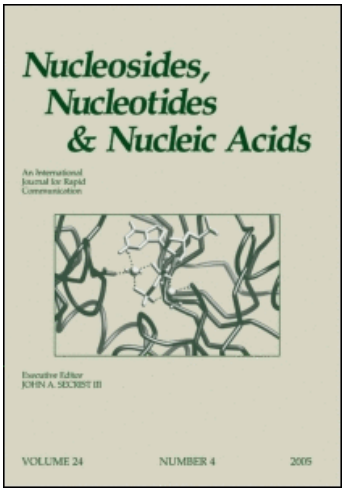


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# Conformatzonal Properties of a Novel Modified Nucleoside, 5-Formylcytidine, Found at the First Position of the Anticodon of Bovine Mitochondrial tRNA$^{Met}$

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# CONFORMATIONAL PROPERTIES OF A NOVEL MODIFIED NUCLEOSIDE, 5-FORMYLCYTIDINE, FOUND AT THE FIRST POSITION OF THE ANTICODON OF BOVINE MITOCHONDRIAL $tRNA^{Met}$

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Abstract: Conformational properties of a novel modified nucleoside, 5-formylcytidine ($f^5C$), which is found at the first position of the anticodon of bovine mitochondrial $tRNA^{Met}$, were analyzed by $^1H$-NMR spectroscopy. $f^5C$ has a normal amino tautomeric form at position 4 of the base moiety. The results indicate the presence of an intramolecular hydrogen bond between the carbonyl of the 5-formyl group and the 4-amino function. $f^5C$ was found to exhibit the C3′-*endo* conformation exclusively and the enthalpy difference ($\Delta H$) between the C2′-*endo* and C3′-*endo* forms was found to be $1.56 \pm 0.13$ kcal/mol, indicating $f^5C$ to be one of the most conformationally rigid nucleosides yet analyzed. The conformational rigidity of $f^5C$ may contribute to regulation of codon recognition by $tRNA^{Met}$.

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## INTRODUCTION

It has been found that most of the posttranscriptional modifications of uridine in the first position of the anticodons of tRNAs regulate codon recognition through control of the conformational rigidity/flexibility of the ribose moiety of the modified residues (1-4). Recently, the conformational properties of several modified cytidines found in tRNAs were analyzed and it was concluded that the cytidine modifications at the first position of the anticodon also serve to regulate codon recognition (5).

In the case of uridine, the functional roles of modification in the first position of the anticodon can be classfied into two groups. In one group, modification results in conformational rigidity, which regulates correct codon recognition by suppression of U · U base pairing; in the other, modification induces enhanced flexibility and serves to maintain the efficiency of tRNA anticodon usage by permitting U · U base pairing (1). By contrast, most modifications of cytidine at the first position of the anticodon make the cytidine residue rigid. The most rigid modified cytidine thus far analyzed is $N^4$-acetyl-2′-*O*-methylcytidine ($ac^4Cm$) (5), which has been found in the tRNAs and 5S rRNA from extremely thermophilic archaebacteria (Archaea) (6-8).

Recently, we have found a novel modified nucleoside, 5-formylcytidine ($f^5C$) in the first position of the anticodon of bovine mitochondrial $tRNA^{Met}$ (9). Because the number of modifications in the mitochondrial tRNAs are exceptionally low (10), the occurrence of $f^5C$ in the anticodon implies an important role in the function of $tRNA^{Met}$, probably in codon recognition. In the present study, the conformational properties of the nucleoside $f^5C$ were analyzed by $^1H$-NMR spectroscopy and the role of the $f^5C$ residue is discussed on the basis of these findings.

## MATERIALS AND METHODS

5-Formylcytidine was synthesized as reported in ref. 9. The synthesized sample (0.5 mg) of $f^5C$ was dissolved in $^2H_2O$ (99.8 atom % $^2H$), evaporated to dryness and then redissolved in 0.4 ml of $^2H_2O$ (99.98 atom % $^2H$). For NMR measurements in $^1H_2O$, the sample (0.5 mg) was dissolved in $^1H_2O$ with 10 % $^2H_2O$.

500 MHz $^1$H-NMR spectra were recorded on a Bruker AMX-500 spectrometer at probe temperatures from 25°C to 60°C. For the NOE experiment in $^2H_2O$, free induction decay (FID) was accumulated with 16K data points with presaturation (5 s) of each of the desired protons and spectra of 32K real data points (spectral width of 7576 Hz) were obtained. For measurements in $^1H_2O$, the water signal was suppressed by a Jump and Return (JR) sequence (11) or by presaturation (1 s). The saturation transfer experiment in $^1H_2O$ was carried out by presaturation (0.5 s) of desired protons and water suppression by the JR sequence. For the precise determination of coupling constants, FIDs were accumulated with 32K data points and spectra consisting of 64K real data points (spectral width of 7576 Hz) were obtained with zero-filling prior to Fourier transformation, resulting in a resolution of 0.1 Hz/point. The spin-coupling constants ($J_{1'2'}$) were determined within 0.1 Hz and were used for estimating the fractional populations of the C2′-*endo* and C3′-*endo* forms with the formula [C2′-*endo*] = $J_{1'2'}/10$ (12), because the coupling constant $J_{3'4'}$ could not determined due to the overlap of the H3′ and H4′ signals. 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 (13).

The UV spectra of f$^5$C and cytidine were measured at several pH values: 0.1 M HCl, 0.1 M sodium citrate buffer (pH 3.0), 0.1 M HEPES-KOH (pH 7.4), 0.1 M sodium carbonate buffer (pH 9.0) and 0.1 M NaOH.

## RESULTS

*Assignments of non-exchangeable proton resonances* — Signals due to ribose protons were assigned on the basis of their chemical shifts and by conventional homonuclear decoupling experiments (data not shown). The resonance at 8.93 ppm was assigned to the H6 proton on the basis of NOEs between the H6 proton and the ribose H1′, H2′ and H3′ protons (Fig. 1c, d). The remaining resonance at 9.49 ppm was, thus, assigned to the H5 proton (in the 5-formyl group). It should be noted that a strong NOE was observed between the H5 and H6 protons (Fig. 1b, c) indicating that the H5 and H6 protons are located spatially close to each other. The

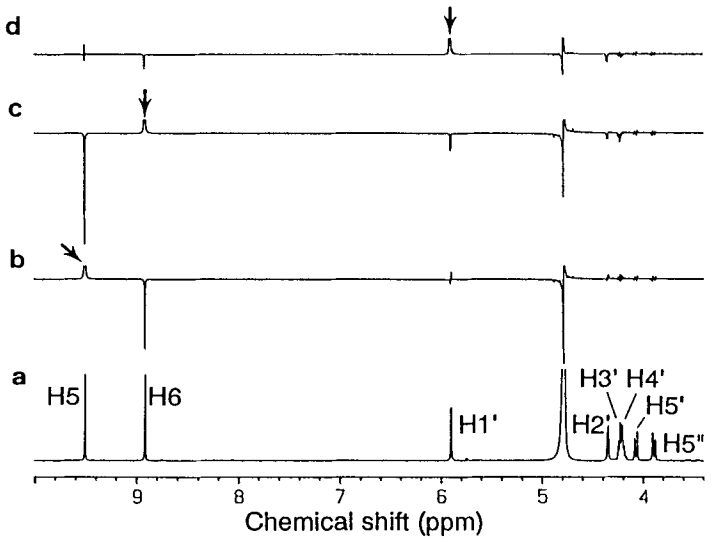


FIG. 1. NOE difference spectra $f^5C$ in $^2H_2O$. (a) normal spectrum, (b-d) NOE difference spectra. Irradiation frequencies are indicated by arrows.



assignments shown in Fig. 1a are consistent with those described in ref. 9.

*Tautomeric form of $f^5C$* — Fig. 2 shows the $^1$H-NMR spectra of $f^5C$ in $^1H_2O$ and in $^2H_2O$. By comparing spectra in $^2H_2O$ and $^1H_2O$ (Fig. 2a and c), two additional resonances (8.33 and 7.49 ppm) were observed in the low-field region in $^1H_2O$ using the JR sequence for water suppression (Fig. 2c). The intensities of these resonances were decreased by presaturation of water signals (Fig. 2b). This indicates that the two resonances at 8.33 and 7.49 ppm correspond to exchangeable protons of the base moiety. LC/MS measurements using deuterated solvents have shown that $f^5C$ has two exchangeable hydrogens in the base moiety (9), thus, each of the two resonances observed is due to an exchangeable proton (not overlapping). In order to establish whether these two resonances are due to an amino group or to an imino structure, a saturation transfer experiment was performed. Fig. 3 shows that when one of the two protons was presaturated, the saturation was transferred

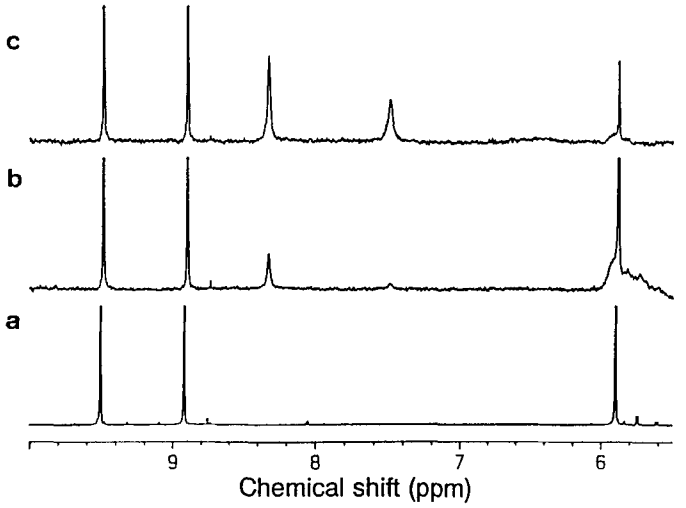


FIG. 2. Low-field region of the NMR spectra of $f^5C$, (a) in $^2H_2O$, (b, c) in $^1H_2O$ with presaturation (1 s) and using the JR sequence for water suppression, respectively.

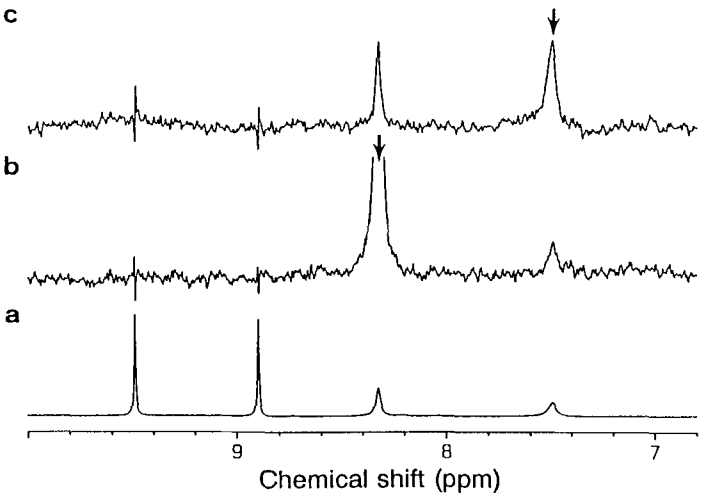


FIG. 3. Saturation transfer experiments in $^1H_2O$. (a) normal spectrum of $f^5C$, (b, c) with presaturations (0.5 s) at the frequencies indicated by arrows. The water signal was suppressed by the JR sequence.

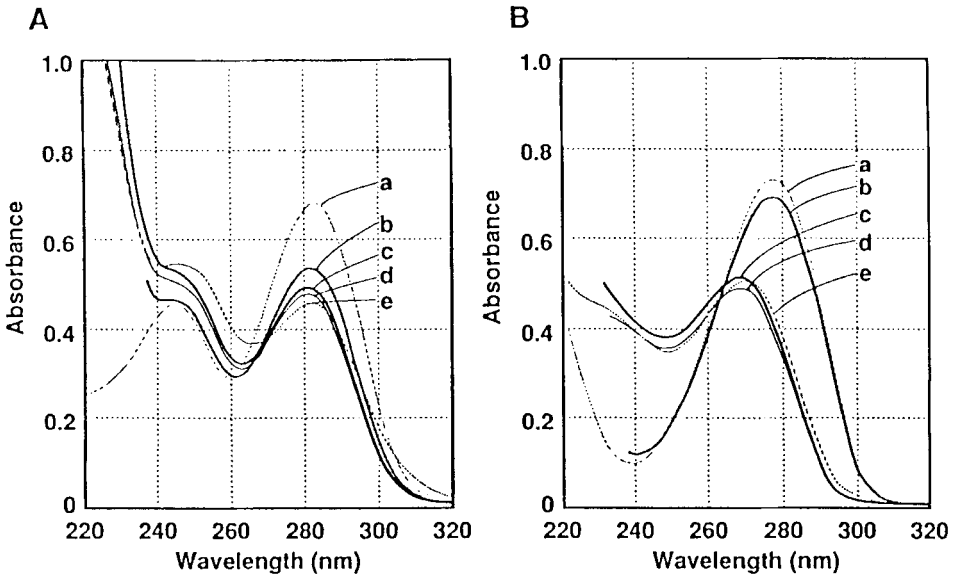


FIG. 4. The pH dependence of the UV spectra of $f^5C$ and cytidine. A. $f^5C$, B. cytidine. (a) 0.1 M HCl, (b) 0.1 M sodium citrate buffer (pH 3.0), (c) 0.1 M HEPES-KOH (pH 7.4), (d) 0.1 M soduim carbonate buffer (pH 9.0) and (e) 0.1 M NaOH



to the other proton, indicating that these protons are interchanging. It should be noted that the water protons are not saturated by presaturation of either of the two exchangeable protons and, thus, the saturation of each exchangeable proton is directly transferred to the other proton by rotation of the amino group. The chemical shifts of these two resonances also suggest that these are due to amino protons because imino protons of nucleic acid bases resonate below 10 ppm (14). Accordingly, it was concluded that these two resonances are due to the 4-amino group and that the tautomeric form of $f^5C$ is the same as that of cytidine.

The $\lambda_{max}$ of UV absorption spectra of $f^5C$ are not changed within the pH range of 1 - 13 (Fig. 4). On the other hand, the intensity of UV absorption increased in the lower pH range, probably due to protonation of $f^5C$. The p*Ka* of $f^5C$ was found to be 2.1 (data not shown), which is

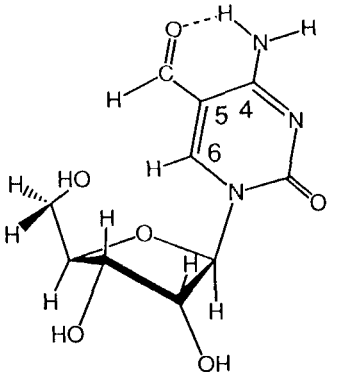

FIG. 5. Preferred conformation of $f^5C$.



significantly lower than that of C (4.2). Thus, $f^5C$ is essentially unprotonated under physiological conditions. The $\lambda_{max}$ of $f^5C$ in neutral or basic solution is longer than that of cytidine probably because of the extended $\pi$-electron system, which includes the formyl moiety.

*Interaction between the 5-formyl and 4-amino groups* — As shown in Fig. 2, the two amino protons resonate at different frequencies (difference 0.84 ppm) and one resonance at lower field (8.33 ppm) was sharper and less exchangeable than the other, suggesting that one of the amino protons is hydrogen bonded, because hydrogen bonding causes a low field shift and decreases the exchange rate, resulting in sharper line shape. The only candidate for such interaction is between the formyl oxygen and amino proton as shown in Fig. 5. In this conformation, the H5 and H6 protons are located close to each other, which is consistent with results from the NOE experiment (Fig. 1) described above.

*Conformational characteristics of the ribose ring* — The sugar pucker was analyzed on the basis of the spin-coupling constant (12). The coupling constants between the H1′ and H2′ protons ($J_{1'2'}$) and the fractional populations of the C2′-*endo* and C3′-*endo* forms at several temperatures are shown in Table 1, and the enthalpy difference ($\Delta H$) and the entropy difference ($\Delta S$) between the C2′-*endo* and C3′-*endo* forms of

TABLE 1. Coupling constants ($J_{1'2'}$) and fractional populations of the C3′-*endo* form ([C3′-*endo*]) of $f^5C$

| temperature (°C) | $J_{1'2'}$ (Hz) | [C3′-*endo*] |
|---|---|---|
| 25 | 2.2 | 0.78 |
| 30 | 2.3 | 0.77 |
| 35 | 2.3 | 0.77 |
| 37 | 2.3 | 0.77 |
| 40 | 2.4 | 0.76 |
| 45 | 2.5 | 0.75 |
| 50 | 2.6 | 0.74 |
| 55 | 2.6 | 0.74 |
| 60 | 2.7 | 0.73 |



$f^5C$ are shown in Table 2, with those of cytidine and a modified cytidine, $N^4$-acetyl-2′-*O*-methyl cytidine ($ac^4Cm$) (5). It was found than $f^5C$ prefers the C3′-*endo* form to a slightly greater extent that $ac^4Cm$, for which $\Delta H$ had been the largest value previously measured (1,3,5). In the C3′-*endo* form of 3′-mononucleotides, rotations about the C4′-C5′ and C3′-O3′ bonds have been found to be fixed in the *gg* and $G^-$ forms, respectively, meaning that the C3′-*endo* form is a rigid conformation (15). Thus, it is concluded that $f^5C$ is one of the most rigid pyrimidine nucleosides, which predominantly adopts the C3′-*endo* conformation as shown in Fig. 5. It should be noted that occurrence of an NOE between H6 and H3′, which is as strong as that between H6 and H1′ and is stronger than that between H6 and H2′ (fig. 1c), indicates predominance of the C3′-*endo*-*anti* form, because only for this conformation do interproton distances fit the observed NOEs (14).

## DISCUSSION

*Mechanism of stabilization of the C3′-endo conformation* —In the case of 5-substituted uridines, Uhl *et al.* (16) have shown that the

TABLE 2. Enthalpy and entropy differences[a] between the C2′-*endo* and C3′-*endo* forms

| compound | enthalpy difference[b] | entropy difference[c] |
|---|---|---|
| $f^5C$ | 1.56 (0.13) | 2.70 (0.40) |
| $ac^4Cm$[d] | 1.53 (0.04) | 2.56 (0.13) |
| C[d] | 0.37 (0.01) | 0.44 (0.04) |

[a] Standard deviations in parentheses.
[b] In kcal · $mol^{-1}$.
[c] In cal · $mol^{-1}$ · $deg^{-1}$.
[d] From ref. 5.



fractional population of C3′-*endo* species is proportional to the Hammett constants for the 5-substituents, and that for 5-formyluridine 80 % of conformers exist in the C3′-*endo* form at 30 °C, compaired with 54 % for uridine at the same temperature. Similar results were reported by Egert and coworkers (17). These are consistent with the present findings: $f^5C$ and C adopt C3′-*endo* conformations to the extent of 77 % and 60 % (5), respectively. Thus, the hydrogen bond-like interaction between H6 and O5′ and/or the interaction between a lone pair at O4′ and the $\pi$ orbital of the C5-C6 double bond may serve to stabilize the C3′-*endo* conformer (16). This type of stabilization has also been observed in the case of 4-substituted cytidines (5), in which case the electron density of H6 was estimated from NMR chemical shifts, and correlated with corresponding C3′-*endo* populations (5). However, in the case of the $f^5C$ the H6 proton resonates at 8.93 ppm, which is more than 1 ppm upfield of unsubstituted cytidine, probably because of the anisotropic deshielding effect of the carbonyl group in the 5-formyl moiety. Therefore, this method for estimation of the electron density of H6 from its chemical shift cannot be applied in the case of the 5-substituted cytidines.

*Function of $f^5C$ in bovine mitochondrial $tRNA^{Met}$* — In the bovine mitochondrial genetic system methionine is coded for by two codons, AUG

and AUA, whereas the codon AUA codes for isoleucine in the universal genetic code (18). Because of the occurrence of $f^5C$ in the first position of the anticodon (9) it is reasonable to assume a functional role in codon recognition by $tRNA^{Met}$. Conformational inflexibility of cytidine in the first position of the anticodon mandates against recognition of either C or U in the third position of the codon (1). Thus, one function of 5-formylation may be to enhance correct codon recognition by prevention of incorrect recognition of codons AUU or AUC. As discussed in ref. 9, $f^5C$ can pair with G in the same manner as unmodified C, and could, in principle, recognize A with the assumption of protonation of A at N1. It is noted that involvement of protonated A with $f^5C$ would require that $f^5C$ remain unprotonated, a reasonable assumption in view of the present findings that the pKa of $f^5C$ is 2.1.

In conclusion, $f^5C$ adopts the C3′-*endo* form predominantly and this conformational characteristic is judged to have functional significance in the regulation of codon recognition. Further additional roles played by formylation, such as recognition by various mitochondrial factors, must await additional studies, including those dealing with *in-vitro* translation analysis, which are needed to clarify the role of $f^5C$ in bovine mitochondrial $tRNA^{Met}$.



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