Determination of fast proton exchange rates of biomolecules by NMR using water selective diffusion experiments

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Abstract We present a new water selective pulse sequence allowing rapid determination of exchange rates of labile protons on the millisecond time scale. Using diffusion measurements, exchange rates of resolved protons can be determined without prior knowledge of relaxation parameters in a short overnight experiment. The use of a sensitive, highly selective and easy to implement water excitation scheme allows for its straightforward application to a wide range of biomolecules. The results obtained for the imino proton exchange rates of a 16 bp DNA are in strong agreement with values obtained by the classical approach of two-dimensional exchange spectroscopy.

Key words: Hydrogen exchange rate; NMR; Diffusion; Water-protein interaction; DNA

1. Introduction

Knowledge of the exchange rates of labile protons is very important in the study of biomolecules; it provides information about local and global flexibility, allows derivation of secondary structure as well as assessment of interaction interfaces, and enables features of great interest such as folding and unfolding of biomolecules to be followed. For slowly exchanging protons showing residence times on the time scale of minutes, the measurement protocol of exchange rates by hydrogen/deuterium isotope exchange is well established [1]. No such simple and practical method exists for measuring exchange rates of rapidly exchanging protons. The methods which have been described are time-consuming [2,3], or require either prior knowledge of the relaxation parameters of each labile proton, or restriction to initial slope analysis [4–8]. Moreover, at high magnetic field, radiation damping is a serious problem for several of those methods [2,5,6]. Only recently, schemes for the selective excitation of water without the need for special equipment have been proposed [9–12]. Two methods bypassing most of the above-mentioned problems have been described [13,14], but they require fully labeled proteins.

Several years ago, Moonen et al. [15] introduced an approach made very attractive by its independence from relaxation parameters. Indeed, separate determination of the relaxation parameters of rapidly exchanging protons is often non-trivial due to their close relationship to the exchange rates [2], and experiments dependent on relaxation parameters are either restricted to initial slope analysis, or require a two-parameter fit to analyze the data [6,8]. To avoid those difficulties, Moonen's approach uses differences in the diffusion behavior of the exchanging protons to measure residence times. This principle relies on the fact that the diffusion constant of a hydrogen atom which is part of a water molecule is much smaller than the diffusion constant of a hydrogen atom which is part of a biopolymer. A hydrogen atom exchanging between those two molecules during the diffusion period will show a weighted diffusion constant, which is a function of its lifetime on each molecule.

The fact that only the 1D extract at the water frequency of each 2D spectrum actually contains the desired information leads us to the development of the one-dimensional experiment selective at the water frequency. We thus propose here a new pulse sequence which maintains the principle of exchange rate determination by diffusion, but which shows the important advantage of being much less time-consuming by the use of water selective excitation. This strategy thus opens the way to the study of larger molecules requiring multidimensional spectra for resolution enhancement. In fact, very recently a similar approach has been used to determine the exchange rates of several amide protons of the Acyl carrier protein [16].

We apply our method to measure the exchange rates of the fast exchanging imino protons of a 16 bp DNA, which is a synthetic operator site of the AlcR DNA binding domain. (AlcR is a transcription factor which is the gene product of *alcR*, a positively acting regulatory gene of the ethanol utilization pathway of *Aspergillus nidulans*, see Felenbok et al. [17], Cerdan et al. [18] and references therein.)

2. Materials and methods

Since the introduction of pulsed field gradients in modern high field NMR, the measurement of diffusion, as introduced by Stejskal and Tanner [19], is no longer a problem. To measure diffusion by NMR, two gradients have to embrace an evolution period, during which diffusion and exchange between the two molecules can take place, as shown by Moonen et al. [15]. The detected signal is a function of the gradient strength G and gradient duration δ, the diffusion time t_{dif} and the effective diffusion coefficient D_{eff}:

$$S = S_0 \exp(-\gamma^2 G^2 \delta^2 D_{eff} t_{dif}). \quad (1)$$

γ is the gyromagnetic ratio of the spin. The effective diffusion coefficient D_{eff} is the weighted sum of the diffusion coefficients of the water D_w, the biopolymer D_b, the lifetime t_w of hydrogen in water before exchange occurs, and of t_b, the lifetime of hydrogen on the biopolymer following exchange:

$$D_{eff} t_{dif} = (D_w t_w + D_b t_b). \quad (2)$$

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Abbreviations: AlcR, transcription factor, product of the *alcR* regulatory gene of *Aspergillus nidulans*; NMR, nuclear magnetic resonance; bp, base pair

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This equation is valid only if the diffusion time t_{dif} is large compared to the lifetime of the proton on the biopolymer. If this condition is not fulfilled, a multidimensional experiment must be performed to distinguish between exchanged and non-exchanged spins [15], and the probability that the spin exchanges at each moment during the diffusion time must be included, leading to the following equation [15]:

$$S = S_0 \frac{\exp(-\gamma^2 G^2 \delta^2 D_w t_{dif}) - \exp(t_{dif}/t_b)\exp(-\gamma^2 G^2 \delta^2 D_b t_{dif})}{(1-\gamma^2 G^2 \delta^2 (D_w - D_b) t_b)(1-\exp(t_{dif}/t_b))} \quad (3)$$

The lifetime of the hydrogen proton on the biopolymer can then be obtained by a fit of the signal intensity of the magnetization observed in a two-dimensional spectrum at the ω_1 frequency of water. These signals contain information about proton magnetization which was excited at the water frequency, and subsequently transferred during the diffusion time by exchange to the biopolymer. However, the selective observation of these signals in a one-dimensional experiment is non-trivial due to the problem of selective excitation at the water frequency at high field, caused by the presence of radiation damping [20,21]. Indeed, radiation damping brings water magnetization back from the z-axis to its equilibrium position within about 100 ms using our standard triple resonance gradient probe at a field of 600 mHz. For the long (and thus weak) selective pulses which are necessary for work with most biomolecules in order to avoid the excitation of other resonances close to the water frequency, the field created by radiation damping is strong enough to act against the pulse, possibly preventing it totally.

To avoid this, we developed a method [10] which is derived from the approach proposed by Otting and Liepinsh [9]. It consists of exciting all spins by a hard pulse, followed by a delay allowing water magnetization to return selectively to the z-axis under the influence of radiation damping. The magnetization is held each second scan on the z-axis by the application of a weak gradient pulse, and the scans are added up in the manner of a difference experiment. Fig. 1 shows the complete pulse sequence for the selective diffusion experiment. The magnetization is inverted using a 160° pulse, rather than a 180° pulse, resulting in the return of the water magnetization to equilibrium under the influence of radiation damping within only 55 ms. Thus, for every odd scan the water magnetization is aligned along the z-axis after the delay t_{RD}. Every even scan, the water magnetization is held

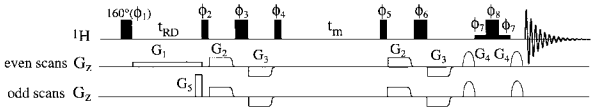

Fig. 1. Scheme of the pulse sequence for the water selective diffusion experiment. The first pulse is a 160° pulse; the remaining narrow and wide pulses correspond to 90° and 180° flip angles. The ^{1}H carrier was set at the H_2O frequency. The 160° pulse is followed by a delay $t_{rd} = 40$ ms allowing most of the water magnetization to return to the z-axis for all odd scans. At the end of t_{rd}, residual transverse magnetization is destroyed by the gradient G_5. For even scans, water magnetization is held down by a weak square gradient pulse G_1 with a strength of 50 mG/cm. The magnetization is defocused by the application of a self-compensating gradient [23] consisting of the gradient pair G_2 and G_3 and the π-pulse. G_2 and G_3 have a square amplitude profile in the first 3/4, followed by a half-Gaussian amplitude profile in the last quarter. The use of this type gradients exhibiting extremely good recovery times is necessary to avoid signal attenuation at high gradient strengths due to non-recovered field homogeneity rather than to diffusion effects. The strength of the gradients is varied between 2 and 17 G/cm, their duration is constant (2·2 ms for each). The magnetization is refocused after the time Δ by a second pair of gradients. (Δ is related to the diffusion time by $t_{dif} = \Delta + 2\delta/3$, where δ is the length of the gradient.) Water suppression is achieved using the WATERGATE sequence [22]. The gradients G_4 have a Gaussian amplitude profile and a strength of 22.5 G/cm at their center. The RF field strength of the water selective pulses of the WATERGATE sequence is 170 Hz. The interscan delay is 5 s. The phases are: $\phi_1 = 2x, 2y, 2(-x), 2(-y)$; $\phi_2 = 2x, 2y, 2(-x), 2(-y)$; $\phi_3 = 2y, 2(-x), 2(-y), 2x$; $\phi_4 = 2(-x), 2(-y), 2x, 2y$; $\phi_5 = 2x, 2y, 2(-x), 2(-y)$; $\phi_6 = 2y, 2(-x), 2(-y), 2x$; $\phi_7 = x$; $\phi_8 = (-x)$; $\phi_{acq} = x, (-x), (-y), y, (-x), x, y, (-y)$.

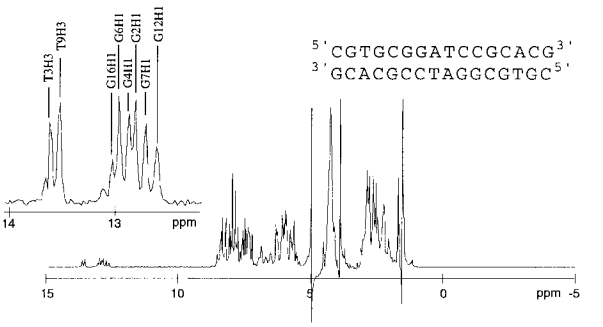

Fig. 2. Sequence of the 16 bp synthetic DNA fragment and its 1D spectrum. The extract shows the imino proton region. The spectrum was recorded on a Bruker AMX600 spectrometer using a triple resonance probe head with a self-shielded z-gradient coil. The sample is a 4 mM solution of the DNA, 90% H_2O and 10% D_2O, at pH = 7.2 and T = 298 K.

by a gradient on the z-axis. After being brought to the xy-plane, the magnetization is defocused by the first gradient pair. Diffusion and exchange of longitudinal magnetization created by the 90° pulse takes place during t_m. At the end of the diffusion time, the magnetization is brought back into the plane and a second gradient pair refocuses part of the magnetization as a function of the diffusion time and the gradient strength. The water suppression is achieved using the WATERGATE sequence [22].

3. Results and discussion

The system of interest is a synthetic 16 bp DNA which represents the palindromic operator site of the DNA binding protein AlcR [17,18]. Its base pair sequence as well as its one-dimensional spectrum are shown in Fig. 2. This DNA acts as a good model system, as all its imino protons are already well resolved in a 1D spectrum.

Fig. 3 shows the extracts of the imino proton region of spectra taken with the pulse sequence shown in Fig. 1 at gradient strengths between 2 and 17 G/cm. Three large signals are observed for the T3H3, T9H3 and G2H1 imino protons exchanging on the millisecond time scale, while G6H1, G7H1 and G12H1 show slower exchange rates, as indicated by the weak signals in the spectrum. The absence of a signal for G4H1 indicates even slower exchange for this proton. The terminal imino protons of the DNA fragment exchange too fast to be observed. Each experiment takes about 45 min; the whole series of 16 experiments can thus be acquired within 12 h.

Fig. 4 shows the signal intensities observed for the imino protons T3H3 (A), T9H3 (B) and G2H1 (C) as a function of the gradient strength. The best least square fits of Eq. 3 to the data were determined using MATLAB. The diffusion constant D_b of the DNA was determined using the pulse sequence shown in Fig. 1, but without the selective excitation. The resulting signals from non-labile protons as a function of gradient strength were fitted to Eq. 1, D_{eff} being in this case the diffusion coefficient D_b of the DNA. A value of 0.11×10^{-9} m^2 s^{-1} was obtained for D_b at 298 K. The diffusion coefficient of water is 2.299×10^{-9} m^2 s^{-1} at 298 K, the proton gyromagnetic ratio is 2.67519×10^9 T^{-1} s^{-1}, the gradient length δ is 4 ms, and the diffusion time 302.7 ms. Using these parameters, the fits result in residence times of 74 ms for T3H3, 285 ms for T9H3, and 45 ms for G2H1 (see Table 1) i.e. their exchange rates are 13.5 s^{-1}, 3.5 s^{-1} and 22.2 s^{-1}, respectively.

As can be expected, these exchange rates show perfectly how the position of the two bases in the sequence and the hydrogen bonding pattern between them influence the stability of the connection between the bases of each strand. G2H1 is the only guanine imino proton exchanging on the millisecond time scale, reflecting the fact that end-fraying takes place at the termini of the double-stranded DNA fragment. The remaining guanine imino protons exchange more slowly than the thymine imino protons, as expected from the higher stability of the guanine-cytosine pair, conditioned by the supplementary hydrogen bond. End-fraying is still felt by the third base pair, as indicated by the four-fold shorter life time of T3H3 when compared to T9H3. The relatively slow exchange rate of T9H3 corresponds well to its position in the center of the double-stranded DNA fragment.

To assess the accuracy of the values obtained by our method, we also measured the residence times using the well established protocol of two-dimensional exchange spectroscopy [2,3]. This approach uses the attenuation of the diagonal signals and the build-up of the cross peaks at the water frequency derived from a series of 2D spectra at different mixing times. A series of 12 two-dimensional spectra of 256×1024 points was recorded with a total acquisition time of 34 h. The lifetimes obtained by this approach were 57, 278, and 38 ms for the T3H3, T9H3, and G2H1 imino proton, respectively (Table 1). These values are very close to those obtained by the diffusion experiments using a diffusion time of 3027 ms, and thus confirm the validity of our measurements.

We also tested our method using longer diffusion times. For diffusion times of 602.7 and 902.7 ms, one observes that the residence time for the T9H3 imino proton stays with a high precision close to the value determined at 302.7 ms. Contrarily, the residence times for the fast exchanging protons seem to increase with increasing diffusion times (Table 1). This can be attributed to the small fraction of imino protons which already have exchanged with water during the water selective pulse of a duration of 40 ms. Those protons then diffuse during the entire diffusion time with the diffusion constant of the biomolecule. One can see this as a contamination of the desired cross peak by the non-desired diagonal peak. However, this contamination gives rise to large errors only at long diffusion times, as exemplified for T3H3: at a diffusion time of 302.7 ms, the small fraction of exchanged protons contributes to the observed residence time with an apparent residence time of 302.7 ms (resulting in an overall residence time of 74 ms), as compared to 902.7 ms (resulting in an overall residence time of 155 ms) at diffusion times of 902.7 ms. For protons showing longer residence times, the exchange occurring during the pulse is negligible, as shown by the constant values observed for the T9H3 imino proton. One can thus conclude that for residence times of the order of 300 ms or longer, correct results are obtained at any diffusion time used. For protons exchanging on the time scale of the water selective pulse, short diffusion times should be used in order to minimize the error in the measured residence times. Typically, at a diffusion time of 302.7 ms, all the values measured by our technique are at least as accurate as those obtained by the original 2D diffusion approach [15].

We have proposed here the use of a selective one-dimensional diffusion experiment to measure residence times of rapidly exchanging protons. This approach allows the determination of the exchange rates of nucleic acids and small proteins

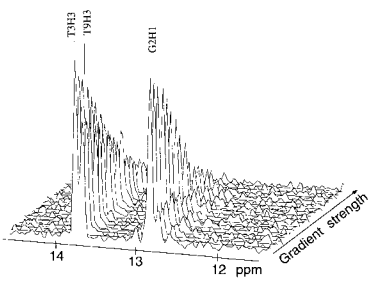

Fig. 3. Extracts of the imino proton region of spectra acquired with the pulse sequence of Fig. 1. Signal attenuation is shown using gradient strengths from 2 to 17 G/cm. The other conditions are the same as given in Fig. 2.

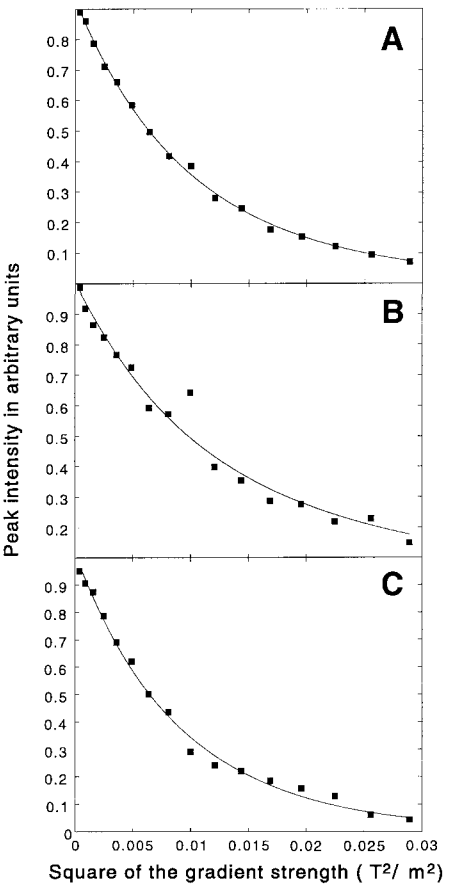

Fig. 4. Plot of signal attenuation versus gradient strength. The data is fitted to Eq. 3 using the parameters described in the text. A: T3H3 imino proton; B: T9H3 imino proton; and C: G2H1 imino proton.

Table 1
Comparison of residence times in ms for three imino protons of the 16 bp DNA measured at different diffusion times and as obtained by two-dimensional exchange spectroscopy

Imino proton	t_{dif} (ms)			2D exchange experiment[a]
	302.7	602.7	902.7	
T3H3	74	108	155	57
T9H3	285	279	284	278
G2H1	45	98	133	38

[a]Measurements according to [2,3].

in a fast experiment. No prior knowledge of relaxation parameters is needed in this method, as the diffusion parameters are independent of relaxation. Residence times can be determined to a high accuracy. The straightforward implementation and the high selectivity of the method should lead to its wide application in the fields of biological macromolecule structure determination, the observation of molecular interactions and the assessment of their dynamics.

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