

Rapid estimation of relative amide proton exchange rates of ^{15}N -labelled proteins by a straightforward water selective NOESY-HSQC experiment

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Received 1 November 1995; revised version received 12 February 1996

Abstract A straightforward heteronuclear pseudo-3D NOESY-HSQC pulse sequence using radiation damping to selectively invert magnetization at the water frequency was developed to estimate the amide proton exchange rates in ^{15}N -labelled proteins. The peak intensities in the resultant 2D spectrum allow a direct classification of amide proton exchange rates according to short (ms), intermediate (ms to s) or long (\geq s) residence times. This method was successfully used for the analysis of amide proton exchange rates in the ^{15}N -labelled FruR DNA-binding domain and pertinent information about its dynamics was obtained.

Key words: Hydrogen exchange rate; FruR; NMR; Protein dynamic; Radiation damping; Water-protein interaction

1. Introduction

In the course of protein 3D structure analysis by NMR, the knowledge of exchange rates between amide and solvent protons reveals important information about the structural fluctuations along the polypeptide chain. Their diversity can be interpreted as a function of local structure and conformational changes, i.e. the dynamics of the protein which is closely related to its biological activity. We recently solved the 3D structure of the DNA-binding domain of Fructose transcription factor by ^1H and ^{15}N NMR [1,2]. In this small domain, the residence times of all amide protons were too short to be measured using the classical deuterium exchange experiment [3] since total exchange occurred in less than 3 min after dissolution of the lyophilized protein in D_2O . Several NMR pulse sequences have been proposed to estimate amide proton exchange rates in the ms to s time scale. The most straightforward is two dimensional exchange spectroscopy [4,5], but this has the disadvantage of being time consuming, and thus is rather rarely used. Selective experiments have been proposed, including isotope filtering methods [6,7] or selective excitation of the water frequency by the DANTE sequence [8] or shaped pulses of the sinc or e-burp type [9–11]. However, these se-

quences require either double $^{13}\text{C}/^{15}\text{N}$ isotope labelling or have to be carefully calibrated and adjusted to avoid spill-over effects of the selective pulse. In addition, especially at high fields, radiation damping (RD) represents a serious problem for selective pulses at the water frequency since it tends to bring water magnetization back to the z -axis even during the pulse.

To avoid the above-mentioned problems, we further developed and applied a new selective pulse sequence following a scheme proposed by G. Otting [12]. The method uses radiation damping to invert magnetization at the water proton frequency selectively. A HSQC sequence is appended in order to identify the exchangeable amide protons of the ^{15}N -labelled DNA-binding FruR domain. The simple measurement of peak intensities in the pseudo-3D NOESY-HSQC spectra obtained gives a good estimate of the relative residence times of amide protons along the polypeptide chain. As expected, we show that most of the amide protons involved in hydrogen bonds found in stable secondary structures of the FruR DNA-binding domain (α -helix and turn) have residence times of several seconds. Conversely, protons localized in aperiodic structures are exchanged on a faster time scale (ms to s) while amide protons at the N-terminal and those of the six histidine residues appended to the C-terminal of the protein show very fast exchange (ms time scale). This reflects the absence of any stable structure for the $6 \times \text{His}$ tag C-terminal extension introduced for overproduction and purification purposes. The results obtained validate the present new method for rapid and simple estimation of amide proton residence times in ^{15}N -labelled proteins.

2. Experimental

Uniformly ^{15}N -labelled DNA-binding domain of Fructose repressor (called FruR(1–57)*) was cloned, overproduced and purified as previously described [1,2]. Spectra were taken at 20°C on a 2 mM ^{15}N -labelled protein sample in solution in 20 mM phosphate buffer (pH 5.9), 0.1 M NaCl, 70 mM NH_4Cl , 5% D_2O and 0.05% sodium azide. Data were recorded on a Bruker AMX-600 spectrometer equipped with a 5 mm triple resonance probe. Gradients were produced by a self-shielded gradient coil built into the probe head. Experiments at 500 MHz were done on the same protein sample using a Varian Unity-*plus* spectrometer equipped with Ultrashims and a standard 5 mm triple resonance probe with gradients. Amide proton resonance assignments are reported elsewhere [2].

3. Results and discussion

Exchange spectra usually require saturation or inversion of the frequency one wants to observe [13]. For water protons, this is non-trivial; as abundant spin species they exhibit a particular behaviour, called radiation damping (RD) [14]. If water magnetization is brought out of equilibrium by applica-

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**Details for the practical implementation on Bruker AMX-600 or Varian Unity-*plus* 500 spectrometer can be obtained from the authors by e-mail upon request.

Abbreviations: FruR, bacterial fructose repressor; FruR(1–57)*, DNA-binding domain of FruR encompassing residues 1 to 57 and a LQHHHHHH C-terminal extension; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; HSQC, ^1H -detected heteronuclear single quantum coherence spectroscopy; RD, radiation damping.

tion of a pulse, no matter whether selective or not, the resulting precessing transverse water magnetization interacts with the detection network and induces a current in the coil. This current creates a magnetic field, which acts back on the spin species by which it has been created. This can be seen as a kind of soft pulse [14,15] which brings water magnetization back into equilibrium within several tens of milliseconds. Whereas this effect is generally considered undesirable, the pulse sequence discussed below makes constructive use of this phenomenon to specifically detect amide protons exchanging with water protons.

If a hard π -pulse is applied to all spins of the sample, protons at the water frequency undergo the phenomenon of RD and thus return back to the z -axis within 30 to 60 ms (using a 600 MHz spectrometer). After that time, a state similar to the one after a selective pulse at the water frequency is reached: water magnetization is aligned along the opposite axis relative to the magnetization of the remaining protons and exchange or NOE takes place during the mixing time. A reference spectrum can be taken by suppressing RD during the mixing time. This is realized in our experiment by the use of a pulsed field gradient of low intensity. In the difference experiment, a net magnetization is observed only for spins showing interactions with spins at the water frequency. In order to resolve the spin systems, several sequences can be appended to the experiment [12,16]. We use here a simple HSQC sequence, resulting in a two dimensional (pseudo-3D) NOESY-HSQC spectrum selective at the water frequency; it shows resonances of amide protons having interactions with protons at the water frequency, resolved by their ^{15}N chemical shift along the ω_1 direction.

The complete pulse sequence is shown in Fig. 1. In more detail, all spins are inverted by the first pulse of 160° , used in order to initiate radiation damping with a predictable phase [12]. In the exchange experiment (even scans), water magnetization undergoes RD; all protons at that specific frequency return to the z -axis within about 50 ms. G_2 serves as a spoiler

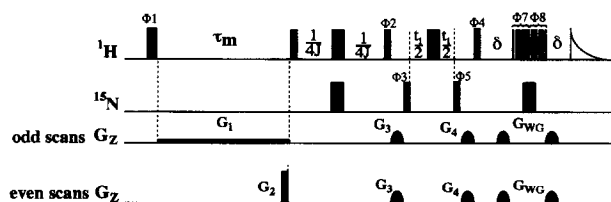


Fig. 1. Pulse sequence of the water selective NOESY-HSQC experiment. The first pulse has a flip angle of 160° . All narrow pulses have a flip angle of 90° , the larger pulses have a 180° flip angle. Pulses for which the phase is not indicated are applied along the x -axis. The ^1H carrier frequency is set to the H_2O frequency. ^{15}N decoupling is accomplished using the garp sequence [21]. τ_m is 100 ms. The gradient G_1 is a square gradient, its duration t_{G1} is τ_m , its strength $G_1 = 25$ mG/cm. It is applied for odd scans. Gradient G_2 is a spoiler gradient of length $t_{G2} = 1$ ms, its strength is set at $G_2 = 2.5$ G/cm to satisfy $G_1 \cdot t_{G1} = G_2 \cdot t_{G2}$ in order to minimise difference artefacts. It is applied for all even scans. The gradients G_3 and G_4 have a Gaussian amplitude profile with a length of 1 ms and a strength of 5 and 0.5 G/cm respectively. The gradients G_{WG} for the WATERGATE sequence [17] have a Gaussian amplitude profile with a length of 800 μs and a strength of 20 G/cm at their centre. The HSQC sequence is according to Sklenár et al. [17]. $\delta = 1/(4J) - 2.5 \cdot 2/\Delta\nu_{\text{max}}$. The phase cycle is as follows: $\Phi_1 = y$; $\Phi_2 = y, -y$; $\Phi_3 = 2(x), 2(-x)$; $\Phi_4 = 4(x), 4(-x)$; $\Phi_5 = 8(x), 8(-x)$; $\Phi_6 = -x$; $\Phi_7 = x$; $\Phi_8 = -x$. Acq. = $x, -x, 2(-x, x), x, -x, -x, x, 2(x, -x), -x, x$.

to defocus the remaining transverse magnetization at the end of the mixing period. In the reference experiment (odd scans), RD is suppressed by the application of a weak pulsed field gradient G_1 during τ_m . Water magnetization and protein proton magnetization stay both aligned along the z -axis. The areas (time * intensity) of G_1 and G_2 are chosen to be equal. We thus avoid problems arising from the use of homospoil pulses, resulting in different amounts of Eddy current present in even and odd scans and thus being a possible source of difference artefacts, as underlined by Otting and Liepinsh [12]. A further advantage in using pulsed field gradients rather than homospoil pulses consists in the possibility of producing very weak or shaped gradients, thus avoiding problems arising from field recovery. Before detection, water is suppressed by the WATERGATE sequence [17] including the last two gradients and the selective π -pulse. By inverting the receiver phase of the odd scans, the two experiments are subtracted scan by scan. Along with the choice of gradients of equal area for the exchange and reference experiment, this scan-by-scan acquisition is essential to the experiment, since the method relies on perfect cancellation between odd and even scans.

The efficiency of this pulse sequence was demonstrated in the course of the 3D structure determination of the DNA-binding domain of FruR (i.e. FruR(1–57)*). A pH of 5.9 was chosen to approach physiological conditions, resulting in fast exchange rates of the protein amide protons. In deuterium exchange experiments, no amide proton resonance could be observed in the first spectrum taken after 3 minutes. Moreover, amide protons of the $6 \times \text{His}$ tag appended to the C-terminal of the overproduced FruR(1–57)* domain were only poorly observed in conventional NMR spectra, even using pulse field gradients for coherence selection without any water suppression. This is illustrated in the ^1H - ^{15}N HSQC spectrum presented in Fig. 2A in which the last five C-terminal histidine amide protons (His 61 to 65, Fig. 3) give rise only to very weak cross peaks while amide protons of other amino acids give intense peaks.

The water selective ^1H - ^{15}N NOESY-HSQC spectrum of FruR(1–57)* using a mixing time of 100 ms is presented in Fig. 2B in comparison to the standard HSQC spectrum (Fig. 2A). The more intense the cross peak, the faster the amide proton exchange rate. The mixing time of 100 ms includes the duration of the selective pulse induced by RD. This time should be short enough to neglect spin diffusion at first approximation. However, a precise measurement of residence times is not straightforward due to exchange processes occurring during the water selective pulse induced by radiation damping, a problem holding true for all selective pulse techniques. Moreover, direct NOE transfer due to proximity of amide protons to hydroxyl protons of Ser, Thr and Tyr may increase cross-peak intensities, i.e. artificially decrease apparent amide proton residence times. Taking into account these restrictions, the cross-peak intensities nevertheless map very well the relative amide proton residence times in the protein.

Intensities of resolved cross peaks are reported in the histogram of Fig. 3B in arbitrary units; the probability of each amide proton forming a hydrogen bond (Fig. 3A) and the secondary structure of FruR(1–57)* as deduced from NMR are shown for comparison. The residence times of the amide protons observed in the spectrum have to be in the range between several ms to several s considering the following two points: (a) resonances of protons exchanging faster

both the dynamics of FruR(1–57)* and the validity and limitations of the proposed method can be extracted from Fig. 3: (i) as expected, most of the slowly exchanging amide protons (several seconds time scale) correspond to protons involved in hydrogen bonds. These protons essentially belong to amino acids found in regular secondary structures of FruR(1–57)*, i.e. α -helices or β -turn. The other ones are located in highly structured parts of the protein: G11 and V12 are components of the turn in the helix–turn–helix motif typical of this DNA-binding protein family [18]; G23, R29 and V30 are involved in the rather tight segment connecting helices 2 and 3 [2]. (ii) amide protons exchanging on the intermediate time scale (ms to s) can be divided into two classes: not surprisingly, the first one is found either in the N-terminal flexible part of FruR(1–57)*, (residue 3) and close to its C-terminal part (residues 47 to 59). The second class is found within the well structured part of FruR(1–57)*, i.e. residues 1 to 47. The most typical examples are the SRTT segment (residues 13 to 16) and the DKT segment (residues 32 to 34). As none of the amide protons for these residues are involved in hydrogen bonds, it is not unexpected that they show high exchange rates. However, these conclusions must be taken with care since amide protons of residues 13, 15, 16, 32 and 34 are situated less than 4 Å away from hydroxyl groups of Ser and Thr residues. The apparently high exchange rates could be at least partly attributed to chemically relayed NOE interactions between water protons, hydroxyl protons and amide

protons [19]. (iii) Amide protons exchanging on a fast time scale are situated at the terminals of FruR(1–57)*: K2 and the 6 \times His tag H60–65 give rise to strong correlation peaks in Fig. 2B while they are only very weakly observed in normal HSQC spectra (using respectively gradient HSQC [20] or the WATERGATE HSQC [17] Fig. 2A). This fast exchange is in agreement with the absence of any stable structure for this 6 \times His tag added at the C-terminal for overproduction and purification purposes. As N-terminal segments of proteins are generally very flexible, it is not surprising to find amide proton K2 in the fast exchange regime.

Due to overlap, no information could be obtained about the intensities of the cross peaks of the amide protons of residues 21, 24, 44, 46 and 50. Residue 49 is a proline and thus does not give rise to any cross peak. The corresponding sites are labelled with an asterisk in the histogram of Fig. 3B.

α -Protons resonating close to the water signal could be directly excited by radiation damping and lead to strong cross peaks due to direct NOE. It is worth mentioning that α -protons of residues H48, V12 and N22, resonating at 4.79, 4.85 and 4.92 ppm respectively, do not give rise to such large intensity signals. This indicates that the “selective” pulse due to radiation damping is indeed highly selective (about 40 Hz). Finally, one should note that the ϵ amide protons of the five arginine residues are all rapidly exchangeable, as expected (data not shown on the figures).

The experiment carried out at 500 MHz resulted basically in

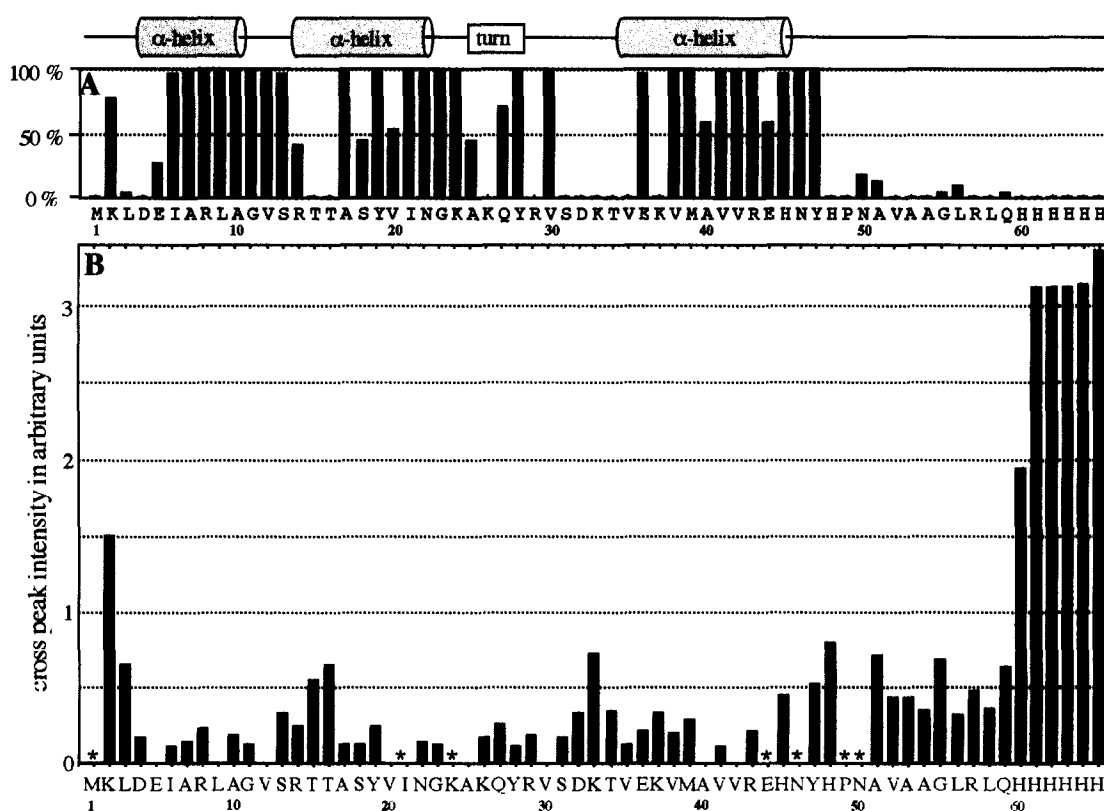


Fig. 3. (A) Histogram of the probability of finding the amide proton involved in hydrogen bonding (calculated using 34 3D structures generated by NMR). (B) Histogram showing cross peak intensities taken from the spectrum in Fig. 2B in arbitrary units. Cross peaks not observable in the spectrum show zero intensity. Amino acids for which the amide proton cross peak intensity could not be measured due to overlap and the proline residue are labelled with an asterisk. Integration of the cross peaks was done using the PARIS integrator of the GIFA program [22]. Primary structure of FruR(1–57)* is shown on the x-axis. Secondary structure of the FruR(1–57)* protein according to Penin et al. is shown above the histograms.

the same spectra, but the mixing time is longer since the length of the pulse induced by RD is increased (about 150 ms). Conversely, one can expect that at magnetic fields higher than 600 MHz, the water selective pulse will be reduced to a few tens of ms, and thus will open the way to more precise measurements of amide proton exchange rates, as processes occurring during the pulse can be neglected at first approximation.

4 Conclusion

The simple straightforward pulse sequence presented here allows a rapid analysis of exchange rates of amide protons in ^1H - ^{15}N -labelled proteins. Although a precise quantification of amide protons residence time is not easily accessible, three different time scales can be distinguished according to fast (ns), intermediate (ms to s) and slow (\geq s) exchange by simple measurement of ^1H - ^{15}N cross-peak intensities. Application of the pulse sequence to FruR(1–57)* differentiates very clearly three groups of amide protons and allows us to verify the method. The best indication of its validity is given by the strong correlation observed between the probability for amide protons to be involved in hydrogen bonds and their exchange rates.

The present method allows a simple access to the knowledge of exchange rates on a time scale which is of particular interest in the study of the dynamic behaviour of a protein at near neutral pH. It should be particularly useful in comparative experiments to study protein folding pathways under various physico-chemical conditions and to study specific protein–ligand interactions. In addition, this method can also be of great interest in identifying amide protons difficult to detect in classical HSQC spectra due to their very rapid exchange rates, a problem generally encountered in the study of peptides in solution.

Acknowledgements: The authors would like to thank G. Otting for the pre-prints of his articles, C. van Heijenoort for interesting discussions, J.-C. Cortay for the overproduction of ^{15}N -labelled FruR, G. Deléage and C. Geourjon for their help in structure analysis, A. Lesage for help concerning the implementation on the Varian spectrometer, Organibio and the German Academic Exchange Service for financial support (A.B., DAAD Doktorandenstipendium aus Mitteln des zweiten Hochschulsonderprogramms).

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