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# Measurement of intrinsic exchange rates of amide protons in a $^{15}$N-labeled peptide

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## Summary

We have used a modified version of a previously proposed technique, MEXICO [Gemmecker et al. (1993) *J. Am. Chem. Soc.*, **115**, 11620], and improved data analysis procedures in order to measure rapid hydrogen exchange (HX) rates of amide protons in peptides labeled only with $^{15}$N. The requirement of $^{13}$C-/$^{15}$N-labeled material has been circumvented by adjusting conditions so that NOE effects associated with amide protons can be neglected (i.e., $\omega_0\tau_c \sim 1$). The technique was applied to an unstructured $^{15}$N-labeled 12-residue peptide to measure intrinsic HX rates, which are the essential reference for examining protein and peptide structure and dynamics through deceleration of HX rates. The method provided accurate HX rates from 0.5 to 50 $s^{-1}$ under the conditions used. The measured rates were in good agreement with those predicted using correction factors determined by Englander and co-workers [Bai et al. (1993) *Proteins*, **17**, 75], with the largest deviations from the predicted rates found for residues close to the N-terminus. The exchange rates were found to exhibit significant sensitivity to the concentration of salt in the sample.

## Introduction

The rates of amide hydrogen exchange (HX) in proteins often vary over orders of magnitude and provide information on structure and dynamics of proteins at the level of individual residues (Englander and Kallenbach, 1984). Slow HX rates of amide protons indicate protection from exchange, usually because of hydrogen bonding and burial in a hydrophobic environment. For a quantitative analysis, the HX rate of an amide proton must be referenced to its HX rates in an 'unprotected' state, or unstructured state, because HX rates are significantly affected by neighboring side chains (Molday et al., 1972; Bai et al., 1993). The degree of protection is usually expressed in terms of the so-called protection factor, $k_{int}/k_{obs}$, where $k_{int}$ is the intrinsic exchange rate for a given amide and $k_{obs}$ is the experimentally observed rate. Therefore, accurate knowledge of $k_{int}$ is essential for determination of a meaningful protection factor, particularly in studies on protein folding intermediates in which protection of amides is usually marginal (on the order of 100 or less; Kim, 1986; Roder et al., 1988; Hughson et al., 1990; Udgaonkar and Baldwin, 1990; Englander and Mayne, 1992).

Englander and co-workers have evaluated side-chain effects by using dipeptide models and measuring slow H-D exchange at low pH (Molday et al., 1972; Bai et al., 1993; Connelly et al., 1993). They have also calibrated the pH dependence of HX rates. Using short (five to six residues) peptides, they have demonstrated that the effects of the side chains of residues i – 1 and i are most important in determining the HX rate of NH(i) and that these effects are independent and additive. Therefore, $k_{int}$ can be predicted using the alanine reference rates at any given pH and temperature, in combination with the correction factors for different side chains. However, because of the 'dead time' ($\geq$ 5 min) imposed by the H-D solvent exchange, the reference measurements had to be performed at acidic pH (pH 5 and lower), where HX rates are sufficiently slow to enable measuring HX reactions by 1D $^1$H NMR (the exchange reaction is catalyzed by $H^+$ and $OH^-$

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ions and HX rates have minima at acidic pH, because of stronger base ($OH^-$) catalysis). Also, it is difficult to examine the accuracy of the prediction method in longer peptides, which would be better references for proteins, because of severe overlap of amide proton signals in unstructured peptides. Because of the importance of $k_{int}$, a direct method to measure $k_{int}$ in longer unstructured peptides at neutral pH, where most HX studies of proteins are performed, would be very valuable.

Several NMR techniques have been proposed to measure rapid proton–proton (H–H) HX rates in $H_2O$ (Spera et al., 1991; Gemmecker et al., 1993; Grzesiek and Bax, 1993b). Although these methods can be used for measuring fast HX rates, most of them require information on the relaxation rates of individual amide protons, which are usually determined under different solution conditions or by separate experiments. For example, the classic saturation transfer method and its variant (Spera et al., 1991) require measurement of an apparent longitudinal relaxation rate to obtain the HX rate and typically assume a simple exponential dependence of HX rates on pH. It is desirable that all measurements be performed under identical solution conditions, in order to minimize experimental errors.

The MEXICO experiment is one of the techniques proposed to measure fast HX rates in proteins (Gemmecker et al., 1993). The original method requires $^{13}C$-/$^{15}N$-labeled proteins. The experiment consists of three components: (i) an isotope filter to selectively remove observable magnetization of all $^{15}N$-attached protons (including, of course, the amide protons) and $^{13}C$-attached protons, while preserving the water magnetization; (ii) a mixing period, during which amide protons recover observable magnetization through the hydrogen exchange with solvent water; and (iii) detection of amide proton magnetization by 2D heteronuclear correlation experiments. The HX rate of a given amide can then be determined by following the buildup of cross-peak intensity in a series of MEXICO spectra as the duration of the mixing time is varied, without the requirement for measurement of relaxation rates for the amide protons. However, the requirement of expensive doubly labeled samples may prohibit the application of the MEXICO experiment. Here we demonstrate the measurement of rapid H–H HX rates in peptides by a modified MEXICO experiment, under conditions that allow the use of peptides labeled only with $^{15}N$. This experiment promises to be extremely valuable for direct measurement of amide exchange rates in small peptides and should find important applications in probing the conformational propensities of linear peptides.

## Materials and Methods

A $^{15}N$-labeled 12-residue peptide, ISMSEEDLLNAK, was prepared by digesting a $^{15}N$-labeled recombinant protein, poplar plastocyanin (S. Koide, M. Reymond and P.E. Wright, manuscript in preparation) with trypsin (sequencing grade; Sigma, St. Louis, MO), followed by purification using a $C_{18}$ reversed-phase HPLC column. The peptide corresponds to residues 55–66 of plastocyanin and has free amino and carboxyl termini. The identity of the peptide was confirmed by subjecting the natural abundance and $^{15}N$-labeled samples to mass spectrometry. The peptide was lyophilized, weighed and then dissolved in potassium phosphate buffer (10 mM, pH 7.0) containing sodium chloride (100 mM), EDTA (50 μM) and $D_2O$ (10%). The pH was finally adjusted to 7.0 by addition of a small amount of concentrated HCl. The pH of the sample was remeasured after the NMR experiments had been performed. The sample concentration was approximately 1 mM, determined from the weight of the lyophilized peptide.

The sequential assignments of the $^1H$ and $^{15}N$ NMR spectra were achieved by standard procedures (Wüthrich, 1986) using homonuclear 2D TOCSY, $\omega_2$ $^{15}N$-edited 2D ($^1H$,$^1H$)-ROESY, $\omega_2$ $^{15}N$-edited 2D ($^1H$,$^1H$)-TOCSY, 2D ($^{15}N$,$^1H$)-HSQC, 2D ($^{15}N$,$^1H$)-HSQC-TOCSY and 2D ($^{15}N$,$^1H$)-HSQC-ROESY spectra (Bodenhausen and Ruben, 1980; Braunschweiler and Ernst, 1983; Bothner-By et al., 1984; Bax and Davis, 1985; Bax et al., 1990; Norwood et al., 1990). Experiments were performed on a Bruker AMX-300 spectrometer with a triple-resonance probe at 5 °C. For the TOCSY experiment, the DIPSI-2rc mixing sequence was used at 12.5 kHz field strength (Cavanagh and Rance, 1992). For the ROESY, a 4 kHz CW field was used for the mixing period. Water suppression was achieved by spin-lock purge pulses (Otting and Wüthrich, 1988; Messerle et al., 1989), except for the 2D ($^1H$,$^1H$)-TOCSY spectrum in which the water resonance was presaturated by weak irradiation during the relaxation period. Data were processed using the program FELIX (v. 2.05, Hare Research, Bothell, WA and Biosym, San Diego, CA) on a Silicon Graphics Indigo workstation. Typically, Lorentzian-to-Gaussian transformation was used in $t_2$ and a shifted sine-bell window in $t_1$. Final matrix sizes were $512 \times 1024$ and $128 \times 1024$ points for ($^1H$,$^1H$) and ($^{15}N$,$^1H$) spectra, respectively.

The pulse scheme of a modified version of the MEXICO experiment is shown in Fig. 1. Experiments were performed at 20 °C. Typically, 32 real points were collected in the $^{15}N$ dimension and 512 complex points in the $^1H$ dimension. Some $^{15}N$ resonances could be folded without introducing overlap, so that the spectral width in the indirect dimension was only 343 Hz. MEXICO spectra with mixing times of 12.5, 25, 50, 75 and 100 ms and a jump-return HMQC spectrum for reference purposes were collected for each data set. A relaxation delay of 2 s was used between transients. Each experiment took approximately 4 h for a sample of ca. 1 mM. The residual water signal was attenuated by deconvolution of the low-

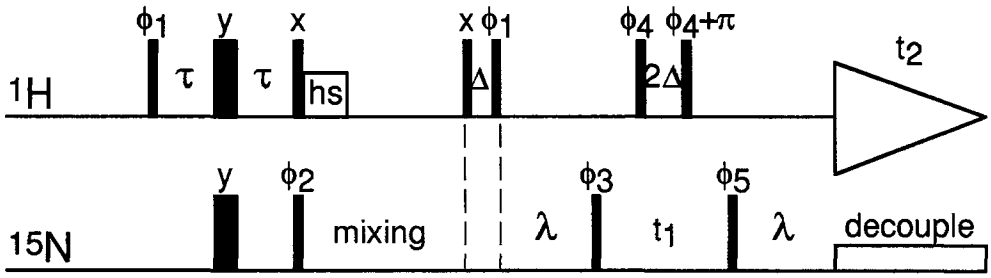


Fig. 1. Pulse scheme of a modified version of the MEXICO experiment. Narrow and wide pulses correspond to 90° and 180° flip angles, respectively. The $^1H$ carrier was set at the $H_2O$ frequency. The $^{15}N$ frequency was set at 121.8 ppm. $^{15}N$ decoupling was achieved using the GARP-1 decoupling sequence (Shaka et al., 1985). A 4-ms homospoil pulse was applied at the beginning of the mixing period at the maximum strength achievable on our AMX-300 spectrometer. For experiments with mixing times shorter than 25 ms, the homospoil pulse was omitted to avoid magnetic field inhomogeneity. Delay durations were $\tau = 2.75$ ms, $\lambda = 5.0$ ms and $\Delta = 250$ μs. Phase cycling was as follows: $\phi_1 = 2(x),2(-x)$; $\phi_2 = x,-x$; $\phi_3 = 4(x),4(-x)$; $\phi_4 = 16(x),16(y),16(-x),16(-y)$; $\phi_5 = 8(x),8(-x)$; acquisition = 2(x),4(−x),2(x),2(−x),4(x),4(−x),4(x),2(−x),2(x),4(−x),2(x). For a reference spectrum, an experiment starting from the fourth $^1H$ pulse was performed; in this case, phase cycling was $\phi_1 = -x$; $\phi_3 = x,-x$; $\phi_4 = 4(x),4(y),4(-x),4(-y)$; $\phi_5 = 2(x),2(-x)$; acquisition = x,2(−x),x,−x,2(x),−x. Quadrature detection in $t_1$ was achieved by incrementing $\phi_3$ in the TPPI manner (Marion and Wüthrich, 1983).

frequency signals in the time-domain data (Marion et al., 1989). Lorentzian-to-Gaussian transformation was applied in the $t_2$ dimension and the data were zero-filled to 1024 complex points, followed by Fourier transformation; the upper halves of the $^1H$ spectra were discarded. Prior to real Fourier transformation in $t_1$, the data were extended to 53 points by linear prediction, multiplied by a 90°-shifted squared sine bell and zero-filled to 1024 real points.

The intensity of the water resonance during the mixing period was calibrated using the following $^1H$ pulse scheme:

$$90°(\phi_1)\text{-}\tau\text{-}180°(\phi_2)\text{-}\tau\text{-}90°(\phi_3)\text{-mixing-}90°(\phi_4)\text{-Acq.}$$

where the phase cycling was: $\phi_1 = 8(x),8(y),8(-x),8(-y)$; $\phi_2 = 4(y),4(-y),4(-x),4(x)$; $\phi_3 = 8(-x),8(-y),8(x),8(y),8(x),8(y),8(-x),8(-y)$; $\phi_4 = x,y,-x,-y$; acq. = 8(x,y,−x,−y),8(−x,−y,x,y). The mixing period was omitted for the calibration of the water intensity immediately after the three pulses. A homospoil pulse was applied during the mixing period in the same way as in the MEXICO experiment, as indicated in Fig. 1. Peak intensities were determined by integrating peak areas.

Predicted HX rates for the amides of the peptide were estimated using the method of Englander and co-workers (Molday et al., 1972; Bai et al., 1993; Connelly et al., 1993). First, the HX rate of an alanine standard (poly-D,L-alanine), $k_{ref}$, at a given pH was calculated by:

$$k_{ref} = k_A 10^{-pH} + k_B 10^{(pH - pK_w)} + k_w$$

where $k_A$, $k_B$ and $k_w$ are rate constants for specific acid catalysis, specific base catalysis and water catalysis, respectively; $K_w$ is the dissociation constant of $H_2O$. The following values were used (Connelly et al., 1993): $k_A = 0.41\ s^{-1}$; $k_B = 1.48 \times 10^8\ s^{-1}$; $k_w = 0$; and $K_w = 14.17$. Note that these are the reference rates for exchange of NH with $H_2O$ rather than with $D_2O$. At neutral pH, the specific base catalysis is predominant. No corrections for temperature dependence were made, because the constants have been determined at 20 °C, i.e., the temperature at which all experiments in the present study were performed. HX rates for individual amides were then calculated from $k_{ref}$ by multiplying with correction factors for side-chain effects determined by Bai et al. (1993).

## Results and Discussion

For the purpose of the present study, we have modified the original MEXICO experiment in the following ways:

*(1) Effects of NOE buildup during the mixing time*

The original sequence uses both $^{13}C$ and $^{15}N$ filters to remove all magnetization except for water, thereby suppressing all NOEs to the amide protons of interest. However, NOEs *from* recovered amide protons to other protons cannot be suppressed by the experiment, which leads to a decay of recovered amide magnetization during the mixing time. Because of this, the experiment would not give a simple buildup curve for amide protons in larger proteins, in which most amide protons are involved in an NOE network. Therefore, for a straightforward analysis of the data it is important to suppress to a negligible level all NOEs involving amide protons, irrespective of the use of $^{13}C$-labeled compounds. It may well be possible that, in an application to peptides, conditions can be adjusted so that $\omega_0\tau_c$ is approximately 1, where $\omega_0$ is the Larmor frequency and $\tau_c$ the rotational correlation time. Under such conditions, NOEs associated with amide protons are negligibly small. In contrast, it is extremely difficult, if not impossible, to find such conditions for proteins, which are usually in the spin-diffusion limit (i.e., $\omega_0\tau_c \gg 1$). In fact, at a $^1H$ frequency of 300 MHz, no NOE cross peaks are found in a 2D NOESY spectrum recorded with

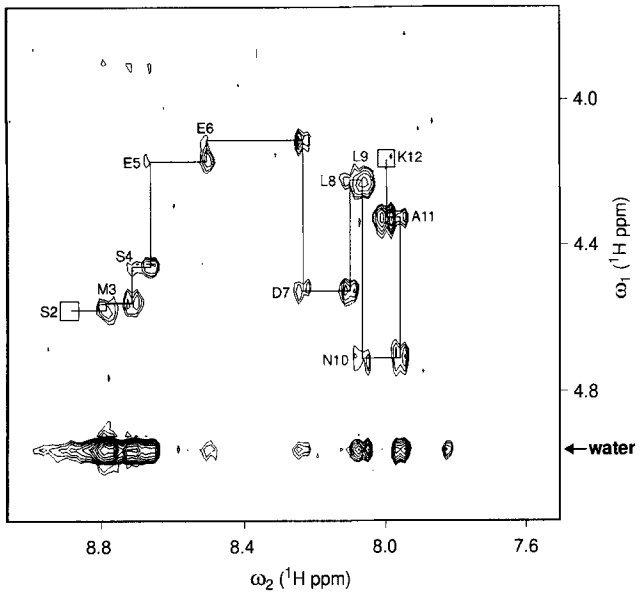


Fig. 2. Fingerprint region of a 2D $\omega_2$ $^{15}$N-edited ROESY spectrum of the 12-residue peptide, ISMSEEDLLNAK, at pH 7.0 and 5 °C. Water suppression was achieved by a spin-lock purge pulse in the HSQC part of the experiment. Assignments of intraresidue $C^{\alpha}H$-$N^{H}$ cross peaks are indicated and sequential αN(i, i+1) connectivities are shown by lines. The cross peaks at the water chemical shift in $\omega_1$ are opposite in sign relative to the diagonal peaks, indicating that they are due to chemical exchange between amide and water protons.

a mixing time of 200 ms for amide protons of the 12-residue peptide used for this study, while negative NOEs were detected at 600 MHz (data not shown). The results indicate that $\omega_0\tau_c$ is close to 1 at $\omega_0 = 300$ MHz. Therefore, contribution of the NOE in the MEXICO experiment can be safely ignored at 300 MHz, which justifies not suppressing the NOE during the mixing period and use of a simple kinetic model for data analysis. It should be emphasized that the contributions of NOE to observable buildup curves can be removed because of the favorable correlation time, not because of the lack of short interproton distances.

*(2) Minimum perturbation of the water signal* It has recently been demonstrated that signal intensities of amide protons that undergo rapid chemical exchange with the bulk water are significantly reduced when the water signal is scrambled by presaturation, spin-lock purge pulses or magnetic field inhomogeneity and the water is not allowed to fully relax to thermal equilibrium (Grzesiek and Bax, 1993a; Stonehouse et al., 1994). Since the focus of the MEXICO experiment is on amide protons that rapidly exchange with water, it is important to properly control the water magnetization in order to obtain maximum sensitivity. Therefore, we have replaced the original detection scheme, which used a spin-lock purge pulse, with the jump-return HMQC experiment (Fig. 1; Roy et al., 1984; Spera et al., 1991). In this way, the water magnetization is kept parallel to the z-axis during most of the experiment. This jump-return HMQC sequence is an alternative to HSQC using pulsed field gradients and a modified choice of proton pulse phases to avoid water saturation (Jahnke and Kessler, 1994; Stonehouse et al., 1994). It should be noted that a higher $^{15}$N resolution can be obtained in an HSQC spectrum compared to an HMQC, because the $^{15}$N line shape in jump-return HMQC suffers from heteronuclear long-range $^{15}$N-$^{1}$H couplings (Bax et al., 1990; Norwood et al., 1990); however, in this application to a short peptide, the HMQC spectrum gives sufficient resolution, even when a course digital sampling is used (Fig. 3). To examine the effectiveness of the modification, the MEXICO spectrum with $\tau_m = 50$ ms and the reference jump-return HMQC spectrum were recorded with variation of the relaxation delay (including the acquisition period) between 1.5 and 10 s; the peak intensities of the amide protons did not change significantly as the delay was varied, suggesting that the majority of the water magnetization is brought back parallel to the z-axis after the last $^{1}$H pulse, so that the contribution of the long $T_1$ of water is small.

*(3) Calibration of the water intensity during the mixing period* The MEXICO experiment uses an isotope filter that is designed to minimally affect the water magnetization, so that peak intensities can be normalized against those in the reference HMQC experiment, which would correspond to a spectrum of completely recovered (exchanged) amide protons. However, the filter involves three $^{1}$H pulses over a period of 5.5 ms, which may de-

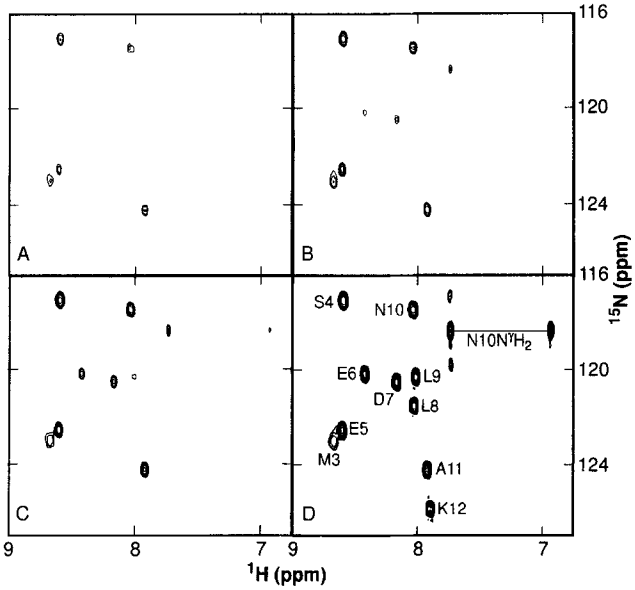


Fig. 3. MEXICO spectra with mixing times of (A) 25 ms, (B) 50 ms and (C) 100 ms, and a reference HMQC spectrum (D) of the 12-residue peptide at pH 7.0 and 20 °C. Assignments of the cross peaks are indicated in (D). The $Asn^{10}$ $N^{\gamma}H_2$ cross peaks are folded in the $^{15}$N dimension. Two weak cross peaks near the low-field $Asn^{10}$ $N^{\gamma}H$ cross peak are due to a small amount of impurities in the sample. All spectra were processed in an identical manner and are plotted at the same contour level.

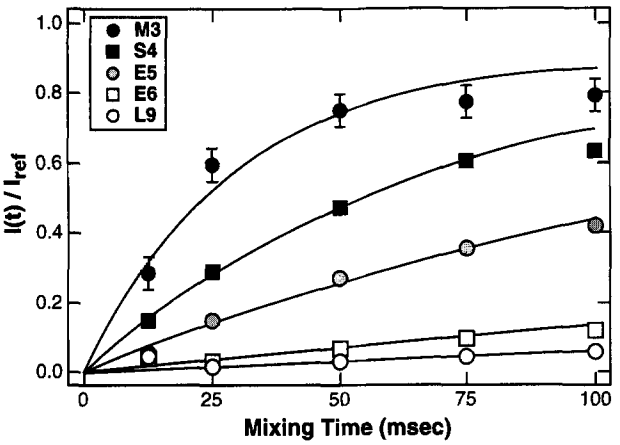


Fig. 4. Peak intensities of amide proton resonances in the MEXICO experiment plotted versus duration of the mixing time. Peak intensities were determined by measuring peak heights and normalized against those in the reference HMQC spectrum. Measurement errors were estimated by measuring noise in cross-peak-free regions of the spectra, which are indicated by bars; where no error bars appear, errors are within the size of the symbols. The curves show the best fit to the function $I(t)/I(\infty) = F(1-\exp(-kt))$, as explained in detail in the text.

crease the water intensity due to rf inhomogeneity, relaxation and diffusion. Thus, it is important to measure the damping factor due to the filter in order to properly normalize peak intensities. We performed a simple experiment to measure the intensity of the water signal after the isotope filter relative to that without the filter, i.e., (90° pulse)-(acquisition). Since this calibration requires measurement of the bulk water magnetization, additional care has been taken to check that the radiation damping effect does not disturb the intensity measurement. The calibration experiment was performed with a buffer solution containing alanine (0.1 M). The intensities of the water and alanine methyl proton resonances measured with the filter were found to be both about 90% relative to those measured without the filter, indicating that the method is not strongly affected by the radiation damping effect of water and that the filter decreases the water intensity by about 10%.

The water intensity in this single-scan calibration experiment may differ slightly from that observed in the steady state in multiple-scan experiments (as in the MEXICO experiment and the reference HMQC experiment). Ideally, the calibration experiment should first establish the steady state by cycling through many scans of the real pulse sequence (without a 90° read pulse, which does not exist in the real sequences; see Fig. 1), and only then apply the calibration sequence, which includes the 90° read pulse. However, the good agreement between the result of calibration and the highest amide proton intensity obtained in MEXICO experiments (80–90%) suggests that not having precisely the same steady state in the MEXICO and calibration experiments does not result in a significant error.

In addition to the measurement of the initial intensity, the water intensity was measured at the end of various mixing times. It was found to decrease almost linearly by approximately 6% after a 100 ms mixing time under the conditions used. The decay of the water due to radiation damping is kept minimal by applying a homospoil pulse at the beginning of the mixing time.

*(4) Use of a single filter* The double filter in the original MEXICO sequence was replaced with a single filter. Reasons for this modification are (i) the $^{1}H$-$^{15}N$ coupling constants as well as the chemical shifts fall within a narrow range, particularly for unstructured peptides, so that sufficient suppression of the 'diagonal' peaks can be achieved by a single filter; and (ii) a double filter inevitably increases the number of pulses and the duration of the filter, resulting in a larger loss of the water intensity due to pulse imperfection, diffusion and relaxation. The peak intensities of amide protons in the peptide used were suppressed by the single filter to less than 2% of those in the reference HMQC spectrum, which is comparable to base-plane noise (0.8–5%).

The HX rates of the amide protons were determined by fitting the measured peak intensities, at mixing times of up to 75 ms, of the amide proton resonances to:

$$I(t)/I_{ref} = F(1-\exp(-kt))$$

where k is the HX rate constant for a given amide proton, I(t) is the peak intensity of a given amide at mixing time t, $I_{ref}$ is its intensity in the reference spectrum, and F is the damping factor for the water signal determined by a separate experiment, as described above. The relaxation of water during the mixing time was not incorporated in the present data analysis, because of the small contribution of this term.

The technique was applied to a 12-residue $^{15}N$-labeled peptide fragment of recombinant plastocyanin. In the ROESY spectrum (Fig. 2), sequential $\alpha N(i, i+1)$ connectivities are present, whereas no NN(i, i+1) and no medium- or long-range connectivities are observed. These results suggest that the peptide takes on an ensemble of 'random' conformations with backbone dihedral angles that are predominantly in the β-region of $\phi,\psi$ space, which is consistent with previous observations (Dyson et al., 1992). It should be noted that the incorporation of $^{15}N$ was essential to resolve ambiguities in the sequence-specific assignments, because of poor dispersion in the $^{1}H$ dimension; this is characteristic of unstructured peptides. For example, all protons of the two leucine residues overlap, but they could be assigned using the distinct $^{15}N$ shifts. No evidence for aggregation has been found at peptide concentrations from approximately 50 μM to 2 mM.

Figure 3 shows MEXICO spectra recorded on the peptide. All detectable cross peaks are well resolved and gradual recovery of amide magnetization due to hydrogen

exchange with solvent $H_2O$ can be readily seen. Figure 4 shows buildup curves of the amide peak intensities as measured from peak heights. At mixing times up to 75 ms, the data are well fitted by a single exponential function. Slight deviation of the 100-ms data points may result from relaxation of the water or amide protons.

The HX rates for the amide protons were determined and are listed in Table 1. The measured HX rates range from 0.4 to 42 $s^{-1}$. The lower limit is largely determined by the sensitivity of the detection method; it may be improved by using longer experiment times or higher sample concentrations. Longer mixing times could also be used, although this would introduce larger errors due to relaxation effects. On the other hand, an upper limit is determined by the line shape of the peaks. As long as the peaks were observed in the spectra, the data obtained were good enough to derive the HX rates. Cross peaks in the reference HMQC spectra become unobservable as their HX rates reach values of approximately 50 $s^{-1}$, i.e., as they approach the $^1J_{^1H^{15}N}$ coupling constant (~90 $s^{-1}$);. for such rapid rates, amide hydrogen exchange becomes significant during the two delays needed to develop the heteronuclear coherence. Although the upper limit cannot be improved, the accuracy for faster HX rates could be improved by sampling more data points with shorter mixing times. Taken together, this method allows determination of a wide range of HX rates from a single sample within a reasonable period of measurement time, typically 4 h for one time point under our experimental conditions.

HX rates were also measured in higher salt concentrations, in order to examine the effects on HX rates (Table 1). The measured HX rates were compared with values predicted using the method of Englander and co-workers (Table 1; Bai et al., 1993; Connelly et al., 1993). The measured rates at 0.5 M NaCl are in good agreement (within a factor of 2.2 for residues 3–12) with the predicted values; the correction factors were determined in the presence of 0.5 M KCl (Bai et al., 1993). The results indicate that the method of Bai et al. is accurate in the prediction of the contributions of side-chain groups to the H-H HX rates at neutral pH. An increase is observed in all measurable HX rates as the salt concentration is increased (Table 1). Because of the sensitivity to salt concentration, the measured rates at 0.1 M and 1.0 M NaCl show larger deviations from predicted values. This tendency can be explained by the shielding of the negative charges in the peptide. A negative charge in general decreases the HX rate of an amide proton in its proximity by decreasing, through electrostatic repulsion, the local concentration of the hydroxide ion, which is predominantly responsible for the catalysis of the HX reaction at neutral pH (Molday et al., 1972; Bai et al., 1993). The increase in the HX rates associated with increasing salt concentration is not restricted to the middle of the peptide, where there is a cluster of negative charges, suggesting that there are long-range effects from charged residues. Such effects have not been taken into account in the prediction method. It should also be noted that the HX rates still increase as the salt concentration is increased from 0.5 M to 1.0 M, indicating that the electrostatic effects are not completely suppressed, even with 0.5 M NaCl. The HX rate for $Ser^2$ could not be measured under any conditions at 20 °C, indicating a larger deviation from the predicted rate. $Met^3$ also has a significantly

TABLE 1
ASSIGNMENT AND MEASURED AND PREDICTED HX RATES OF THE AMIDE PROTONS IN ISMSEEDLLNAK (pH 7.0, 20 °C)

| Residue | Resonance assignment (ppm) | | HX rates ($s^{-1}$) at NaCl concentrations of: | | | Predicted[a] (0.5 M[a]; $s^{-1}$) |
|---|---|---|---|---|---|---|
| | $^1H$ | $^{15}N$ | 0.1 M | 0.5 M | 1.0 M | |
| $Ile^1$ | n.d.[b] | n.d. | – | – | – | 78 |
| $Ser^2$ | (8.74)[c] | (120.6)[c] | > 50[c] | > 50[c] | > 50[c] | 14 |
| $Met^3$ | 8.67 | 123.0 | 35 (4) | 42 (11) | 40 (16) | 20 |
| $Ser^4$ | 8.59 | 117.1 | 14.9 (1.0) | 23 (2) | 26 (3) | 30 |
| $Glu^5$ | 8.60 | 122.5 | 6.8 (0.3) | 11.3 (0.7) | 15.6 (1.2) | 6.2 |
| $Glu^6$ | 8.42 | 120.2 | 1.47 (0.11) | 2.87 (0.18) | 4.1 (0.3) | 2.2 |
| $Asp^7$ | 8.17 | 120.5 | 2.06 (0.12) | 4.0 (0.2) | 5.1 (0.3) | 3.6 |
| $Leu^8$ | 8.03 | 121.5 | 0.54 (0.09) | 1.12 (0.12) | 1.67 (0.16) | 1.8 |
| $Leu^9$ | 8.01 | 120.3 | 0.67 (0.09) | 1.31 (0.13) | 1.79 (0.18) | 1.6 |
| $Asn^{10}$ | 8.04 | 117.5 | 5.4 (0.3) | 9.7 (0.5) | 11.9 (0.8) | 19 |
| $Ala^{11}$ | 7.92 | 124.2 | 5.8 (0.3) | 9.9 (0.7) | 12.0 (0.7) | 21 |
| $Lys^{12}$ | 7.90 | 125.8 | 0.41 (0.08) | 0.36 (0.26) | 0.62 (0.36) | 0.16 |

The HX rates were determined as described in the text. Numbers in parentheses indicate errors determined from Monte Carlo simulations (Press et al., 1988).

[a] Predicted HX rates were calculated using the poly-D,L-alanine reference rate in $H_2O$ and correction factors in the presence of 0.5 M KCl as determined by Englander and co-workers (Bai et al., 1993; Connelly et al., 1993).

[b] The amino proton of $Ile^1$ was not observed under any conditions, thus its HX rates could not be determined.

[c] The amide proton was not detectable at 20 °C, and the chemical shifts were measured at 5 °C. A lower limit of the HX rate at 20 °C was estimated from HX rates determined at 5–15 °C.

higher HX rate than that predicted. These results suggest that the HX rates of the two residues are enhanced, presumably by the positive charge at the N-terminus, which increases the HX rates in general. On the other hand, the HX rates for $Asn^{10}$ and $Ala^{11}$ are significantly smaller than those predicted, which is consistent with effects of the negative charge at the C-terminus. Because the terminal charges are in the backbone, they may have larger long-range effects than charges on side chains. It can be concluded that the prediction method, although quite accurate, may have a larger error in the estimation of the HX rate of an amide if the amide is close to a cluster of charged residues or to the peptide termini, or if a salt concentration significantly different from 0.5 M is used. Systematic studies are obviously required to address long-range effects of charged groups and for such studies the MEXICO experiment should be very useful.

## Conclusions

The technique presented in this paper is well suited for measuring rapid HX rates of amide protons in small peptides labeled only with $^{15}N$. The method does not require changes in solvent conditions ($D_2O$ concentration, pH) or the use of pulsed field gradients or shaped pulses, and it can be used at neutral pH, close to physiological conditions. It should, however, be borne in mind that the technique gives accurate results only under conditions where HX rates are much faster than cross-relaxation rates (NOE) for the amide protons of interest. This requirement can be met, at least in the case of short peptides, by adjusting experimental conditions so that $\omega_0\tau_c \sim 1$.

Application of the technique is not limited to measurement of *intrinsic* HX rates in *unstructured* peptides. Our preliminary results show that it works equally well in solutions containing denaturant (urea and guanidine hydrochloride), which may be used to unfold peptides with secondary structure propensities for measurement of intrinsic HX rates. Alternatively, the technique may be used to detect the small changes in HX rates that are associated with hydrogen-bond formation in small populations of folded structures in peptides in aqueous solution (Goodman and Kim, 1991; Rohl and Baldwin, 1994).

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## References

Bai, Y., Milne, J.S., Mayne, L. and Englander, S.W. (1993) *Proteins*, **17**, 75–86.

Bax, A. and Davis, D.G. (1985) *J. Magn. Reson.*, **63**, 207–213.

Bax, A., Ikura, M., Kay, L.E., Torchia, D.A. and Tschudin, R. (1990) *J. Magn. Reson.*, **86**, 304–318.

Bodenhausen, G. and Ruben, D.J. (1980) *Chem. Phys. Lett.*, **69**, 185–189.

Bothner-By, A.A., Stephens, R.L. and Lee, J. (1984) *J. Am. Chem. Soc.*, **106**, 811–813.

Braunschweiler, L. and Ernst, R.R. (1983) *J. Magn. Reson.*, **53**, 521–528.

Cavanagh, J. and Rance, M. (1992) *J. Magn. Reson.*, **96**, 670–678.

Connelly, G., Bai, Y., Jeng, M.-F. and Englander, S.W. (1993) *Proteins*, **17**, 87–92.

Dyson, H.J., Sayre, J.R., Merutka, G., Shin, H.-C., Lerner, R.A. and Wright, P.E. (1992) *J. Mol. Biol.*, **226**, 819–835.

Englander, S.W. and Kallenbach, N. (1984) *Quart. Rev. Biophys.*, **6**, 521–655.

Englander, S.W. and Mayne, L. (1992) *Annu. Rev. Biophys. Biomol. Struct.*, **21**, 243–265.

Gemmecker, G., Jahnke, W. and Kessler, H. (1993) *J. Am. Chem. Soc.*, **115**, 11620–11621.

Goodman, E.M. and Kim, P.S. (1991) *Biochemistry*, **30**, 11615–11620.

Grzesiek, S. and Bax, A. (1993a) *J. Am. Chem. Soc.*, **115**, 12593–12594.

Grzesiek, S. and Bax, A. (1993b) *J. Biomol. NMR*, **3**, 627–638.

Hughson, F.M., Wright, P.E. and Baldwin, R.L. (1990) *Science*, **49**, 1544–1548.

Jahnke, W. and Kessler, H. (1994) *J. Biomol. NMR*, **4**, 735–740.

Kim, P.S. (1986) *Methods Enzymol.*, **131**, 136–156.

Marion, D. and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun.*, **13**, 967–974.

Marion, D., Ikura, M. and Bax, A. (1989) *J. Magn. Reson.*, **84**, 425–430.

Messerle, B.A., Wider, G., Otting, G., Weber, C. and Wüthrich, K. (1989) *J. Magn. Reson.*, **85**, 608–613.

Molday, R.S., Englander, S.W. and Kallen, R.G. (1972) *Biochemistry*, **11**, 150–158.

Norwood, T.J., Boyd, J., Heritage, J.E., Soffe, N. and Campbell, I.D. (1990) *J. Magn. Reson.*, **87**, 488–501.

Otting, G. and Wüthrich, K. (1988) *J. Magn. Reson.*, **76**, 569–574.

Press, W.H., Flannery, B.P., Teukolsky, S.A. and Vetterling, W.T. (1988) *Numerical Recipes in C. The Art of Scientific Computing*, Cambridge University Press, Cambridge, p. 548.

Roder, H., Elöve, G.A. and Englander, S.W. (1988) *Nature*, **335**, 700–704.

Rohl, C.A. and Baldwin, R.L. (1994) *Biochemistry*, **33**, 7760–7767.

Roy, S., Papastavros, M.Z., Sanchez, V. and Redfield, A.G. (1984) *Biochemistry*, **23**, 4395–4400.

Shaka, A.J., Barker, P.B. and Freeman, R. (1985) *J. Magn. Reson.*, **4**, 547–552.

Spera, S., Ikura, M. and Bax, A. (1991) *J. Biomol. NMR*, **1**, 155–165.

Stonehouse, J., Shaw, G.L., Keeler, J. and Laue, E. (1994) *J. Magn. Reson.*, **A107**, 178–184.

Udgaonkar, J.B. and Baldwin, R.L. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 8197–8201.

Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York, NY.