NMR diffusion and nuclear Overhauser investigation of the hydration properties of thymine: influence of the methyl group

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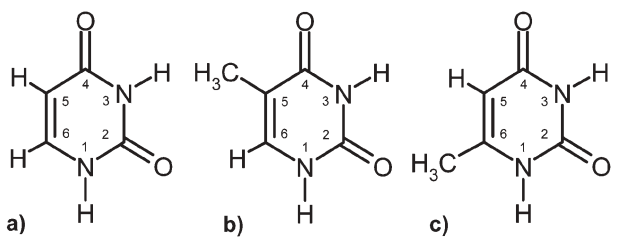
The absence of preferential hydration in thymine and its lowest water accessibility with respect to uracil were evidenced by NMR diffusion and HOESY experiments; the hydration differences observed between these pyrimidine bases were attributed to the electronic rather than steric properties of the methyl group.

Water has long been shown to exert a notable influence on the hydrogen bonding involved in the DNA and RNA structures.[1] For this reason, knowledge of the exchange rates of labile protons with water is of extreme importance in the study of biomolecules. As a matter of fact, Snoussi and Leroy have shown that the exchange rate of the amido proton of thymine in DNA is lower than that of uracil in RNA.[2] In the meantime, Vakonakis and Liwang have evidenced the weaker interactions involved in the adenine–thymine pair with respect to the adenine–uracil one.[3]

In the past decades NMR spectroscopy has become a powerful tool for investigating the solvation properties of both small and large molecules.[4] Meanwhile, NMR diffusion,[5] later extended as Diffusion Ordered SpectroscopY (DOSY),[6] has proved its strength for probing intermolecular interactions.[7] Recently, we described the benefits of NMR diffusion experiments for measuring the exchange rates of the amido protons of uracil with bulk water in the slow exchange regime (Scheme 1).[8] These protons play key roles in the interactions present in nucleic acids, especially proton H_3 which is directly involved in the hydrogen bonding between uracil and adenine in RNA. Significantly different exchange rates were found for H_1 and H_3, the latter being much more labile than the former. In this context, because H_3 is also deeply involved in the hydrogen bonding between thymine and adenine in DNA, we decided to investigate the hydration properties of thymine.

In the present communication, NMR diffusion experiments were used for measuring the exchange rates of the labile protons of thymine and Heteronuclear Overhauser SpectroscopY (HOESY) experiments[9] were applied to evidence specific intermolecular interactions between thymine and water.



Scheme 1 Structures of (a) uracil, (b) thymine and (c) 6-methyl-uracil.

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All NMR experiments were carried out at 300 K on a Bruker AVANCE 500 MHz spectrometer fitted with a $^1H/^{13}C/^{15}N$ triple resonance cryoprobe optimized for ^1H detection and equipped with a 55 G cm^{-1} gradient coil.† ^{15}N–^1H and ^{13}C–^1H HOESY experiments were acquired using the pulse sequence proposed by Bigler and Köver.[10] Moreover, a series of NMR diffusion experiments at distinct diffusion time (Δ) were recorded by using the bipolar pulse pair longitudinal eddy-current delay (BPPLED) sequence[11] and by paying attention to the radiation damping effect.[12] For each experiment, the durations (δ) of the gradient pulses (g) were optimized whereas the LED was kept equal to a low value (5 ms).[13] The exchange rates of the amido protons were calculated from their experimental diffusion decays as described elsewhere.[8,14]

NMR diffusion experiments allowed the exchange rates for the H_1 and H_3 protons of thymine to be calculated, and values of 5 s^{-1} and 7 s^{-1} were found, respectively. With respect to the values obtained for the amido protons of uracil, 8 s^{-1} and 18 s^{-1}, respectively, two conclusions could be drawn. Firstly, the amido protons in thymine exchange more slowly than in uracil, suggesting that thymine has a lower water accessibility. Secondly, in thymine, the exchange rates for H_1 and H_3 were similar whereas, in uracil, H_3 was shown to exchange much more rapidly than H_1. Therefore, in contrast to uracil, there seems to be no preferential hydration in thymine.

Moreover, because the intensity of heteronuclear NOEs is proportional to the inverse of the sixth power of the intermolecular distance, HOESY experiments can be used for determining the local position of water in the first thymine hydration shell. Specifically, this hydration shell has been defined up to 2.5 Å from previous studies.[15,16] Therefore, because heteronuclear NOEs between ^{15}N and ^1H nuclei are observed when the internuclear distance is lower than 3 Å, ^{15}N–^1H HOESY experiments are appropriate to investigate the innermost thymine hydration layer. Moreover, it has been reported that the residence time of the water molecules in the first thymine hydration shell can be roughly estimated by using the Einstein relationship for one-dimensional diffusion.[17] By assuming a translational diffusion model where water and thymine molecules are represented by hard spheres, the closer approach distance is limited by the van der Waals radii of the hydrogens. Thus, the residence time of a given water molecule at its hydration site may be obtained from the water diffusion coefficient. By assuming an average displacement of 2.5 Å and a water diffusion coefficient of 6.6×10^{-10} m^2 s^{-1}, as measured in

our experimental conditions, we can estimate the water proton residence time to be about 1×10^{-10} s. Fig. 1 shows the ^{15}N–^{1}H HOESY spectrum of a ^{15}N-enriched thymine sample recorded with a mixing time of 400 ms. The 1D trace along the water chemical shift is also shown. This trace illustrates the intermolecular interactions between water and thymine. With respect to the corresponding trace obtained for uracil in the same experimental conditions,[15] weaker cross-peaks intensities were evidenced for thymine. Therefore, water molecules are more distant to thymine than uracil in the first hydration shell of the respective pyrimidine bases. This agrees with the lower water accessibility of thymine observed by NMR diffusion experiments. In addition, in thymine, the ratio between the intensities of the N_3/water and N_1/water cross-peaks was 1.2 whereas, in uracil, a value of 2.0 was found.[8,15] This confirmed the absence of preferential hydration in thymine as suggested by NMR diffusion experiments.

All these results suggest that the presence of the methyl group strongly influences the hydration properties of thymine. A first possible explanation for this effect is related to the steric hindrance of the hydrophobic methyl group. This may alter the distribution of the water molecules in the first hydration shell of thymine, especially around the carbonyl group in position 4, hereby reducing correspondingly the H_3 lability.

Remarkable differences between uracil and thymine have already been reported in the literature. Hobza *et al.* have used *ab initio* computations for characterizing the atomic charges of the oxygen atoms of thymine and uracil in the gas phase.[18] They showed that O_4 has a higher atomic charge in thymine, which may correspondingly reduce its attraction to water. In contrast, no difference was found for O_2. In the meantime, by analyzing fluorescence spectra of hydrated nucleic acid bases, Gustavsson *et al.*[16] have demonstrated that the excited state properties of thymine as well as other uracil derivatives, are influenced by the nature and position of substituents. In this case both studies have shown that the methyl group has, in addition to its steric effect, an electronic influence which may modify the hydration properties of thymine. To better ascertain the role played by the methyl group, we studied 6-methyl-uracil, where the methyl group is in position 6 instead of position 5 (Scheme 1).

To qualitatively describe the local distribution of water molecules, ^{13}C–^{1}H HOESY experiments‡ were recorded on thymine and 6-methyl-uracil samples with a mixing time of 6 s. Fig. 2 shows the 1D traces along the water chemical shift extracted from the ^{13}C–^{1}H HOESY spectra of thymine and 6-methyl-uracil (Fig. 2a and 2b, respectively). From these traces, the ratio between the C_4 and C_2 integrals could be calculated. With respect to the $\approx$ 0.5 value reported for uracil in the same experimental conditions,[15] a lower value was obtained for thymine ($\approx$ 0.3) whereas no value could be calculated for 6-methyl-uracil (Fig. 2b). All together, these data showed that, although the steric hindrance around C_4 in uracil and 6-methyl-uracil is virtually equivalent, no significant intermolecular interactions could be detected between water and C_4 for 6-methyl-uracil. This clearly demonstrates that the CH_3 group influences electronically, rather than sterically, the hydration properties of thymine. In addition, Fig. 2 shows that this perturbation mainly takes place on C_4, which may in turn explain the H_3 lability difference evidenced between thymine and uracil.

In conclusion, by combining NMR diffusion and HOESY experiments, substantial differences in the hydration properties of uracil and thymine have been highlighted. In addition, by studying 6-methyl-uracil, it has been possible for the first time, at least to the

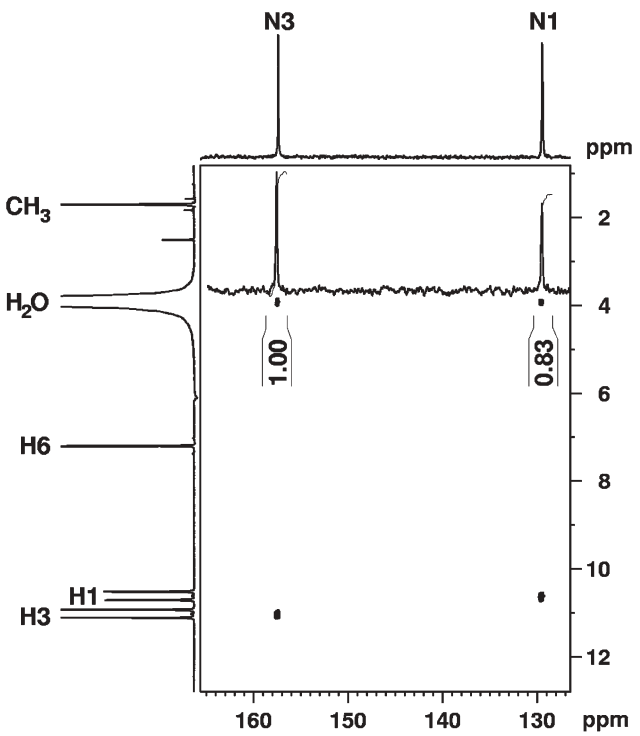

Fig. 1 ^{15}N–^{1}H HOESY NMR spectrum of ^{15}N-enriched thymine recorded at 50.7 MHz with a mixing time of 400 ms. The horizontal and vertical projections show the corresponding $\{^{1}H\}^{15}N$ and ^{1}H spectra, respectively. Intermolecular NOEs are shown between water and nitrogens N_1 and N_3 (labels follow Scheme 1). The 1D trace along the water chemical shift is also reported. The N_3–water/N_1–water cross-peaks ratio is 1.2.

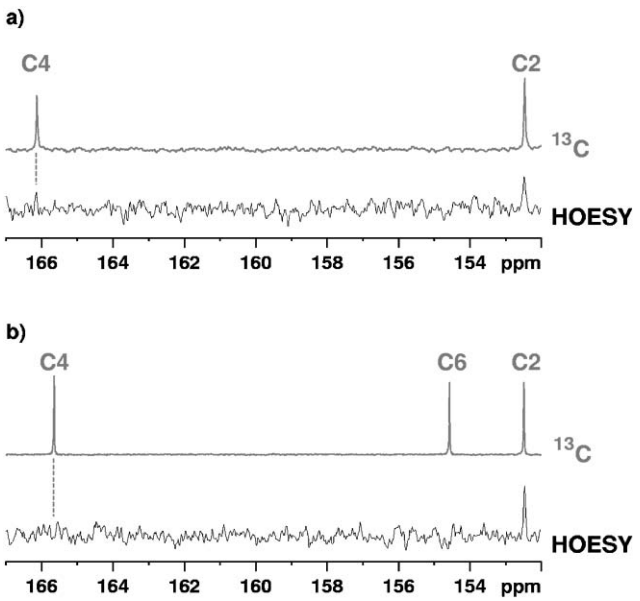

Fig. 2 1D traces (in black) along the water chemical shift extracted from the ^{13}C–^{1}H HOESY spectra recorded on unenriched samples of (a) thymine and (b) 6-methyl-uracil. The respective ^{13}C spectra§ are shown above.

best of our knowledge, to experimentally demonstrate the electronic influence played by the methyl group in the hydration of pyrimidine bases. All these results should contribute to better understanding the differences observed for the hydrogen bonding strength in DNA and RNA.[19]

Notes and references

† 12.3 mg of a ^{15}N-doubly enriched thymine sample from Cambridge Isotope Laboratories, Inc., were dissolved in 0.5 mL of DMSO-d_6, and 0.1 mL of H_2O was added to provide a large excess of water *vs.* thymine (molar ratio of about 60 : 1).

‡ Because ^{15}N-enriched 6-methyl-uracil was unavailable, ^{15}N–^{1}H HOESY experiments could not be performed in a reasonable experimental time on our spectrometer. Therefore, in order to probe the local distribution of water molecules near the carbonyls, ^{13}C–^{1}H HOESY experiments were recorded on unenriched thymine and 6-methyl-uracil samples, which were prepared as described in the previous note.

§ Note that the ^{13}C spectra shown in Fig. 2 were not recorded with the same number of scans.

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