Multiquantum Chemical Exchange Saturation Transfer NMR to Quantify Symmetrical Exchange: Application to Rotational Dynamics of the Guanidinium Group in Arginine Side Chains

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ABSTRACT: Chemical exchange saturation transfer (CEST) NMR experiments have emerged as a powerful tool for characterizing dynamics in proteins. We show here that the CEST approach can be extended to systems with symmetrical exchange, where the NMR signals of all exchanging species are severely broadened. To achieve this, multiquantum CEST (MQ-CEST) is introduced, where the CEST pulse is applied to a longitudinal multispin order density element and the CEST profiles are encoded onto nonbroadened nuclei. The MQ-CEST approach is demonstrated on the restricted rotation of guanidinium groups in arginine residues within proteins. These groups and their dynamics are essential for many enzymes and for noncovalent interactions through the formation of hydrogen bonds, salt-bridges, and π-stacking interactions, and their rate of rotation is highly indicative of the extent of interactions formed. The MQ-CEST method is successfully applied to guanidinium groups in the 19 kDa L99A mutant of T4 lysozyme.

A key strength of NMR spectroscopy is its ability to quantify the dynamics of molecules with atomic level resolution. Within biomolecules, conformational exchanges often occur on milli- to microsecond time scales, and these exchanges can be critical for function.1,2 A number of NMR-based approaches for characterizing exchange on these time scales now exist and provide important insights into conformations that are transiently populated, invisible to other high-resolution methods, and also broadened beyond detection in traditional NMR experiments.3–6 Although chemical exchange saturation transfer (CEST) methods have traditionally been used within the MRI field,7–9 CEST approaches have recently emerged as powerful tools also for studying biomolecular dynamics on a time scale from 20 to 200 s−1.10,11 In these experiments, first developed in the 1960s,12 saturation of magnetization by a weak pulse is transferred by exchange events within a network of exchanging conformers, and in particular, magnetization is transferred from invisible species to visible species in order to report on chemical shifts and rates of exchange.

However, for symmetrical exchange, where all the exchanging species are broadened in NMR spectra, a quantification of the exchanging system becomes challenging. This scenario is, for example, encountered for the rotational exchange about the C=–N bond in arginine side chains in proteins, Figure 1A. The importance of the arginine side chain for a range of protein functions, such as protein folding, catalysis, and noncovalent interactions, is well-established.13–15 It is the arginine guanidinium group, with its delocalized π system, that confers the functionality by allowing for a large range of interactions.16–18 Recently, it was shown that 13C-detection NMR provides an excellent tool to probe arginine side chains in proteins, and it was also shown that in favorable cases the rate of rotational exchange can be determined to provide a measure for the interactions formed by arginine guanidinium groups within proteins.23 Below, we present a multiquantum CEST (MQ-CEST) NMR experiment that is ideally suited to characterizing dynamics in symmetrically exchanging groups, and we apply this methodology to quantify the rotational dynamics of guanidinium groups in the side chains of arginine residues within proteins.

In the multiquantum CEST (MQ-CEST) experiment, the CEST pulse is applied to a longitudinal multispin order spin-density element in order to quantify symmetrical exchange. The restricted rotation about the C=–N bond in arginine side chains, Figure 1A, corresponds to a symmetrical exchange of the two 15N nuclei. The MQ-CEST approach applied to a density operator that spans both of the 15N species is able to capture the rate of restricted rotation as well as the chemical shifts of the sometimes severely broadened 15N signals.

For the MQ-CEST approach applied to arginine guanidinium side chains, two equally sized dips are typically observed corresponding to the CEST pulse being resonant with one of the two exchanging species.
the two $^{15}$N$^\alpha$ chemical shifts, Figure 1B and Figure S1. When the exchanging nuclei are in the so-called slow-exchange regime ($k_{ex} \approx \Delta \omega^{(15N^\alpha)} = |\omega^{(15N^\alpha)}| = |\Delta \omega|$), two well-separated dips are visible in the MQ-CEST profile and the individual chemical shift of the two $^{15}$N$^\alpha$ nuclei can be directly identified from the center of the dips, Figure 1B and Figure S1. When the nuclei are in the intermediate chemical exchange regime ($k_{ex} \approx \Delta \omega$), either because of fast rotation or because of a smaller chemical shift difference, the two dips coalesce into a single dip centered at the average of the two $^{15}$N$^\alpha$ chemical shifts. It is particularly near the intermediate chemical exchange regime that single-quantum $^{15}$N$^\alpha$ signals become severely broadened in NMR spectra.

For the MQ-CEST approach, the CEST intensities are encoded onto $^{13}$C chemical shifts from MQ-CEST experiments. In such cases of very slow exchange, the $k_{ex}$ can be determined using the previous longitudinal exchange method, which provided that the $^{13}$C resonance is isolated. It is interesting to note that the range of chemical exchange rates, $k_{ex}$ accessible with the MQ-CEST approach is substantially larger than what is accessible from typical CEST experiments ($< 5$ $s^{-1}$).

This larger range of $k_{ex}$ accessible with the MQ-CEST approach is mainly due to the symmetrical exchange of the $^{15}$N$^\alpha$ bond that causes the symmetrical exchange of the two $^{15}$N$^\alpha$ nuclei. The position of the two dips shows the $^{15}$N$^\alpha$ chemical shifts, and a least-squares fit to the profile provides the rate of exchange. The simulations show that, for $\Delta \omega \gtrsim 1$ ppm, the MQ-CEST approach provides accurate exchange rates over the large range of $k_{ex}$ from 5 to 1500 $s^{-1}$, which covers almost the full range of possible arginine guanidinium rotation rates at 293 K. Generally, the larger the chemical shift differences are, $\Delta \omega$, the larger exchange rates $k_{ex}$ are accessible, Figure S2, whereas accurate $\Delta \omega$ values can be obtained for the full range of possible $k_{ex}$ values from 5 to 1500 $s^{-1}$, Figure S3, as long as $\Delta \omega \gtrsim 0.5$ ppm. For arginine side chains involved in very strong interactions and thus experiencing very slow rotational rates ($< 5$ $s^{-1}$), it is possible to set an upper bound for $k_{ex}$ and accurately determine the two $^{15}$N$^\alpha$ chemical shifts from MQ-CEST experiments.
can be simply obtained from inspection of the MQ-CEST profiles.

Finally, the simulations show that it is highly desirable to collect data with different $B_z$ CEST saturation field strengths and/or at different static magnetic field strengths, $B_0$; Figures S2 and S3. It is interesting to note that MQ-CEST profiles with multiple $B_z$ field strengths provide essentially as accurate $k_\text{ex}$ rates and $\Delta \omega$ as MQ-CEST profiles at multiple $B_0$ fields. Recording MQ-CEST profiles at multiple static $B_0$ fields, particularly at higher field strengths, gives access to higher rotational rates and smaller chemical shift differences, since the exchange is moved toward the slow-chemical exchange regime. A disadvantage of ultrahigh magnetic fields ($B_0 \gtrsim 19$ T), however, is an increased $^{13}$C transverse relaxation due to chemical shift anisotropy (CSA), which can reduce signal-to-noise in the resulting spectra.

The pulse sequence derived for obtaining MQ-CEST profiles to characterize the symmetric exchange of arginine guanidinium groups in proteins is shown in Figure 1D. Briefly, equilibrium longitudinal magnetization residing on $^1$H, $^1$H$_e$ is initially excited and transferred via an INEPT step between $a$ and $b$ to a $^1$H$\rightarrow^1$N$^\text{ex}$ longitudinal two-spin order density element, $2H^\text{ex}N^\text{ex}$. Using the magnetization on the $^1$H proton as the source brings two main advantages compared to methods where $^1$C magnetization is used as the source. First, the higher gyromagnetic ratio of $^1$H provides additional signal-to-noise, even though this is partly mitigated by the longer sequence. Second, $^1$H nuclei have substantially faster longitudinal $R_1$ relaxation rates compared to $^1$C, which means that more scans can be acquired within a given time unit. Between $b$ and $c$, the one-bond scalar coupling between $^1$H and $^{15}$N and between $^{15}$N and $^{13}$C is evolved to generate the $^{13}$C$\rightarrow^{15}$N longitudinal two-spin order element $2\text{C}_\text{N}N^\text{ex}$, while concomitantly encoding the $^{15}$N chemical shift. The two selective $^{13}$C pulses between $b$ and $c$ ensure that $^{13}$C$\rightarrow^{15}$N and $^{13}$C$\rightarrow^{15}$N scalar couplings are refocused and evolved, respectively. Between $c$ and $d$, a further INEPT is used to evolve $^{13}$C$\rightarrow$N$^\text{ex}$ and $^{13}$C$\rightarrow$N$^\text{ex}$ scalar couplings, yielding a density element proportional to $4\text{C}_\text{N}N^\text{ex}$ at point $d$. The MQ-CEST period between points $d$ and $e$ is carried out with the $^{15}$N carrier frequency being varied, providing the CEST intensities, $I$. A reference spectrum is also recorded without the CEST element ($T_{\text{CEST}} = 0$ s), but including the gray block in Figure 1D. The reference spectrum provides $I_0$ and the final MQ-CEST profiles are calculated as $I/2I_0$. The effects of scalar couplings between $^{15}$N and $^1$H are minimized through the application of high-power composite decoupling during the CEST period as described previously. As the $^{15}$N CEST pulse is applied to a $4\text{C}_\text{N}N^\text{ex}$ density element, no decoupling is applied to $^1$C as this would deteriorate the signal. Instead, the effects of the $^{13}$C$\rightarrow^{15}$N scalar couplings ($\sim 20$ Hz) are explicitly included in the analysis of the CEST profiles as described previously. It should be noted that several density elements are present during the $^{15}$N CEST pulse, including Zeeman order (4C$_\text{N}$N$_{\text{ex}}$), zero-quantum (e.g., 4C$_\text{N}$N$_{\text{ex}}$), single-quantum (e.g., 4C$_\text{N}$N$_{\text{ex}}$), and double-quantum (e.g., 4C$_\text{N}$N$_{\text{ex}}$) coherences. Whereas the double-quantum coherences are insensitive to the exchange, the zero-quantum coherence will report on the exchange process. Finally, after point $e$, the density element of interest is transferred to transverse in-phase $^{13}$C$^\text{ex}$ magnetization for detection.

Several variations to the pulse sequence in Figure 1D have been developed (Figure S4). Of particular note is the $^1$H detected version (Figure S4B) in which magnetization, via additional INEPT steps, is transferred back to the $^1$H$^\text{ex}$ proton for detection. If relaxation is ignored, detecting $^1$H gives an 8-fold increase in signal-to-noise compared to $^{13}$C detection; however, in practice the additional delays required as well as exchange of $^1$H with the bulk solvent mean that this approach is only advantageous when the site in question has a local correlation time of less than approximately 10 ns (Figure S5). The guanidinium groups of greatest interest are often those that form interactions, making them more rigid, and so for the applications shown below on the 19 kDa T4L99A, the $^{13}$C detect sequence, Figure 1D, provides the best signal-to-noise.

In order to experimentally validate the MQ-CEST approach, experiments were carried out on a sample of free [$^{13}$C$_6$,$^{15}$N$_4$]-l-arginine at a high concentration (50 mM) and dissolved in a 50/50% vol mixture of H$_2$O and MeOH under acidic conditions. With this sample, experiments can be carried out at temperatures below 0 °C, where the symmetrical exchange rate, $k_\text{ex}$ for free arginine is slow enough to be quantified by classical longitudinal exchange methods, such as zz-EXSY. This system therefore forms an ideal basis for validating and benchmarking the performance of the MQ-CEST approach.

The MQ-CEST profiles for free arginine measured at four temperatures between −15 and 2.4 °C are shown in Figure 2A. The chemical shifts of the $^{15}$N nuclei can be easily identified from the position of the dips at low temperatures, and the exchange rate can be obtained from a least-squares analysis at each temperature (see Supporting Information). The correlation between the obtained exchange rates, $k_\text{ex}$ from MQ-CEST and from longitudinal exchange is excellent, Figure 2C, validating the MQ-CEST approach. It is important to note that the longitudinal exchange approach is only applicable when the $^{15}$N nuclei give rise to diagonal and cross-peaks in single-quantum NMR spectra and these peaks can be accurately quantified. This is not the requirement for the MQ-CEST approach, since the CEST intensities are encoded onto the $^{13}$C$\rightarrow^{15}$N cross-peaks.

Having demonstrated the validity of the MQ-CEST approach, both theoretically and experimentally, for extracting the rate for symmetrical exchange of the arginine guanidinium group, we turned our attention to arginine side chains within the 19 kDa L99A mutant of T4 lysozyme (T4L99A). T4 lysozyme is a challenging test case since a large range of exchange rates spanning more than 3 orders of magnitude have been observed. Previously, D-evolution and longitudinal exchange were used to characterize the rotational dynamics of arginine guanidinium groups in T4L99A; however, these measurements rely on single-quantum $^{13}$C$\rightarrow^{15}$N spectra, and only five of the 13 arginine residues in T4L99A could previously be characterized. On the contrary, the MQ-CEST approach relies on $^{13}$C$\rightarrow^{15}$N$^\text{ex}$ (or $^1$H$\rightarrow^{15}$N$^\text{ex}$) spectra, where well-separated cross-peaks are observed, e.g., Figure 1C. Thus, the MQ-CEST approach shows substantial improvements over the existing D-evolution method, since nearly every arginine residue can be resolved, resulting in an exchange rate for 11 out of the 13 arginine residues in T4L99A at 293 K. Four of these MQ-CEST profiles are shown in Figure 3, while all data is provided in Figure S6 and Table S2. For the arginine residues, where $k_\text{ex}$ could previously be obtained from the D-evolution approach, there is an excellent agreement with the rates derived from the MQ-CEST profiles.
The obtained $k_{ex}$ rates confirm that the rate of rotation is a very good indicator of interactions formed by a particular arginine side chain within the protein environment. For example, from the crystal structure of T4L99A, various interactions are observed for R52, R95, and R96, Figure 3, and these residues show a large range of rates, albeit all substantially slower than free arginine. The guanidinium group that shows the slowest rotational rate is in R95 that forms cation−π and π−π interactions with the large aromatic system of tryptophan W126.

In order to further assess the robustness of the MQ-CEST analysis for the extraction of exchange rates, the relaxation rates, $R(4C\eta\eta, N\eta)$ and $R_z(C\eta)$, were measured experimentally for T4L99A. First, the experimentally measured relaxation rates were compared to the corresponding rates obtained from an analysis of the MQ-CEST profiles, where those rates were allowed to vary, however, independent of the static magnetic field (Figure S7). Second, two-dimensional $\chi^2(k_{ex}, R_z)$ grid plots were generated, Figure S8, to quantify the influence of the relaxation rates on the derived $k_{ex}$. For nuclei with slower exchange rates, accurate transverse $^{15}N\eta\eta$ relaxation rates can be obtained. In cases of faster rotational exchange rates, the exchange rate is uncorrelated with the transverse relaxation rate, Figure S8, meaning that the transverse relaxation rate can be safely fixed to a sensible value in the fitting process. In all cases, accurate rotational exchange rates, $k_{ex}$, can be obtained (Figure S9).

In summary, we have described a multiquantum CEST NMR experiment, which is ideally suited for characterizing the rate of symmetrical exchange and the chemical shifts of the involved nuclei. The MQ-CEST approach was applied to quantify the rotation of guanidinium groups in arginine side chains in proteins, and it is shown that the MQ-CEST approach can accurately provide the rate of exchange over a very large range of time scales and also report on sites that previously remained undetected.
symmetric exchange over a large range of time scales and in many sites.

**EXPERIMENTAL METHODS**

Sample preparations are described in the Supporting Information. All spectra were processed using NMRPipe and visualized with NMRFAM-Sparky. Peak intensities were quantified using FuDa. MQ-CEST profiles were simulated and analyzed using an in-house program that numerically propagates the Bloch–McConnell equations. A detailed description is provided in the Supporting Information.

**ASSOCIATED CONTENT**

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