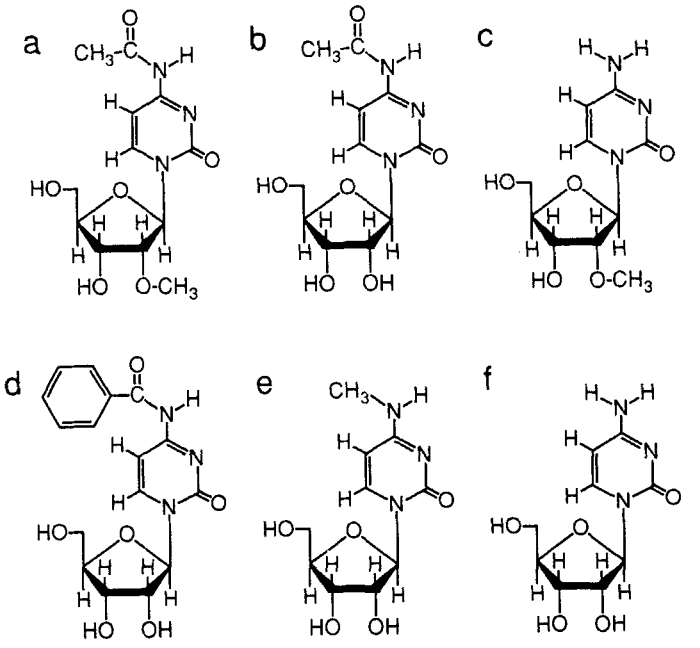


FIG. 1. Structures of (a) $ac^4Cm$, (b) $ac^4C$, (c) Cm, (d) $bz^4C$, (e) $m^4C$ and (f) C.



methyluridine and their derivatives regulate dynamic properties of tRNA, and thus contribute to its various biological functions [1-11]. There are several examples of biologically important modifications of cytidine, such as the hypermodified nucleoside lysidine in the first position of the anticodon of an *E. coli* isoleucine tRNA which contains a modification that changes both the codon and amino acid specificities of the tRNA [12, 13].

$N^4$-Acetyl-2'-*O*-methylcytidine ($ac^4Cm$, FIG. 1a) is widely distributed in the tRNA of hyperthermophilic archaebacteria, including *Thermococcus* sp. (optimal growth 98°C), *Pyrobaculum islandicum* (100°C) and *Pyrodictium occultum* (105°C) [14, 15]. While $ac^4Cm$ appears to be phylogenetically specific for high temperature archaebacteria, $N^4$-acetylcytidine ($ac^4C$, FIG. 1b) and 2'-*O*-methylcytidine (Cm, FIG. 1c) are more common and occur widely in various other tRNA species. The nucleoside $ac^4C$ has been found in the first position of the anticodon of the elongator

methionine tRNA from *Escherichia coli* and the glutamine, glutamate, lysine, proline and serine tRNAs from halobacteria, and in position 12 of the D-stem of eukaryotic leucine and serine tRNAs [16]. On the other hand, 2'-*O*-methylation of pyrimidine nucleotides occurs in several positions of tRNA from a wide range of organisms [16]. It has been proposed that 2'-*O*-methylation of pyrimidine nucleotides stabilizes the *C3'-endo* form and contributes to thermostability of the tRNA molecule as well as to correct codon recognition, especially in positions 54 and 56 of the T-loop, and 32 and 34 (the first position of the anticodon) of the anticodon loop [6]. The crystal structure of $ac^4C$ has been analyzed [17], whereas the conformational characteristics in solution have not previously been examined.

In the present study, the conformational characteristics of $ac^4Cm$, $ac^4C$, Cm and cytidine were analyzed by proton nuclear magnetic resonance ($^1$H-NMR) spectroscopy. It was found that $N^4$-acetylation and 2'-*O*-methylation each independently stabilize the *C3'-endo* form of the ribose moiety, and that these stabilizations occur additively. Further, in order to elucidate the mechanism of stabilization of the *C3'-endo* form by $N^4$-acetylation, the conformational properties of $N^4$-benzoylcytidine ($bz^4C$, FIG. 1d) and $N^4$-methylcytidine ($m^4C$, FIG. 1e) were also analyzed. On the basis of these conformational characteristics, the functional roles of these modified cytidines are presently discussed.



## MATERIALS AND METHODS

Nucleosides $ac^4C$, $bz^4C$, Cm, cytidine, and 4-thiouridine were purchased from Sigma, St. Louis, MO. The remaining compounds were prepared as follows.

The synthesis of $ac^4Cm$ was carried out by acetylation of Cm in anhydrous ethanol solution: freshly distilled $Ac_2O$ (150 $\mu$l, 1.5 mmol) was added to a refluxing solution of Cm (25.7 mg, 0.1 mmol) in anhydrous ethanol (3.5 ml). Refluxing and stirring were continued for 30 min and then cooled at 4°C. Crystals formed were collected by filtration, washed with a small quantity of cold MeOH, and then dried, affording 28.4 mg (95 %) of $ac^4Cm$: UV max ($H_2O$) 247 nm ($\varepsilon$ 14860), 298 nm (8570); thermospray LC/MS m/z 300 ($MH^+$), 154 ($BH_2^+$), 164 ((sugar-H)$NH_4^+$) [18]; HPLC Rt 22.9 min [18]; $^1$H-NMR ($D_2O$) is shown in FIG. 2.

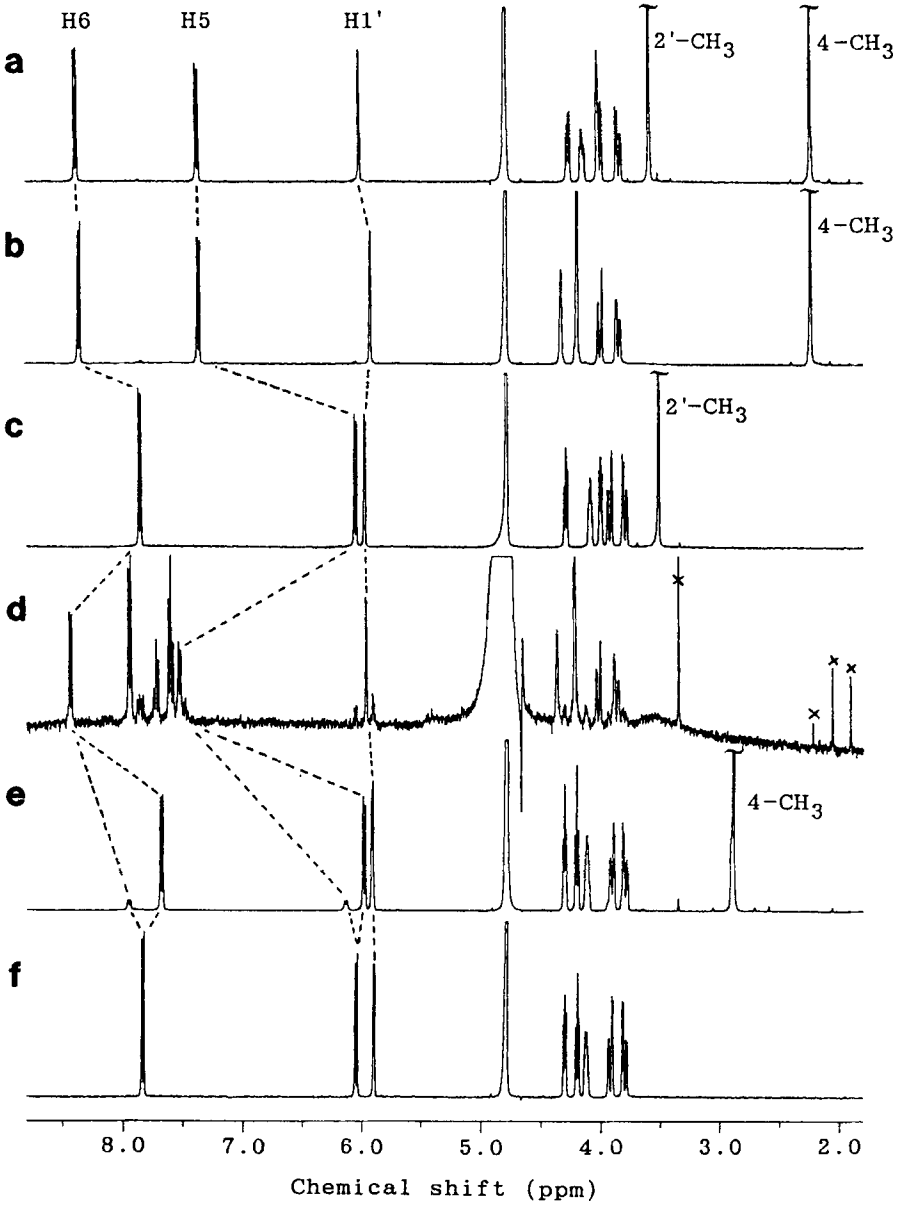


FIG. 2. 400-MHz proton NMR spectra of (a) ac$^4$Cm, (b) ac$^4$C, (c) Cm, (d) bz$^4$C, (e) m$^4$C and (f) C at 25°C.

The synthesis of $m^4C$ was done by a procedure similar to that reported by Ueda and Fox [19] for the synthesis of $N^4$-methylcytosine. 4-Thiouridine (30 mg, 0.12 mmol) was dissolved in 1 ml of MeOH saturated with methylamine gas at 0°C. The solution was heated at 100°C for 15 h in a sealed tube. After cooling, the solution was evaporated. The resinous residue was crystallized from ethanol and recrystallized from methanol: yield 26 mg (87.7 %); mp 235°C (dec. 237°C [20]); thermospray LC/MS m/z 258 ($MH^+$), 126 ($BH_2^+$), 150 ((sugar-H)$NH_4^+$) [18]; HPLC Rt 12 min [18]; $^1$H-NMR ($D_2O$) is shown in FIG. 2.

Each sample (1mg) of $ac^4Cm$, $ac^4C$, Cm, $bz^4C$, $m^4C$ or C was dissolved in $^2H_2O$ (99.8 %), evaporated to dryness and then redissolved in 0.4 ml of $^2H_2O$ (99.98 %). The pH value of each sample was 6.4, 6.3, 6.6, 6.7 or 7.1 (direct pH meter reading), respectively. Sample concentrations were about 10 mM, except the concentration of $bz^4C$ was 0.5 mM because of low solubility. 400-MHz $^1$H-NMR spectra were recorded on a Bruker AM-400 spectrometer at probe temperatures from 25°C to 80°C. Free induction decay of each nucleoside was accumulated with 32K data points and spectrum of 64K data points (spectral width of 4000 Hz) were obtained with zero-filling prior to Fourier transformation, resulting in a resolution of 0.1 Hz/point. Chemical shifts and spin-coupling constants were determined within 0.1 Hz and vicinal coupling constants ($J_{1',2'}$ and $J_{3',4'}$) were used for estimating the fractional populations of the *C2'-endo* and *C3'-endo* forms with the formula [*C2'-endo*] = $J_{1',2'}/(J_{1',2'} + J_{3',4'})$ [21]. For $ac^4Cm$, $ac^4C$ and $bz^4C$, the coupling constant $J_{3',4'}$ could not determined, and the formula [*C3'-endo*] = 1 - [*C2'-endo*], where $J_{1',2'} + J_{3',4'}$ were assumed to be 10 Hz [21], was used instead. The temperature dependence of equilibrium constants [*C2'-endo*]/[*C3'-endo*] was subjected to a least squares treatment, and the enthalpy difference ($\Delta H$) and the entropy difference ($\Delta S$) between the *C2'-endo* and *C3'-endo* forms were obtained together with their standard deviations.

## RESULTS

***NMR spectra of modified cytidines*** — The 400-MHz proton NMR spectra at 25°C of $ac^4Cm$, $ac^4C$, Cm, $bz^4C$, $m^4C$ and cytidine are shown in Fig. 2. Resonance assignments were performed mainly on the basis of their chemical shifts and were confirmed by double resonance experiments. For $m^4C$,

two sets of resonances for H6, H5, H5' and H5" protons were observed at low temperatures, and the largest chemical shift difference was observed for H6 resonances (0.3 ppm). A two dimensional exchange spectrum [22] showed that these resonances arise from the same molecule (data not shown), indicating that two conformers of $m^4C$ are slowly exchanging as have been shown in Ref. 23. This is due to the restricted rotation about the exocyclic C-N bond [24]. However, the averaged resonances were observed for H1', H2' and H3' protons of ribose moiety and we could determine the coupling constants $J_{1',2'}$ and $J_{3',4'}$ from these resonances. It should be noted that the resonances due to the H6 and H5 protons are broadened at above 50°C because of faster exchange (data not shown). For other nucleosides, only one set of sharp resonances was observed at all temperatures between 25 and 80°C.

Lowfield shifts were observed for the H6 and H5 resonances of cytosine base in the presence of $N^4$-acetylation, as has been reported in Ref. 17. The same effect was also observed for $N^4$-benzoylation and, by contrast, an opposite effect was observed for $N^4$-methylation. These effects were stronger for the H5 resonance than the H6 resonance. Because these shifts are thought to be due to changes in shielding effect by the s-electron, it is concluded that the $N^4$-substituent affects the electron density of the H6 and H5 atoms of cytidine base.

***Conformational characteristics of modified cytidines*** — The enthalpy and entropy differences between the *C2'-endo* and *C3'-endo* forms of the ribose ring in modified cytidines are shown in TABLE 1. For cytidine, the enthalpy difference of 0.37 kcal · $mol^{-1}$ indicates that the *C3'-endo* form is slightly more stable than the *C2'-endo* form. The enthalpy difference of cytidine is the same as that of uridine [6], within experimental error. By contrast, for $ac^4Cm$, the enthalpy difference of 1.53 kcal · $mol^{-1}$ indicates that $N^4$-acetyl-2'-*O*-methylation stabilizes the *C3'-endo* form by as much as 1.2 kcal · $mol^{-1}$. The enthalpy difference for the $ac^4Cm$ is the largest among the nucleosides which have thus far been analyzed [4, 6]. For $ac^4C$, the enthalpy difference is 1.22 kcal · $mol^{-1}$, indicating that the $N^4$-acetylation of cytidine stabilizes the *C3'-endo* form by 0.9 kcal · $mol^{-1}$. This stabilization is as strong as that resulting from 2-thiolation of uridine, which has also been found to provide *C3'-endo* stabilization of 0.8 kcal · $mol^{-1}$ [4]. On the other hand, for Cm, the enthalpy difference of 0.65 kcal · $mol^{-1}$ indi-

TABLE 1. Enthalpy and entropy differences[a] between the *C3'-endo* and *C2'-endo* forms

| compound | enthalpy difference[b] | entropy difference[c] |
|---|---|---|
| $ac^4Cm$ | 1.53 (0.04) | 2.56 (0.13) |
| $ac^4C$ | 1.22 (0.05) | 2.07 (0.15) |
| Cm | 0.65 (0.01) | 0.98 (0.04) |
| $bz^4C$ | 1.50 (0.15) | 3.02 (0.48) |
| $m^4C$ | 0.12 (0.02) | -0.12 (0.06) |
| C | 0.37 (0.01) | 0.44 (0.04) |

[a] Standard deviations in parentheses.
[b] In kcal · $mol^{-1}$.
[c] In cal · $mol^{-1}$ · $deg^{-1}$.



cates that the 2'-*O*-methylation only slightly stabilizes the *C3'-endo* form, by 0.3 kcal · $mol^{-1}$. It should be noted that *C3'-endo* stabilization by $N^4$-acetylation and by 2'-*O*-methylation is additive; 0.9 ($N^4$-acetylation) and 0.3 (2'-*O*-methylation) yield 1.2 kcal · $mol^{-1}$ ($N^4$-acetyl-2'-*O*-methylation).

The enthalpy differences for $bz^4C$ and $m^4C$ are found to be 1.50 and 0.12 kcal · $mol^{-1}$, respectively. Thus, $N^4$-benzoylation stabilizes the *C3'-endo* form by 1.1 kcal · $mol^{-1}$ and, by contrast, $N^4$-methylation destabilizes the *C3'-endo* form by 0.3 kcal · $mol^{-1}$. These results indicate that $N^4$-substitution in the base can exert significant influence on the conformational characteristics of the ribose moiety.

## DISCUSSION

***Effect of $N^4$-acetylation on the conformation of cytidines*** — In the present study, $N^4$-acetylation was found to confer rigidity to cytidine, by providing significant stabilization of the *C3'-endo* form over that of the more flexible *C2'-endo* form [25]. Although the stabilization of the *C3'-endo* form has been found to result from 2-thiolation, 5-substitution

and 2'-*O*-methylation of uridine [1-6], the stabilization by $N^4$-acetylation was unexpected because the position of this modification in the base is spatially far from the ribose ring and cannot interact directly with the ribose moiety. In addition, the crystal structure of $ac^4C$ has been determined as the *C2'-endo* form [17].

As discussed above, the $N^4$-substituent affects the electron density of the H6 atom of the cytidine base, probably due to the electron withdrawing property of the $N^4$-functional group. On the other hand, it was also clear that $N^4$-substitution in base moiety affects the conformational characteristics of ribose. FIG. 3 shows a correlation between the chemical shifts of H6 protons and the enthalpy differences for $bz^4C$ (d), $ac^4C$ (b), cytidine (f) and $m^4C$ (e). This type of effect has been previously observed in the case of 5-substituted uridines [26]. As discussed in Refs. 26 and 27, the electron withdrawing groups enhance the bonding interaction between a lone pair at O4' and the $\pi^*$ orbital of the C5-C6 double bond, and change the glycosidic torsion angle $\chi$ (C2-N1-C1'-O4') to ca. $-180^\circ$ which may in turn result in stabilization of the *C3'-endo* form due to steric repulsion between the 2-carbonyl and 2'-hydroxy groups in the *C2'-endo* form. Decrease of the electron density of H6 by the electron attracting $N^4$-substituent may also provide a probable mechanism for the stabilization of the *C3'-endo* form through stabilization of the hydrogen bond-type interaction between C6-H6 and O5' in pyrimidine nucleotides [28, 29]. FIG. 3 also shows that the enthalpy differences for $ac^4Cm$ (a) and Cm (c) are higher than those expected from chemical shifts of H6 protons, indicating that the 2'-*O*-methylation stabilizes the *C3'-endo* form by a different mechanism than from the $N^4$-substitution.

It should be noted that there is no intermolecular interaction, because 10-fold dilution of the $ac^4C$ sample did not affect the the $^1$H-NMR spectrum at 25°C (data not shown).

***Effect of 2'-O-methylation on the conformation of cytidines*** — In the present study, 2'-*O*-methylation was found to stabilize the *C3'-endo* form of cytidine by 0.3 kcal · $mol^{-1}$. Thus, it was found that 2'-*O*-methylation as well as $N^4$-acetylation leads to enhance conformational stability of cytidine. 2'-*O*-Methylation was earlier found to stabilize the *C3'-endo* form of uridine 3'-monophosphate (Ump) by 0.8 kcal · $mol^{-1}$, due to the steric repulsion between the 2'-*O*-methyl and 2-carbonyl groups,

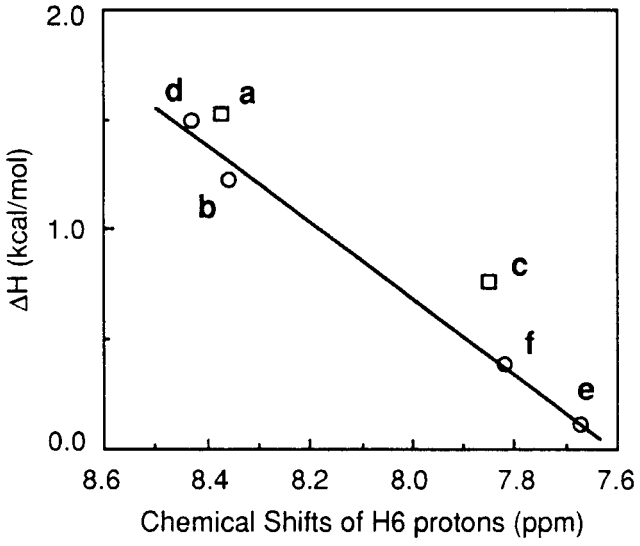


FIG. 3. Chemical shifts of H6 protons and enthalpy differences (ΔH). (a) $ac^4Cm$, (b) $ac^4C$, (c) Cm, (d) $bz^4C$, (e) $m^4C$ and (f) C.



thus providing a precedent for the present cytidine results [6]. For Ump, the 3'-phosphate group pushes the 2'-*O*-methyl group toward the 2-carbonyl group, and enhances the steric repulsion; accordingly, the stabilization for Ump is significantly stronger than that for uridine (0.1 kcal · $mol^{-1}$) [6]. In the case of cytidine, the effect is 0.3 kcal · $mol^{-1}$ without the 3'-phosphate group, whereas the enthalpy difference between the *C2'-endo* and *C3'-endo* forms for uridine [6] and cytidine is the same (0.37 kcal · $mol^{-1}$) within experimental error. The difference between effect of the 2'-*O*-methylation for cytidine and uridine is not known and is yet to be established.

***Additivity of the effects of $N^4$-acetylation and 2'-O-methylation*** — As noted above, the effects of the $N^4$-acetylation and the 2'-*O*-methylation on the conformation of the ribose moiety are additive. This may mean that the molecular mechanism of the stabilization by $N^4$-acetylation and 2'-*O*-methylation are independent of each other, and thus leads to exceptional stability in $ac^4Cm$.

This kind of additivity has also been observed for 5- and 2-substituted uridines [4]. For instance, in the case of uridine, -0.2 (5-meth-

ylation) and 0.8 (2-thiolation) yield 0.6 kcal · $mol^{-1}$ (5-methyl-2-thiolation). Thus, the combination of modifications appears to be an efficient means to generate greater overall stabilization. In fact, in addition to $ac^4Cm$, hyperthermophilic organisms produce a significant number of novel base-ribose multiply modified nucleosides: 5,2'-*O*-dimethylcytidine, 2-thio-2'-*O*-methyluridine, $N^2$,2'-*O*-dimethylguanosine, $N^2$,$N^2$,-2'-*O*-trimethylguanosine [14, 15], and 1,2'-*O*-dimethylinosine [30].

***Role of modified cytidines*** — As described above, $N^4$-acetylation and 2'-*O*-methylation was found to make cytidines more rigid, thus effectively stabilizing the A-type helical conformation (with the *C3'-endo* form of the ribose moiety) of RNA. Some of the biological consequences of these modifications in cytidine are as follows.

The nucleoside $ac^4C$ has been found in the first position of the anticodon of an *E. coli* tRNA specific for methionine (AUG), and Cm has also found in the first position of the anticodon of tRNAs specific for leucine (UUA/UUG), methionine (AUG) and tryptophan (UGG) from several organisms [16]. In this position, both $N^4$-acetylation and 2'-*O*-methylation will stabilize the A-type conformation of cytidine residues. It has been proposed that this form stabilization of pyrimidine residues in the first position of the anticodon avoids misrecognition of codons terminating in pyrimidine nucleotides [4]. Thus, we conclude that $N^4$-acetylation and 2'-*O*-methylation of cytidine residues at position-34 contributes to the correct codon recognition. In the case of tRNAs specific for methionine (AUG) and tryptophan (UUG), the cytidine residues in the first position of the anticodon have been found to be almost always either 2'-*O*-methylated or $N^4$-acetylated except for initiator methionine tRNAs, or tRNAs from chloroplasts or mitochondria [16]. Thus, these modifications may serve to prevent base pairing with adenosine in the third position of the codon. It should be noted that the $N^4$-acetyl group has been found to be proximal to C5 and will not block Watson-Crick base pairing [17].

Because, within the archaeal domain, both $ac^4C$ and Cm occur in the first position of the anticodons in halobacteria [16], it appears likely that $ac^4Cm$ is also located at this position in archaeal hyperthermophiles. In the case of mesophiles, the conformational rigidity of $ac^4C$ and Cm is sufficient for the correct codon recognition. However, for the extremely thermophilic organisms which grow optimally at tempera-

tures near the boiling point of water [31], the exceptional stability of $ac^4Cm$ may be required to maintain correct codon recognition, due to conformational fluctuations at higher temperatures. It should be noted that $ac^4Cm$ is the most conformationally rigid nucleoside thus far analyzed [4, 6].

The contribution of the conformational rigidity to correct codon recognition has also been found in the case of 2-thiolation and 2'-*O*-methylation of uridine [4, 6]. It is therefore concluded that post-transcriptional modifications found in the first position of the anticodon of tRNAs are indispensable for correct codon recognition, which is achieved by the regulation of the conformational characteristics as a result of modification.

Cm has been found not only in the first position of the anticodon (position 34) but also at position 32 (the anticodon loop) and position 56 (the T loop) [16]. The importance of these positions (32, 34 and 56) to the thermostability of tRNA can be considered in terms of the structural similarity of the two loops, in which positions 32 and 34 in the anticodon loop are analogous to positions 54 (in which modified uridines are highly conserved) and 56 in the T loop, respectively [1, 6, 11, 32]. The only known modification of cytidine in the T-loop of archaebacteria is by methylation of C-56 at O-2', which occurs in all 59 reported archaeal tRNA sequences [16]. Accordingly, we conclude that the Cm residues in these loop positions contribute to the thermostability of the tRNA molecules and these are especially important for the extreme thermophiles. The $ac^4C$ in position 12 of the D-stem, which has been exclusively found in eukaryotic tRNAs specific for leucine and serine, may also contribute to the stability of the stem conformation and is possibly recognized by leucyl and seryl-tRNA synthetases of eukaryotes.

In conclusion, through the regulation of conformational properties, all these post-transcriptional modifications contribute to the biological functions of tRNA species, and in the extremely thermophilic archaebacteria, exceptional conformational rigidity is achieved by combinations of modifications.

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