Measurement of Fast Proton Exchange Rates in Isotopically Labeled Compounds
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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 deter-
mination of fast amide proton exchange rates in uniformly 12C- 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.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),8 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 T1 relaxation.9 The main advantage of this method is the
good resolution achieved by using a two-dimensional 1H,15N-
HMOC 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 T1 times of the amide
protons or the assumption of uniform T1 values for the whole
molecule. Additional sources of error are potential NOE effects
from the water, from hydroxyl groups, and from H2O resonances
that are also saturated by the rf irradiation.
Alternatively, Liepinsh et al. have used build-up 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-dimensionsal 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: ϕ1 = 8(x), 8(-x), ϕ2 = x, -x; ϕ2 = 2(x), 2(-x);
ϕ3 = x, ϕ3 = 16(x), 16(-x); ϕ4 = 4(x), 4(-x); Aα = α(2, 8(x), 8
(-x), 4(x), 4(-x)); Aβ = 4(-x). The delay values were Δ3 = 5.5 ms, Δ4' = 5.4 ms,
Δ5 = 3.9 ms, Δ6' = 3.6 ms, Δ7 = 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
rates 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 HMOC, 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 HMOC 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 H2O 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.
especially 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-HMOC experiment.13 Alternatively, an HSQC experi-
can also be used for detection, yielding a higher resolution
than the HMOC, 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 T1 relaxation, which tends to restore the Boltzmann equilibrium
magnetization during the mixing time, so that practically the
complete HMOC 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 T1 relaxation always creates positive signals.
Subtraction of alternating scans then eliminates the T1 contribu-
tions, leaving only signals caused by exchange with the solvent

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the observed cross peaks.

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 $90^\circ$ pulse on $^1$H 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 $^{13}$C filter simultaneous to the one on $^{15}$N). Of course this feature can only be applied to proteins that are uniformly labeled with $^{15}$N and $^{13}$C. For proteins merely labeled with $^{15}$N, 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 $^{13}$C and $^{15}$N labeled sample of the mannose permease domain P13 from *E. coli*, a homodimer of ca. 3 1 kDa currently under investigation. The measurements were performed at 50 K and pH 7.5, and the measuring time was 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 $^{15}$N 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 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 $^{15}$N-HMQC after water irradiation. 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 $^{15}$N- and $^{13}$C-labeled compounds, without the use of additional model assumptions. Furthermore, this method is quite sensitive and can be run on dilute protein samples in a reasonable time. It could also prove useful for exchange measurements of different classes of compounds, e.g. the determination of nucleic acid duplex stability.

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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.