Determining chemical exchange rates of the uracil labile protons by NMR diffusion experiments†

Pierre Thureau, Bernard Ancian,* Stéphane Viel and André Thévand

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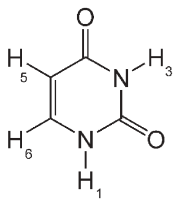
The exchange rates of the amido-protons of uracil with water were determined by NMR diffusion experiments and the results showed a factor 2 difference in lability between them, which was confirmed by more classical 2D-NMR exchange experiments.

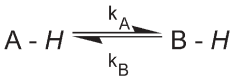
NMR diffusion[1] and, more recently, diffusion ordered spectroscopy (DOSY)[2] have become invaluable tools for studying the size and shape of molecules[3,4] as well as aggregation and binding processes.[5–7] These methods have also been used for calculating association constants between enzyme–inhibitor,[8] ligand–protein[9] and macrocyclic guest–host complexes.[10]

The effects of chemical exchange in NMR diffusion experiments were described by Johnson[11] using a two-site model. More recently, Cabrita *et al.* have qualitatively estimated the exchange rates of the hydroxyl protons of sucrose[12] and butanol[13] with water, by varying the diffusion time in a series of DOSY experiments. However, the quantitative determination of exchange rates remains extremely difficult because many other factors, such as T_1 and T_2 relaxation, overlap with water resonance, radiation damping, *etc.*, may interfere.[6,8] Therefore only few applications have been reported in the literature, such as the study of the amide protons of the acyl Carrier Protein,[14] the labile protons of a 16 bp DNA[15] and the NH protons of viomycine.[16]

In this communication, NMR diffusion experiments were used for measuring quantitatively the exchange rates of the 2 amido-protons of uracil, H_1 and H_3 (Scheme 1), with water. These protons play a key role in the interactions present in the nucleic acids, especially H_3 because it is involved in the hydrogen bonding between uracil and adenine in RNA. Here the exchange is assumed to be described by a two-site model (Scheme 2), where a labile proton exchanges exclusively between uracil (site A) and water (site B). In addition the exchange is slow on the chemical shift timescale, as can be seen in the ^{1}H spectrum of uracil (Fig. 1a).



Scheme 1 Molecular structure of uracil.



Scheme 2 Two-site exchange model.

All NMR experiments were carried out at 300 K on a Bruker AVANCE 500 MHz spectrometer fitted with a ^{1}H/^{13}C/^{15}N cryoprobe equipped with a 55 G cm^{-1} gradient coil. 11 mg of ^{15}N-enriched uracil (Cambridge Isotope Laboratories, Inc.) were dissolved in 0.5 mL of DMSO-d_6 and 0.1 mL of H_2O were added to have a large excess of water *versus* uracil. Note that both nitrogens were labelled. A series of 10 NMR diffusion experiments were recorded by increasing the diffusion time (Δ) from 50 to 900 ms, using the bipolar pulse pair longitudinal eddy-current delay (BPPLED) sequence.[17] For each experiment, the durations (δ) of the gradient pulses (g) were optimized and the LED was kept equal to a low value (5 ms).[18] Although previously performed,[19] the assignments of the ^{1}H and ^{15}N spectra were confirmed by conventional two-dimensional NMR experiments.‡

Fig. 1 shows the DOSY maps§ of uracil for 3 distinct Δ values, namely 50, 200 and 900 ms. Typically, in NMR diffusion experiments, all the nuclei belonging to the same molecule are characterized by the same diffusion coefficient. This may not be the case, however, for the nuclei that undergo chemical exchange. In fact, if the spins exchange between the uracil and water sites during the diffusion time, then their measured diffusion coefficient is an average between the uracil and water diffusion coefficients, weighted by the exchange rate. For Δ = 50 ms (Fig. 1a), the diffusion coefficients of the amido and ethylenic protons are clearly different, although these protons belong to the same molecule. A difference can even be detected between the diffusion coefficients of the amido-protons themselves. This qualitatively indicates that H_1 and H_3 are in exchange with water and that H_3 is more water like than H_1, *i.e.* its exchange rate is expected to be greater. For Δ = 200 ms (Fig. 1b), the difference between the diffusion coefficients of H_1 and H_3 increases, which confirms their difference in exchange rate, and both tend to the value of the water diffusion coefficient. For Δ = 900 ms (Fig. 1c), H_1 and H_3 have the same diffusion coefficient as water, meaning that they are totally located on the water solvent.

To calculate the exchange rates, the intensities of the H_1 and H_3 resonances were plotted as a function of the square of the gradient pulse area, as shown in Fig. 2 for a diffusion time of 300 ms. A two-parameter non-linear least-squared fitting procedure based on the analytical solutions of the diffusion-modified Bloch equations[11]

Jeune Equipe 2421 TRACES, Université de Provence et Paul Cézanne, Centre de St Jérome, 13397 Marseille cedex 20, France.
E-mail: bernard.ancian@univ.u-3mrs.fr; Fax: 0033491282897;
Tel: 0033491288643

† Electronic supplementary information (ESI) available: Fig. A1–A3. See DOI: 10.1039/b513580j

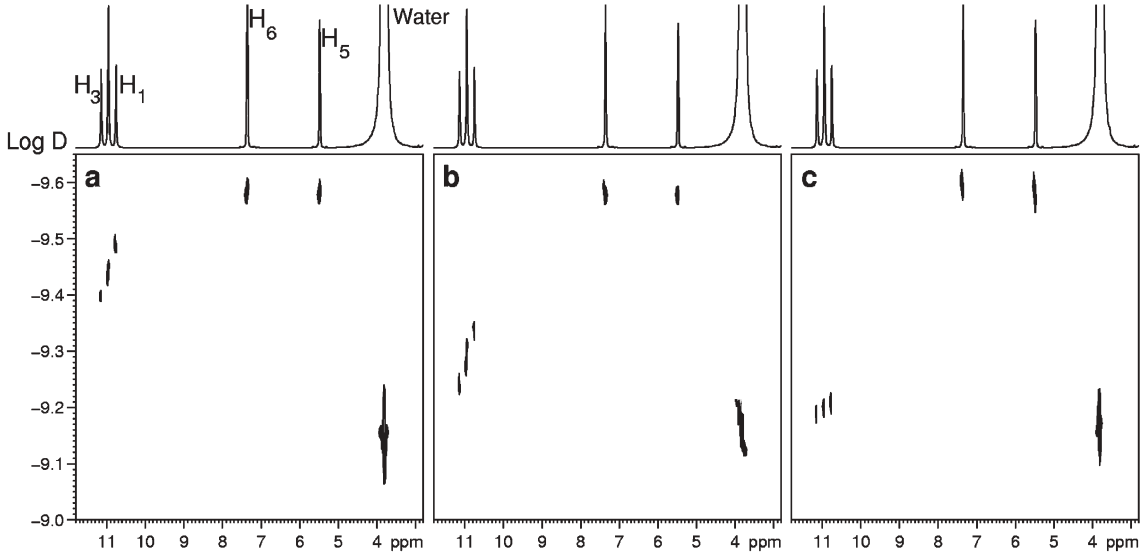


Fig. 1 DOSY maps of uracil for a diffusion time Δ of (a) 50 ms, (b) 200 ms and (c) 900 ms. The projection in 1a shows the ^{1}H spectrum along with the corresponding assignment.[19] Diffusion coefficients are expressed in $m^2\ s^{-1}$.

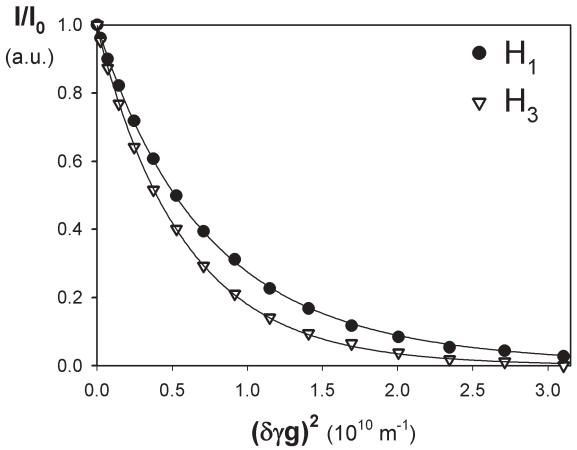


Fig. 2 Evolution of the intensity of the H_1 (•) and H_3 (▽) resonances as a function of the square of the gradient pulse area for a diffusion time Δ of 300 ms. The curves are best fit obtained with a regression coefficient close to 0.99, and the exchange rates are 8 s^{-1} and 18 s^{-1} for H_1 and H_3, respectively.

was then applied to the experimental decays to extract the exchange rates k_A and k_B. To fit the data, the true diffusion coefficients of uracil and water, *i.e.* in the absence of chemical exchange, were required as input parameters. The first diffusion coefficient was obtained by analysing the ethylenic protons of uracil and averaging the values obtained from all experiments performed with different Δ values. The second diffusion coefficient was obtained by analysing the water resonance in an independent experiment recorded under the same experimental conditions, but without uracil. These calculations were performed for each NMR diffusion experiment, leading to average k_A values of 8 s^{-1} and 18 s^{-1} for H_1 and H_3, respectively, with a standard deviation of $\pm 6\%$.

To our knowledge, except for the studies by Bellon *et al.* and Engler *et al.*, who considered 1-cyclohexyluracil in a different system,[20,21] no exchange data are available for the amido-protons of uracil. Therefore, to confirm NMR diffusion data, a series of exchange spectroscopy (EXSY)[22] experiments at different mixing times were performed, and k_A values of 10 s^{-1} and 20 s^{-1} were obtained for H_1 and H_3, respectively. This very good agreement confirmed the robustness of NMR diffusion experiments for estimating rate constants in the case of slow exchange. Moreover, because only one experiment is in principle required, the NMR diffusion approach is much faster.

Finally, Chahinian *et al.* have recently studied ^{15}N enriched uracil in a DMSO-d_6/H_2O mixture by one- and two-dimensional NMR techniques.[19] In particular, by using HOESY experiments at short mixing times, they showed that, in the first solvatation shell of uracil, nitrogen N_3 is surrounded by twice as many water molecules as nitrogen N_1.¶ This result may explain the 2-fold lability of the H_3 proton evidenced in this study by NMR diffusion and NOESY experiments.

Notes and references

‡ See Fig. A1–A3. Because of their ^1J coupling to ^{15}N, both H_1 and H_3 should appear as doublets in the ^{1}H spectrum. However, because of their close chemical shifts (10.84 and 11.03 ppm) and their relatively large ^{1}H–^{15}N coupling constants (95 and 90 Hz), one component of each doublet almost perfectly overlaps, which leads to the observation of a 'pseudo-triplet' in the ^{1}H spectrum (actually, H_1 is also ^3J coupled with H_6: $^3J \approx$ 6 Hz).

§ The DOSY maps were obtained by mono-exponential fitting. This is the reason why the central line of the 'pseudotriplet' due to H_1 and H_3 is characterized by a diffusion coefficient (D) that lies between the diffusion coefficients of H_1 and H_3. Clearly, only the external lines of the 'pseudotriplet', *i.e.* the non overlapping components of the H_1 and H_3 doublets, were used to determine $D(H_1)$ and $D(H_3)$.

¶ Because we worked on an almost equivalent system (only a slight difference in the DMSO-d_6/H_2O ratio) and because the HOESY experiments that we performed gave identical results, Fig. A1, we can safely assume that the conclusion of Chahinian *et al.* is also valid in our case.

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