Determination of Amide Exchange Rates by Measurement of 2D NMR Line-Broadening

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Abstract: A novel NMR method is presented that allows direct quantitative measurement of hydrogen exchange rates from a single 2D NMR experiment. The method is based on the fact that first-order exchange of amide protons with deuterons results in line-broadening in the t1 dimension of a 2D experiment. The time scale of the experiment can be varied to optimize the measurement of exchange rates that are slower than 1 min^{-1}. The method is demonstrated on a small peptide, angiotensin II, and it is shown that rates, on the order of 10^{-1}–10^{-2} min^{-1}, are obtained by the 2D line-broadening method and that these agree with the rates obtained by sequentially acquired 1D spectra.

Introduction

Measurement of hydrogen exchange rates for individual amide protons in proteins and peptides is a widely used NMR method for obtaining information about structure and dynamics.[1] A wide range of hydrogen exchange rates have been measured by 1D NMR methods including line-shape analysis, saturation transfer, and acquisition of sequential NMR spectra.[2] For more complicated systems, hydrogen exchange rates are measured with 2D NMR methods in order to overcome the problem of overlapping resonances.

Direct measurement of very fast exchange rates (>1 min^{-1}) are obtained by dynamic NMR methods such as 2D exchange spectroscopy and magnetization transfer.[3] These methods rely on establishing equilibrium exchange conditions between the labile amide protons and the bulk water and then measuring the rate of exchange between the two. Slow rates (<10^{-2} min^{-1}) are usually obtained by acquiring sequential 2D NMR spectra and measuring the change in the observed peak intensity of the amide proton as a function of time.[4] Direct measurement of intermediate hydrogen exchange rates, such as 1–10^{-2} min^{-1}, is often difficult with 2D methods.

In addition to direct measurement of hydrogen exchange rates, indirect methods, employing hydrogen exchange trapping techniques, have been developed. Using indirect methods, a range of exchange rates can be obtained for proteins placed in D_2O.[5]

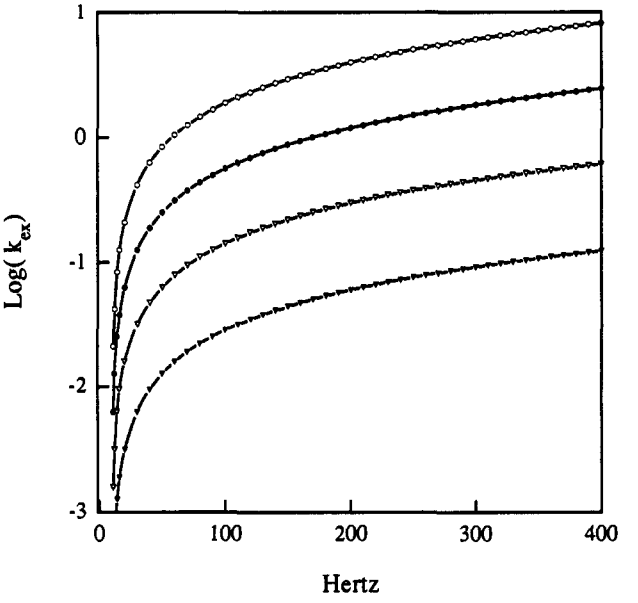

Figure 1. Theoretical curves showing the hydrogen exchange rate vs line-broadening (Hz) as a function of t_x. The value t_x corresponds to the delay time between each t1 increment and is varied by changing the number of transients per t1 increment and/or the recycle delay time of the experiment. For example, with a recycle delay of 1 s, 2 scans/t1 increment and 96 scans/t1 increment correspond to t_x values of 0.03 and 1.6 min, respectively. Curves representing different values of t_x are as follows: (○) t_x of 0.03 min, (●) t_x of 0.1 min, (▽) t_x of 0.4 min, (▼) t_x of 2 min.

The limitations of these exchange trapping methods are 2-fold. One, hydrogen exchange information is limited to the subset of proton resonances that are slowly exchanging under the observation conditions, and two, intensity artifacts may arise due to incomplete trapping. For these reasons, direct measurement of exchange rates is generally preferable when possible.

In this paper, we demonstrate a novel direct method in which quantitative hydrogen exchange rates are obtained by measuring line-broadening in the t1 dimension of a single 2D experiment such as TOCSY,[6] NOESY,[7] or HMQC.[8] The method is based on the fact that first-order exchange of amide protons with deuterons results in line-broadening in the t1 dimension of a 2D experiment.[9] The time scale of the experiment can be varied to optimize the measurement of exchange rates that are slower than 1 min^{-1}. This method will be particularly useful for measuring

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exchange rates ($1–10^{-3}$ min^{-1}) that are too fast to measure by sequential methods and too slow by dynamic methods.

Background

In a 2D experiment, the proton decay rate that arises from hydrogen exchange is superimposed on the oscillation frequency in the t1 dimension and acts as an exponential window function as seen in eq 1. We assume that the observed hydrogen exchange rate (k_{ex}) is a first-order exponential decay. Upon Fourier transformation of the t1 dimension, the signal takes the Lorentzian form[10] shown in eq 2. Equation 2 contains a line-broadening term, α, in the t1 dimension. $1/T_2^*$ is due to relaxation processes and field inhomogeneity,[11] k_{ex} (min^{-1}) is the hydrogen exchange rate, A describes the amplitude, and $t_x/\Delta t_1$ is a time scale conversion factor containing Δt_1 (s), the dwell time in t1, and t_x (min), the experimental time interval between each t1 increment.

$$S(\omega_1) = A\int_0^\infty e^{-\alpha t}e^{-i\omega_1 t}dt \tag{1}$$

$$S(\omega_1) = A\frac{1}{\alpha + i\omega_1}$$

$$\text{where} \quad \alpha = k_{ex}t_x/\Delta t_1 + 1/T_2^* \tag{2}$$

The value of t_x can be varied either by changing the number of scans per t1 increment or by changing the recycle delay time of the experiment. By measuring the half-width of the signal, one can determine k_{ex} from the following equation:

$$k_{ex} = \frac{\Delta t_1}{t_x}\pi(\nu_{obs} - \nu_{const}) \tag{3}$$

where ν_{obs} is the observed peak half-width (in Hz) of the exchangeable proton, and ν_{const} is the intrinsic peak half-width of the proton under nonexchanging conditions. Figure 1 displays a theoretical curve showing exchange rates versus line-broadening for a number of values of t_x. It is clear from this figure that choosing the proper value of t_x is critical for observing a given exchange rate. Rates on the order of 10^{-1} and 10^{-2} min^{-1} have a line-broadening of only 100–200 Hz for a number of values of t_x and can, therefore, be easily measured. Slower rates can be obtained with larger values of t_x, which correspond to longer experiments.

Hydrogen exchange rates can be measured either with a single 2D experiment or with a series of 2D experiments. If the rate is obtained from a single experiment, then, as seen in eq 3, the inherent line width of the resonances under nonexchanging conditions must be measured in addition to their line widths under exchanging conditions in D_2O. A second method is to perform three or four different experiments with different values of t_x under exchanging conditions. The rate of exchange is obtained from the slope ($m = \pi\Delta t_1/k_{ex}$) plotted of the observed line width versus t_x, with the intercept equivalent to the inherent line width. The second method offers advantages over the first because multiple measurements with different values of t_x cover a broader range of exchange rates while simultaneously improving accuracy.

Experimental Section

Materials. Angiotensin II was purchased from Sigma Chemical Co.

Hydrogen Exchange Studies. Samples were prepared for hydrogen exchange by dissolving the peptide in H_2O, adjusting the pH to 2.00 with HCl, and then drying the sample by lyophilization. When the sample is then dissolved in D_2O at a concentration of 5 mM, the pH values are 2.20 ± 0.04. The pH measurements in D_2O were uncorrected for isotope effects. All exchange measurements were acquired at 22 °C (the ambient room temperature), which eliminated the need for temperature equilibration. Data collection was begun 2–4 min after dissolving the sample in 99.99% D_2O. The sample pH was checked after each experiment.

NMR Spectroscopy. All spectra were acquired on a Varian 500-MHz spectrometer. Exchange rates from 1D experiments were obtained by acquiring sequential 1D NMR spectra and measuring the decay of the peak height with time. Exchange rates from 2D experiments were obtained with the standard homonuclear TOCSY pulse sequence using a spin-lock period of 50 ms and a recycle delay of 1 s. To obtain the rate, four standard TOCSY experiments with different values of t_x were performed, the observed line width of each resonance in the peptide was plotted against t_x, and the exchange rate was obtained from the slope of the line. TOCSY data sets, consisting of 1024 data points in the t2 dimension and 256 t1 increments in the t1 dimension, were collected using the hypercomplex method.[12] The experimental time between each t1 increment, t_x, is determined by the number of scans and the recycle delay. To collect different experiments with different values of t_x, the recycle delay is kept fixed and the number of scans per t1 increment is varied. Four separate TOCSY experiments were collected with 4, 8, 16, and 32 scans per t1 increment corresponding to t_x, values of 0.2, 0.39, 0.78, and 1.56 min. Spectral data was processed off line using FELIX software (Hare Research Inc.). Zero-filling and mild apodization were used in the t2 dimension; however, no zero-filling or apodization was employed in the t1 dimension. Peak slices were taken from the t1 dimension of each 2D spectrum and fit with one or more Lorentzian line shapes, depending upon the proximity of the neighboring resonances. The program Sigma Plot (Jandel Scientific) was used to perform a nonlinear least squares fit to the data.

Results and Discussion

The line-broadening analysis of 2D NMR spectra to obtain the direct quantitative measurement of hydrogen exchange rates is demonstrated on a small peptide, angiotensin II (ERVYIHPF). Angiotensin II is an eight-residue peptide that contains seven amide protons. Of these seven amides, three are resolved in the 1D NMR spectrum (Val3, Ile5, Phe8), two of the resonances are overlapping completely in the 1D spectrum and are resolved in the 2D spectrum (His6, Tyr4), and two of the amides are so rapidly exchanging that they do not appear in the 1D spectrum (Glu1, Arg2). 2D TOCSY experiments were used to determine the hydrogen exchange rates of the five observable amide protons.

To measure line-broadening in the t1 dimension, either the amide to backbone or amide to side chain cross peaks of the TOCSY experiment can be used. We have used the NH–αCH cross peaks in all cases except Phe8 where the NH–αCH peak was close to the water frequency; for Phe8, the NH–βCH_2 cross peak was used. In Figure 2, TOCSY cross sections as a function of t_x are shown for Val3 along with the Lorentzian curve fits. As can be seen, the cross sections are approximated by a Lorentzian function, and line-broadening is easily discerned even in the most slowly exchanging residue of angiotensin II. Figure 3 shows the exchange broadening for the different amide protons in angiotensin II as a function of t_x. The exchangeable amide cross peaks broaden linearly, in the t1 dimension, as a function of t_x. The exchange rate for each amide proton is easily extracted from the slope of each line. For the resonances that are resolved in the 1D spectra, we compare, in Table I, rates obtained by our 2D method with rates obtained from sequentially acquired 1D spectra. Clearly, the rates obtained by both methods are essentially identical,

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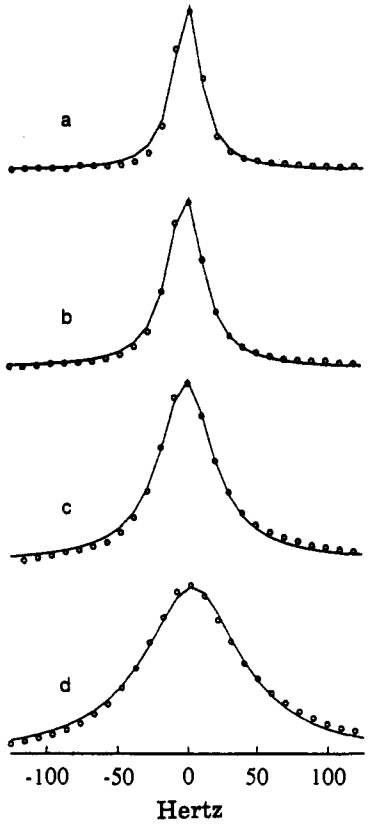

Figure 2. Cross section of Val3 NH–αH of angiotensin II (pH 2.2, 5 mM) obtained from the t1 dimension of four 2D TOCSY spectra acquired with different values of t_x. Four 50-ms TOCSY experiments were collected with 4, 8, 16, and 32 transients per t1 increment and a 1-s recycle delay corresponding to t_x values of (a) 0.2, (b) 0.39, (c) 0.78, and (d) 1.56 min.

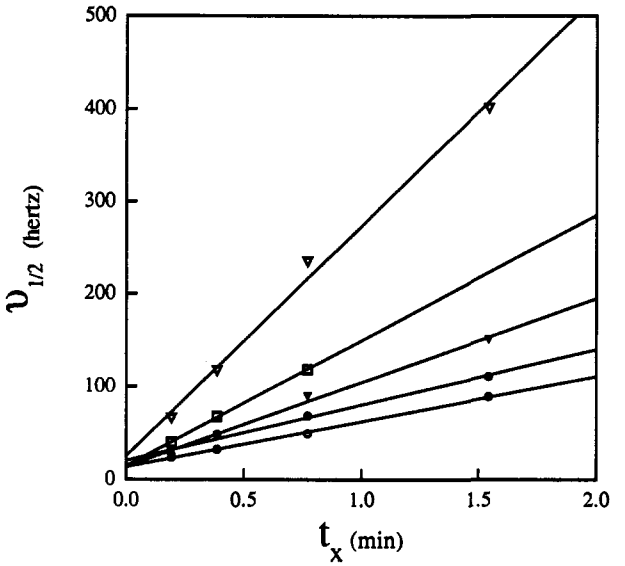

Figure 3. Experimentally observed line widths of the amide protons in angiotenisin II plotted against t_x. The curves are as follows: (▽) Phe8, (□) His6, (▼) Tyr4, (●) Ile5, and (○) Val3.

indicating that quantitative hydrogen exchange rates can be obtained directly from simple 2D experiments via the observed line-broadening. In addition, the exchange rates of the overlapping peaks His6 and Tyr4, which cannot be determined easily by 1D methods, can be obtained separately in the 2D experiments.

Table I. 1D and 2D Hydrogen Exchange Rates

residue	1D rate[a,b]	2D rate[a,c]
Val3	3.2×10^{-2}	3.0×10^{-2}
Tyr4	*d*	5.7×10^{-2}
Ile5	3.5×10^{-2}	3.6×10^{-2}
His6	*d*	8.5×10^{-2}
Phe8	1.5×10^{-1}	1.6×10^{-1}

[a] Exchange rates are reported in min^{-1}. [b] Measurements obtained by integration of sequential 1D NMR spectra. [c] Measurements obtained by 2D line-broadening method. [d] Resonances His6 and Tyr4 are overlapping in the 1D spectra.

The exchange rates for angiotensin II show no evidence of solvent protection under the conditions employed. Residues 3–6 exchange at rates that are approximately 2 times slower than the rate calculated for the theoretical random coil form of the sequence.[13] The terminal residues Glu1, Arg2, and Phe8 all display exchange rates faster than expected for random coil residues. In particular, the amino terminal residues Glu1 and Arg2 exchange within the dead time of the 1D and 2D NMR experiments (~3 min), indicating that they undergo exchange in excess of 1 min^{-1}. These rates are significantly faster than predicted; however, Phe8, the carboxy terminal residue, exchanges only twice as fast as expected. The fast amino terminal rates may be due to end effects. Our results agree with previous studies which show that angiotensin II is predominantly random coil in solution.[14]

By measuring line-broadening in a 2D experiment, a range of hydrogen exchange rates, slower than 1 min^{-1}, can be obtained. The limitation of our experiment lies in the extent to which broadened peaks can be measured and the speed with which the experiment can be started. In the experiments on angiotensin II, line widths on the order of 400 Hz could be measured fairly easily, and acquisition was typically started within 3 min. Measurement of line-broadening in a single experiment may sometimes provide an alternative to sequential acquisition of 2D spectra to obtain slow rates on the order of 10^{-3}–10^{-4} min^{-1}. In addition, the method is complementary to sequential 2D NMR methods as line-broadening analysis can be routinely applied to the first spectrum in a series of sequential 2D spectra in order to extend the range of rates measured. The line-broadening method can be implemented easily with simple 2D experiments and promises to be of particular value for direct measurement of hydrogen exchange rates between approximately 1 and 10^{-2} min^{-1}.

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Note Added in Proof. During the review of the paper, Led and co-workers[15] independently published a similar method for measuring hydrogen exchange rates.

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