

Measurement of Fast Proton Exchange Rates in Isotopically Labeled Compounds

Gerd Gemmecker,* Wolfgang Jahnke, and Horst Kessler

Organisch-Chemisches Institut, TU München
Lichtenbergstrasse 4, D-85747 Garching, Germany
Received June 7, 1993

In the past decade, multidimensional NMR has developed into the premier method for the determination of structures of biomacromolecules in solution. In addition to the commonly used structural parameters derived from dipolar and scalar coupling, the measurement of hydrogen exchange rates has recently gained increasing importance for providing additional information about the structural and dynamical features of molecules, such as solvent accessibility, complexation sites, and hydrogen bonding.¹⁻³ We propose here a new NMR technique for the convenient determination of fast amide proton exchange rates in uniformly ¹³C- and ¹⁵N-labeled compounds.

Proton exchange can take place in different time regimes, with rates ranging from reciprocal milliseconds to reciprocal months. Several methods have been published to determine these exchange rates by NMR spectroscopy.^{2,4-7} However, the most commonly used techniques can only measure slow exchange rates, since they monitor H-D exchange that cannot resolve rates faster than the time required for acquiring an NMR snapshot of the molecule (i.e., a 2D spectrum for reasonably large compounds),⁵ unless the H-D exchange process can be quenched, as in folding studies.⁸

Only a few techniques are yet available to determine fast proton exchange rates. Spera et al. have presented a technique that allows the measurement of fast exchange rates of >1 Hz by saturating the water resonance and observing the intensities of the amide resonances in the steady state of saturation transfer and *T*₁ relaxation.⁹ The main advantage of this method is the good resolution achieved by using a two-dimensional ¹H,¹⁵N-HMQC representation for detection, making it suitable for most (¹⁵N labeled) proteins that can be studied by NMR. However, it requires the knowledge of the individual *T*₁ times of the amide protons or the assumption of uniform *T*₁ values for the whole molecule. Additional sources of error are potential NOE effects from the water, from hydroxyl groups, and from H^α resonances that are also saturated by the rf irradiation.

Alternatively, Liepinsh et al. have used buildup rates from 2D exchange spectra to determine hydrogen exchange rates of 5-50 Hz between water and hydroxyl groups in small peptides as well as in BPTI.^{10,11} However, this method is only applicable to labile protons with nonoverlapping signals in a one-dimensional proton spectrum and therefore is only of limited use for larger proteins.

For this reason we have developed a new method, MEXICO (Measurement of EXchange rates in Isotopically labeled COmpounds), for the determination of fast amide proton exchange rates that avoids most of the drawbacks of other techniques and

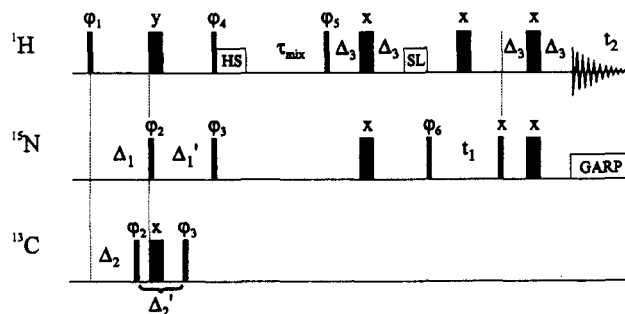


Figure 1. Pulse sequence of the MEXICO experiment. The following pulse phases were used: $\phi_1 = 8(x), 8(-x)$; $\phi_2 = x, -x$; $\phi_3 = 2(x), 2(-x)$; $\phi_4 = -x$; $\phi_5 = 16(x), 16(-x)$; $\phi_6 = 4(x), 4(-x)$; Acq. = $4(x), 8(-x), 4(x), 4(-x), 8(x), 4(-x)$. The delay values were $\Delta_1 = 5.5$ ms, $\Delta_1' = 5.4$ ms, $\Delta_2 = 3.9$ ms, $\Delta_2' = 3.6$ ms, $\Delta_3 = 2.25$ ms; HS denotes a 10-ms homospoil and SL a 3-ms spinlock pulse.

can be easily applied to even larger proteins that are uniformly labeled with ¹⁵N and ¹³C.

The most straightforward way to measure fast exchange processes is the evaluation of buildup rates of signals that are caused by the exchange process itself. Additionally, the detection should occur in the form of a 2D spectrum, preferably a ¹H,¹⁵N-correlation, to ensure sufficient spectral resolution even for large proteins. This requires that all contributions from the natural Boltzmann magnetization of the amide protons have to be filtered out prior to the acquisition of the HMQC, with a mixing time inserted in between to allow for magnetization transfer by exchange from the water back to the labile amide protons. The result will be an HMQC spectrum where only labile amide proton resonances are visible, with an intensity correlated to their exchange rate. Essentially, this corresponds to the observation of exchange after a selective excitation of the water resonance (without underlying signals such as H^α etc.). In addition, it is necessary also to saturate all carbon bound protons to reduce unwanted NOE effects during the buildup of amide magnetization.

For this purpose, the MEXICO pulse sequence (Figure 1) starts with a double ¹⁵N filter and a double ¹³C filter,¹² followed by a homospoil pulse, to ensure quantitative suppression of all protons attached to nitrogen or carbon. All other protons, i.e. essentially the water protons, are turned back onto the *z* axis by the second 90° proton pulse. In the following variable mixing time exchange of *z* magnetization from water to the labile amide protons can occur, which is subsequently detected in a standard ¹H,¹⁵N-HMQC experiment.¹³ Alternatively, an HSQC experiment can also be used for detection, yielding a higher resolution than the HMQC, but also requiring a slightly longer phase cycle because of the additional pulses.¹⁴

There are some potential sources of error when observing exchange effects that have to be addressed: longitudinal relaxation and NOE could also give rise to amide cross peaks and interfere with the exchange buildup. First, the effect of exchange with water magnetization along the +*z* axis would normally be obscured by *T*₁ relaxation, which tends to restore the Boltzmann equilibrium magnetization during the mixing time, so that practically the complete HMQC spectrum (cf. Figure 2a) is observed with reduced intensity (not shown). Therefore in our experiment the water signal is alternatively aligned along the +*z* and -*z* axis in subsequent scans by appropriate phase cycling (Figure 1). This leads to the alternating exchange of positive and negative *z* magnetization, while *T*₁ relaxation always creates positive signals. Subtraction of alternating scans then eliminates the *T*₁ contributions, leaving only signals caused by exchange with the solvent

(1) Englander, S. W.; Kallenbach, N. R. *Q. Rev. Biophys.* 1984, 16, 521-655.

(2) Henry, G. D.; Sykes, B. D. *Biochemistry* 1990, 29, 6303-6313.

(3) Linse, S.; Teleman, O.; Drakenberg, T. *Biochemistry* 1990, 29, 5925-5934.

(4) Waelder, S.; Lee, L.; Redfield, A. G. *J. Am. Chem. Soc.* 1975, 97, 2927-2928.

(5) Gooley, P. R.; Caffrey, M. S.; Cusanovich, M. A.; MacKenzie, N. E. *Biochemistry* 1992, 31, 443-450.

(6) Led, J.; Gesmar, H.; Abildgaard, F. In *Methods in Enzymology*; Oppenheimer, N. J., James, T. L., Eds.; Academic Press: San Diego, CA, 1989; Vol. 176.

(7) Olsen, H. B.; Gesmar, H.; Led, J. *J. Am. Chem. Soc.* 1993, 115, 1456-1460. Bracken, W. C.; Baum, J. *J. Am. Chem. Soc.* 1993, 115, 6346-6348.

(8) Roder, H.; Elöve, G.; Englander, S. W. *Nature* 1988, 335, 700-704. Baldwin, J. E. *Nature* 1990, 346, 409-410.

(9) Spera, S.; Ikura, M.; Bax, A. *J. Biomol. NMR* 1991, 1, 155-165.

(10) Otting, G.; Liepinsh, E.; Farmer, B. T., II; Wüthrich, K. *J. Biomol. NMR* 1991, 1, 209-215.

(11) Liepinsh, E.; Otting, G.; Wüthrich, K. *J. Biomol. NMR* 1992, 2, 447-465.

(12) Gemmecker, G.; Olejniczak, E. T.; Fesik, S. W. *J. Magn. Reson.* 1992, 96, 199-204.

(13) Bax, A.; Griffey, R. H.; Hawkins, B. L. *J. Magn. Reson.* 1983, 55, 301-315.

(14) Bodenhausen, G.; Reuben, D. *J. Chem. Phys. Lett.* 1980, 69, 185-189.

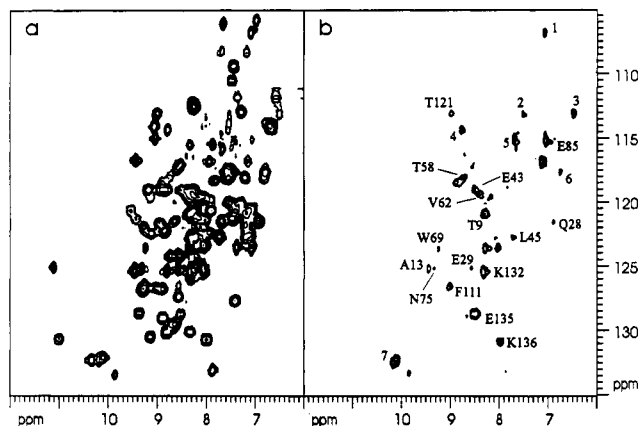


Figure 2. 2D spectra of 1 mM mannose permease domain P13 in H₂O/10% D₂O (pH 7.5) acquired at 310 K on a Bruker AMX600 with triple resonance equipment: (a) ¹H,¹⁵N-HMQC; (b) MEXICO spectrum with 200-ms mixing time, only exchange is observed, leading to a drastically reduced spectrum when compared to part a. The signal intensities (relative to the HMQC) correspond directly to the exchange rates.

(Figure 2b). Because of the nonlinear dependence of T_1 on the magnitude of the z magnetization, this is only true for exchange that causes only minor changes in the intensity of the previously saturated amide protons (compared to the Boltzmann equilibrium polarization). This means that the initial buildup rate is not disturbed by T_1 relaxation and can be used for a quantitative evaluation of exchange rates.

Then, NOE effects between the amide protons and other protons (amide or carbon bound) will also occur during the mixing time. The importance of these contributions was checked in a modified experiment where the water signal was not realigned with the z axis (by skipping the second 90° pulse on ¹H in sequence 1), so that exchange with the water resonance is suppressed and all signals that appear must be due to other mechanisms. It was found that these spectra are essentially empty up to ca. 300 ms of mixing time, so that NOE contributions can be safely neglected in this time range. Therefore exchange rates from ca. 50 Hz down to as low as ca. 0.05 Hz can be measured reliably.

However, this requires that, in order to minimize NOE effects with the bulk of carbon-bound protons, these also have to be saturated prior to the mixing time (which is accomplished with the double ¹³C filter simultaneous to the one on ¹⁵N). Of course this feature can only be applied to proteins that are uniformly labeled with ¹⁵N and ¹³C. For proteins merely labeled with ¹⁵N, NOE between amide protons and carbon-bound protons leads to the appearance of weak cross peaks for practically all amides, thus obscuring the true exchange peaks even for short mixing times.

As in all exchange studies, NOE effects between amide protons and water molecules or rapidly exchanging hydroxyl groups cannot be ruled out completely, but will not contribute significantly to the observed cross peaks.

The MEXICO experiment has been applied to a 1 mM uniformly ¹³C and ¹⁵N labeled sample of the mannose permease domain P13 from *E. coli*, a homodimer of ca. 31 kDa currently under investigation.¹⁵ The measurements were performed at 310 K and pH 7.5, and the measuring time was ca. 6 h for each 2D spectrum. In comparison to the normal HMQC spectrum (Figure 2a), only very few exchange signals appear in the spectra with mixing times of 25, 50, 100, and 200 ms (Figure 2b), as expected for a native protein. This means that the chance of signal overlap in these spectra is much reduced when compared to a full ¹H,¹⁵N-correlation (as needed for the technique of Spera et al.⁹). Figure 3 displays for some typical resonances the peak intensities as a function of mixing time, calibrated to the intensity of a regular HMQC spectrum. The evaluation of buildup rates from these

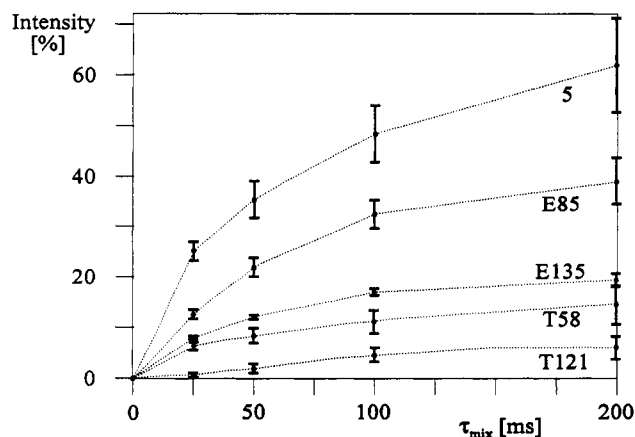


Figure 3. Selected buildup curves of amide signal intensity in MEXICO spectra of P13 (cf. Figure 2) with mixing times of 25, 50, 100, and 200 ms. Intensities have been normalized to the corresponding HMQC signals, and the labels correspond to Figure 2b. Error bars represent the accuracy of the peak integrals.

exponential curves is straightforward and allows the precise determination of fast proton exchange rates (Table I, supplementary material).

It is interesting to note that many of the peaks visible in the MEXICO spectra belong to the small fraction (<10%) of all peaks of P13 that could not be assigned yet. We suspect that the reason for this is the absence of cross peaks in triple resonance spectra, due to the fast exchange of these amide protons that are probably located in regions without regular secondary structure. This might equally be true for the C-terminal residues (E135, K136) that also show fast amide exchange, but very narrow and intense signals in the HMQC.

In order to investigate the reliability of the MEXICO technique, we measured the amide exchange rates of P13 independently with the method published by Spera et al. observing intensity changes in a ¹H,¹⁵N-HMQC after water irradiation.⁹ Unfortunately several amide resonances could not be evaluated because of signal overlap in these HMQC spectra. Also, for a precise quantitative evaluation this method requires the knowledge of the individual amide T_1 relaxation rates that were not available for P13. However, the assumption of a uniform amide T_1 time of ca. 600 ms (as suggested by 1D inversion recovery experiments) results in a good correspondence with our MEXICO data (Table I, supplementary material), if one takes into account an experimental error of up to 20% for both techniques (essentially from integration of cross peaks with partial overlap or low signal to noise).

In conclusion, the MEXICO technique described above allows the precise and straightforward measurement of fast amide exchange rates in ¹⁵N- and ¹³C-labeled compounds, without the use of additional model assumptions. Furthermore, this method is quite sensitive and can be run on dilute protein samples in reasonable time. It could also prove useful for exchange measurements of different classes of compounds, e.g. the determination of nucleic acid duplex stability.

Acknowledgment. We thank Prof. B. Erni and K. Flückinger (University of Bern) for the preparation of the labeled sample of P13 and Jochen Balbach, Stephan Seip, and Stefan Behrens for the preliminary assignments. This work was supported by fellowships from the Studienstiftung des Deutschen Volkes (W.J.) and the Fonds der Chemischen Industrie (G.G.); G.G. also acknowledges financial support from the Dr.-Ing. Leonhard Lorenz-Stiftung.

Supplementary Material Available: Table of fast amide exchange rates of *E. coli* mannose permease domain P13 (1 page). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(15) Erni, B.; Zanolari, B.; Graff, P.; Kocher, H. P. *J. Biol. Chem.* 1989, 264 (31), 18733-18741.