Published in final edited form as:

J Magn Reson. 2017 August ; 281: 125–129. doi:10.1016/j.jmr.2017.05.016.

Optimized fast mixing device for real-time NMR applications

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Abstract

We present an improved fast mixing device based on the rapid mixing of two solutions inside the NMR probe, as originally proposed by Hore and coworkers (*J. Am. Chem. Soc.* 125 (2003) 12484–12492). Such a device is important for off-equilibrium studies of molecular kinetics by multidimensional real-time NMR spectroscopy. The novelty of this device is that it allows removing the injector from the NMR detection volume after mixing, and thus provides good magnetic field homogeneity independently of the initial sample volume placed in the NMR probe. The apparatus is simple to build, inexpensive, and can be used without any hardware modification on any type of liquid-state NMR spectrometer. We demonstrate the performance of our fast mixing device in terms of improved magnetic field homogeneity, and show an application to the study of protein folding and the structural characterization of transiently populated folding intermediates.

Keywords

Real-time NMR; Fast mixing; SOFAST; Protein folding; β 2-microglobulin

1 Introduction

Real-time NMR spectroscopy is a powerful technique for the study of off-equilibrium molecular kinetics at atomic resolution [1,2]. One-dimensional (1D) real-time NMR is widely used to follow the fate of small molecules both in-vitro and in-vivo. With the advent of fast multidimensional data acquisition techniques [3,4], two-dimensional (2D) and three-dimensional (3D) real-time NMR techniques have been developed that now allow monitoring the conversion between different conformational states of biological macro-molecules, e.g. proteins and nucleic acids, separated by energy barriers interconverting on the time scale of a few minutes or more [5–7]. This provides valuable information on the energy barriers involved in the kinetic process, as well as transiently accumulated intermediate states along the kinetic pathway(s) (Fig. 1a). Fast multidimensional real-time NMR spectroscopy has been successfully applied to the study of hydrogen/deuterium (H/D) exchange kinetics [8], protein and RNA folding [7,9], as well as phosphorylation, acetylation, and other chemical modifications of proteins [10,11]. In the most favorable cases, real-time NMR spectroscopy allows the characterization of local structure and dynamics in transiently accumulated

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protein folding intermediates. This has been demonstrated recently for folding intermediates of the amyloidogenic protein β 2-microglobulin [12,13], and ribonuclease T1 [14].

Real-time NMR requires an experimental setup that allows initiation of the reaction of interest directly inside the NMR spectrometer in order to reduce the experimental dead time in which no data can be recorded. One of the most versatile and widely used approaches to real-time NMR is the sudden change in sample buffer conditions, e.g. change of pH, dilution of denaturant, or addition of co-factors, metal ions, or other molecules. This is experimentally realized by a so-called fast mixing device that allows fast injection of one solution into another in-situ in the NMR tube, where the molecules of interest can be dissolved in either one of the two solutions. In the setup of Hore and coworkers [15], shown in Fig. 1b, the fast mixing device consists of an injector that is placed in a Shigemitsu NMR tube (5 mm diameter) in a central position, and with the injector tip slightly inserted into solution 1. Rapid mixing of the two solutions is ensured by the fluidic turbulences that are caused by rapid injection of solution 2 onto the flat bottom of a Shigemitsu NMR tube. With this setup, a homogeneous mixture is typically reached within less than 100 ms. An interesting feature of this fast-mixing device is that it is easy to build, cheap, and highly portable allowing to use the same device on different NMR instruments. However, for minimal disturbance of the magnetic field homogeneity, the tip of the injector needs to be positioned outside the NMR detection volume. On a conventional Bruker 5 mm probe, this requirement imposes a lower limit of about 350 μ l for the volume of solution 1 in the NMR tube. Here we present an optimized injection device that allows to remove the injector from the NMR detection volume after injection, and therefore to overcome some of the volume restrictions on the solutions to be mixed.

2 Materials and Methods

2.1 Fast mixing device

Pieces required for the construction of the optimized fast mixing device: (1) the base element and mobile support pieces have been designed as STL files and printed in white plastic (polyamide) using the web service Sculpteo. The STL files are available from the authors upon request. (2) Plastic 1 ml Inject-F syringe (B Braun). (3) HPLC syringe connector (20 mm in height, 7 mm in width) with screw thread of $\frac{1}{4}$ " -28 UNF of 8 mm height. (4) PTFE tubing with diameter of 0.5 mm. (5) 2 plastic rods of 3 mm diameter and about 14 cm length.

2.2 NMR spectroscopy

All NMR experiments were performed on a Bruker 700 MHz instrument equipped with an Avance IIIHD console and a cryogenically cooled triple-resonance probe. For the real-time B2M-W60G folding experiments, 1.5 mg of 15 N-labeled protein were dissolved in 300 μ l of an unfolding buffer (95% H_2O , 5% D_2O , pH 2.0, 1.5 M urea). Refolding was initiated by injecting 100 μ l of refolding buffer (95% H_2O , 5% D_2O , 300 mM HEPES, pH 7.7), and the final protein concentration was 320 μ M. The injector was removed immediately after the mixing. The B_0 -field homogeneity (shims) was optimized by pre-shimming on a reference sample. The refolding reaction was then monitored by a series of SOFAST-HMQC

experiments [16,17], with an experimental time of 28 s per experiment, repeated for about 2 h (3 h at 10 °C). After completion of the refolding reaction a pure N-state spectrum was recorded in an experimental time of ~1 h using the same SOFAST-HMQC experiment and acquisition parameters. Kinetic traces of the N-state buildup were obtained by extracting peak intensities of about 30 well-resolved N-state cross peaks along the kinetic dimension. The sum of these kinetic traces was then fitted to the function $A(1 - \exp(-kt)) + B$ to obtain the folding rate constant k at a given temperature. SNR-optimized pure I-state spectra were obtained by adding SOFAST-HMQC spectra recorded during the folding reaction, as long as the peak signal is higher than the noise level. The N-state peaks are then removed from this real-time sum-spectrum by subtracting the intensity-normalized steady-state (N-state) spectrum from the real-time spectrum [12].

3 Results

3.1 Design and construction of optimized injection device

A schematic drawing of the design, and the individual pieces required for the improved fast mixing device are shown in Fig. 2. The basic idea of the device is to introduce an additional air syringe with the barrel fixed to the NMR sample holder, and the piston connected to the injector. By pumping air into the syringe the piston, which is connected to the injector, is pushed away from the NMR sample holder, and thus the NMR detection coil. Two of the pieces required for this device, the “base element” and the “mobile support”, were manufactured by means of 3D printing, while the other pieces are commercially available plastic syringes, PTFE tubing, HPLC and plastic connectors. Detailed drawings of the printable pieces with exact dimensions are shown in Fig. 2c. Once the individual pieces are available, the device is assembled in the following way: (i) A PTFE tubing of sufficient length to fit into the NMR magnet is cut and fixed to the HPLC connector that is screwed into the thread of the base element; (ii) The piston of the air syringe, as well as the 2 guiding rods are inserted into the mobile support; (iii) The top part (mobile support, plus guiding rods, plus air syringe) is connected with the base element; (iv) The whole apparatus is then bonded on top of a standard Bruker 5 mm (blue) spinner. Photographs of the assembled fast mixing device, with and without the injector present, are shown in Fig. 3a–c. The injector can be moved up by about 2–3 cm by air injection using a second syringe connected at the opposite end of the PTFE tubing. In order to avoid that the piston of the pneumatic syringe is pushed out of the barrel, a small hole is drawn into the plastic barrel of the pneumatic air syringe that acts as a pressure relief once the injector has reached its upper limit.

3.2 Experimental applications

In order to characterize the performance of this optimized fast mixing device, we have first compared the magnetic field homogeneity achieved under different experimental conditions (volumes of solutions to be mixed). Without removal of the injector, we found that a minimal volume of 350 µl for the solution placed in the NMR sample tube was required to obtain a homogeneous magnetic field profile. The interesting question thus was whether removal of the injector now allows to work with smaller sample volumes. Fig. 3d shows the results of a magnetic field mapping experiment (along the z direction) after injecting a buffer solution of 100 µl (solution 2) into 250 µl (solution 1) before (blue line) and after (green

line) removing the injector. The field maps were recorded after optimization of the shim coil currents using an automated gradient-shimming method (topshim) implemented in the Bruker software TopSpin. Without removing the injector, a significant deviation from homogeneity is clearly observed at the position of the injector tip inside the NMR detection volume (the position shifts as a function of the volume chosen for solution 1 – data not shown). As expected, this remaining field inhomogeneity is not present after injector removal. The improved field homogeneity results in NMR line narrowing, and more importantly, in good water suppression performance when using frequency-selective pulse sequences. The magnetic field profile can be further improved by increasing the total sample volume (after injection) to 400 μl by injecting a volume of 150 μl into 250 μl (red line in Fig. 3d). The magnetic field profiles shown in Fig. 3d correspond to an ideal scenario where the B_0 -field homogeneity (shimming) is directly optimized for the sample of interest. Though, for practical applications of real-time NMR, using an injection device for fast mixing, shimming is typically realized on a reference sample containing the same sample volume as the “real” sample after the injection. This so-called pre-shimming procedure is much more robust in the absence of the injector from the detection volume, as even slight differences in the injector position will have a measurable effect on the shim quality.

An interesting side effect of this improved fast mixing device is that it can be easily placed into a high-field NMR magnet, because the device mounted on top of the NMR spinner provides an additional guide for inserting the sample tube in the NMR probe. In previous applications using the conventional injector, we had to use a combination of 2 spinners, one placed on top of the other in order to ensure proper insertion of the sample into the NMR probe.

Finally, we used our new injection device for probing the refolding of $\beta 2$ -microglobulin (B2M), the light chain of the human class I major histocompatibility complex. This protein forms amyloid fibrils in the joints and connective tissues of patients undergoing long-term dialysis. B2M has been studied extensively as a model system in order to better understand the relationship between protein folding and amyloid formation [18–22]. In particular, a long-lived folding intermediate (I-state) has been identified that shows a higher propensity for oligomerization than the N-state [13], and that is believed to be involved in the onset of amyloidosis [18]. Recently, we have obtained partial NMR assignments of the I-state of the B2M mutant W60G at 15 °C. Here we have measured the real-time folding of B2M-W60G for a range of temperatures (10 °C, 15 °C, 20 °C and 25 °C) in order to obtain information on the activation energy for the transition from the I to N-state, and to derive residue-specific amide proton temperature coefficients that can be related to the presence/absence and thermal stability of a hydrogen bond [23].

The results of these real-time protein folding experiments are shown in Fig. 4. The spectral quality achieved using the new fast mixing device is illustrated by the first SOFAST-HMQC spectrum recorded after injection (Fig. 4a). Even in the absence of any phase cycling, residual signals from urea (1.5 M) and water do not disturb the amide ^1H spectral region, and therefore high-quality kinetic data are obtained from these real-time data with a time resolution of $\sim 2 \text{ min}^{-1}$. The peaks detected in this spectrum are characteristic of the transiently populated folding intermediate of B2M-W60G that has been formed during the

dead time (a few seconds) of the real-time NMR experiment. According to the simplified free energy landscape drafted in Fig. 1a, the life time of this I-state is determined by the energy barrier (E_a) towards the native state and the reaction temperature. The N-state buildup kinetics extracted from our real-time NMR data are plotted in Fig. 4b. Non-linear data fitting to a mono-exponential function yields the following folding rate constants (half-life times) for the transition from the folding intermediate (I-state) to the native state conformation: $k = 3.0 \cdot 10^{-4} \text{ s}^{-1}$ ($t_{1/2} = 38 \text{ min}$) at 10°C , $k = 5.7 \cdot 10^{-4} \text{ s}^{-1}$ ($t_{1/2} = 20 \text{ min}$) at 15°C , $k = 12.0 \cdot 10^{-4} \text{ s}^{-1}$ ($t_{1/2} = 10 \text{ min}$) at 20°C , and $k = 21.2 \cdot 10^{-4} \text{ s}^{-1}$ ($t_{1/2} = 5 \text{ min}$) at 25°C . The temperature dependence of the observed folding rates allows to estimate the activation energy (E_a) required to cross the transition state barrier. An Arrhenius plot of the temperature dependence of the measured kinetic rates is shown in the insert of Fig. 4b, and linear regression results in an activation energy of 90 kJ/mol. This value is in good agreement with the hypothesis that the main contribution to this energy barrier comes from the *cis-trans* isomerization of the peptide bond between residues H31 and P32 that is known to be in *trans*-conformation in the I-state, and needs to convert to *cis* in the N-state. An overlay of I-state spectra, reconstructed from the real-time NMR data recorded at different temperature, is shown in Fig. 4c. These data provide residue-specific information on the hydrogen-bond strengths in this transiently formed protein conformation. Temperature coefficients of individual amide protons, calculated as the slope of a linear fit of amide ^1H chemical shifts versus the sample temperature, are color-coded on the ribbon structure of B2M-W60G (N-state) using -4.5 ppb/K as a threshold value to distinguish between amides engaged in hydrogen bonds ($> -4.5 \text{ ppb/K}$), and those most likely not part of a stable H-bond ($< -4.5 \text{ ppb/K}$). Temperature coefficients in the I-state are very similar (within the experimental uncertainty) to those measured for the corresponding residues in the N-state (data not shown), confirming that the I-state backbone conformation is native-like, and only little destabilized, at least in the part of the protein that is visible by NMR.

Acknowledgments

We thank Isabel Ayala for the production of the labeled B2M-W60G samples, and Lionel Imbert for technical support. We acknowledge access to the high-field NMR and protein labeling platforms of the Grenoble Instruct Centre (ISBG: UMS 3518 CNRS-CEA-UJF-EMBL) with support from FRISBI [ANR-10-INSB-05-02] and GRAL [ANR-10-LABX-49-01] within the Grenoble Partnership for Structural Biology (PSB).

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Fig. 1.

(a) Schematic drawing of a one-dimensional energy landscape for protein folding, involving an ensemble of unstructured states (U) that spontaneously undergo a conformational transition to the native state (N) via a transiently populated intermediate state (I) conformation. (b) Drawing of a fast mixing device as proposed by Mok et al. [15]. The device consists in a standard 5 mm Shigemi sample tube and an injector that is either built using glass capillaries, or plastic tubing.

Fig. 2.

(a) and (b) Device designed to remove the injector from the NMR sample volume after injection and mixing of the 2 solutions. The two major parts of the device (c) were manufactured by a 3D printer.

Fig. 3.

Photographs of the improved injection device. (a) Injector-removal device mounted on NMR spinner; (b) injection device and air syringe before injection of solution 2, (c) injection device and air syringe after injection and removal of the injector tip from the NMR detection volume. (d) Magnetic field maps recorded along the static magnetic field direction (z) using a water imaging sequence under different conditions: situation after injecting 100 µl into 250 µl, and injector in place (blue line), after injecting 100 µl into 250 µl, and removal of the injector (green line), and after injecting 150 µl into 250 µl, and removal of the injector (red

line). In all cases the B_0 -field homogeneity has been optimized prior to recording the field map. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

B2M-W60G refolding probed by a series of SOFAST-HMQC experiments [16,17]. Refolding was initiated by mixing a refolding buffer with the protein solution at pH 2 inside the NMR magnet using the fast mixing device shown in Fig. 3. (a) ^1H - ^{15}N correlation spectrum recorded without any phase cycling (NS = 1) a few seconds after the mixing of the solutions (at 20 °C), illustrating the quality of the B_0 field homogeneity achieved. The observed cross peaks belong to the transiently populated folding intermediate (I-state). (b) N-state buildup kinetics measured at different temperatures. The plotted intensities

correspond to the normalized sum of peak intensities of about 30 well resolved N-state peaks in the SOFAST-HMQC spectra. The results of the non-linear data fitting are shown by straight lines. Note that the first time-points recorded in the 25 °C series are missing due to a lock problem. The insert in (c) shows an Arrhenius plot of the dependence of the folding rate constant on the sample temperature. Linear regression gives an activation energy E_a of 90 kJ/mol. (c) Overlay of reconstructed I-state spectra measured at temperatures of 10 °C, 15 °C, 20 °C, and 25 °C, annotated by their residue number and amino-acid type. The observed temperature coefficients have been classified in two categories using -4.5 ppb/K as a threshold value. Residues with less negative temperature coefficients, supposed to be involved in hydrogen bonds, are color coded in blue on the ribbon structure of the native state (PDB entry 2Z9T) [24], while residues with larger negative temperature coefficients are color coded in yellow. In addition, residues for which no ^1H - ^{15}N peak is detected (and assigned) in the I-state are color-coded in dark red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)