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Amide-resolved hydrogen–deuterium exchange measurements from membrane-reconstituted polypeptides using exchange trapping and semiselective two-dimensional NMR

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SUMMARY

Amide-resolved, hydrogen–deuterium exchange from bee venom melittin reconstituted in fully hydrated vesicles suspended in D_2O buffer was measured using a technique involving (1) trapping samples throughout an exchange time course by rapid freezing and lyophilization; and (2) dissolving the dried peptide/lipid mixtures in deuteromethanol to record high-resolution spectra using semiselective excitation pulses to select peptide amide signals in the presence of large excess lipid signals. Two-dimensional, amide-selective GaussNOESY and fingerprint-selective off-diagonal PingCOSY spectra are shown to be suitable for rapid acquisition of amide-selective spectra, obtained throughout a time course of amide exchange in the membrane-bound state. Membrane-reconstituted melittin is shown to contain two sequences of exchange-stable amides, corresponding to helical regions on either side of the single proline residue.

Some insight into the structural and dynamic properties of membrane proteins in membranes has come from measurements of the rates of bulk exchange of hydrogen in the backbone polypeptide amides with aqueous solvent deuterium or tritium (Englander et al., 1982; Downer et al., 1986; Jung et al., 1986; Alvarez et al., 1987; Mantsch et al., 1988; Sami and Dempsey, 1988; Zhang et al., 1992). The ability to resolve rates of individual amides of membrane proteins has the potential for more detailed analysis, including the identification of stable amides that are hydrogen-bonded within the transmembrane helices of these proteins. High-resolution NMR spectroscopy is a powerful method for resolving signals from individual amides of proteins in solution, but is severely limited in membrane systems because of the extreme line broadening arising from dipolar interactions and chemical-shift anisotropy in systems that undergo slow anisotropic motions on the NMR time scale.

An exchange trapping method was recently described which permits the application of high-

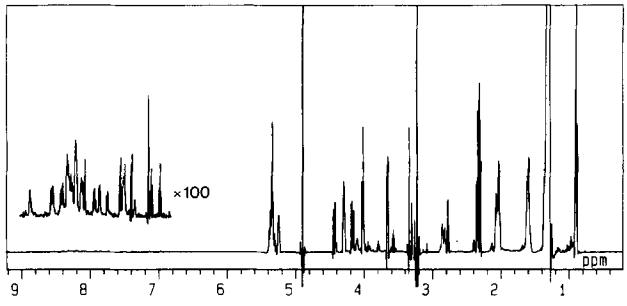

Fig. 1. ^{1}H NMR spectrum of a melittin:lipid (2:100 mM) preparation, obtained by freezing and lyophilizing reconstituted vesicles suspended in 20 mM sodium acetate buffer, pH 4.0 and dissolving the dried residue in deuteromethanol (CD_3OD). The peptide amide signals in the insert are shown at 100-fold vertical expansion. The lipid composition of the vesicles was egg phosphatidylcholine:egg phosphatidylglycerol, 88:12 (M:M). The peptide and membrane lipids dissolve as monomers in methanol, giving rise to narrow linewidths.

resolution NMR to measuring amide-resolved hydrogen–deuterium exchange from small membrane-reconstituted polypeptides (Dempsey and Butler, 1992). The hydrogen–deuterium exchange that occurs on transferring a reconstituted vesicle population into a buffer made in D_2O is trapped at various periods throughout an exchange time course by rapid freezing and lyophilization to remove all water. The extent of exchange of each amide at each time point is measured after transferring the dried peptide–lipid complexes into a solvent, where the amide signals in the 1D high-resolution NMR spectrum are assigned and where amide exchange is intrinsically slow. Extension of this method to larger membrane-reconstituted polypeptides and small membrane proteins requires the use of multidimensional NMR methods to resolve peptide amide signals which, for largely helical polypeptides, fall within a narrow spectral region. In this report we show that 2D NMR spectra, obtained using semiselective excitation pulses (Bauer et al., 1984; Freeman, 1992), can be used for monitoring amide exchange rates from membrane- bound polypeptides using exchange trapping. The use of semiselective pulses allows the acquisition of amide-selective subspectra, suppressing large excess signals from the dried membrane lipids that are dissolved with the peptide; it also permits rapid (on the amide exchange time scale) spectral acquisition.

The interaction of bee venom melittin (Dempsey, 1990) with fully hydrated phospholipid bilayer membranes is being studied using exchange trapping to determine its conformational, dynamic and topological properties in the membrane-bound state. This 26-amino acid peptide adopts an amphipathic helical conformation on binding to membranes (Dawson et al., 1979; Vogel, 1981), but the difficulties in direct determination of structural and orientational information from membrane-associated peptides and proteins has limited understanding of the voltage-gated ion channel and lytic activities of the peptide. To minimize membrane-disrupting effects and to retain physiological relevance, significant molar excesses of membrane phospholipids (50:1 lipid:melittin) are preferred when studying the properties of membrane-bound melittin. Under these conditions, the high-resolution ^{1}H NMR spectrum of exchange-trapped and lyophilized membranes dissolved in deuteromethanol is dominated by signals from the lipids (Fig. 1) which have up to 10^5-fold greater signal intensity compared to the partially exchanged peptide amide signals. The amide signals (7–9 ppm) are well separated from lipid signals (0.8–5.5 ppm), permitting semiselective excitation pulses to be used to acquire amide-selective subspectra, and their use is beneficial to the acquisition of acceptable 2D spectra under these conditions.

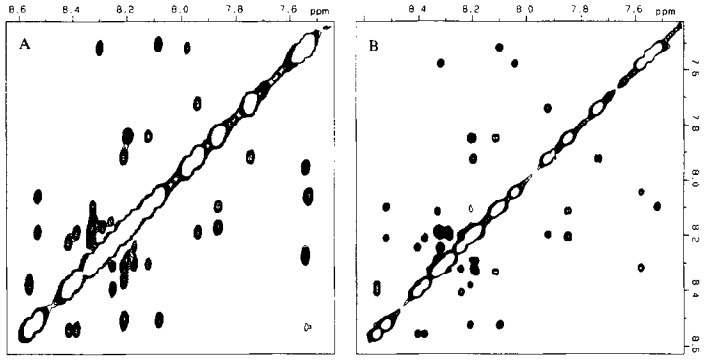

Fig. 2. (A) Phase-sensitive NOESY spectrum of melittin (without lipids) in CD_3OH, using standard acquisition conditions (Jeener et al., 1979; States et al., 1982); and (B) amide-selective subspectrum of a cosolubilized melittin/lipid complex similar to that in Fig. 1, obtained using a phase-sensitive GaussNOESY experiment. The spectra were obtained at 500 MHz ^1H frequency at 20 °C on a JEOL alpha 500 NMR spectrometer with a mixing time of 300 ms. 90° Gaussian-shaped pulses of 1.8 ms duration, centred at 7.9 ppm, were used for the GaussNOESY, for which an f2 frequency dimension of 4000 Hz was used to accommodate small residual folded signals from solvent and lipids. These were additionally suppressed by reducing the Besel filter width to ± 1000 Hz; the f1 frequency dimension was 1250 Hz. The nonselective NOESY was obtained with a frequency range of 5000 Hz in f1 and f2. Forty-eight scans per 400 f1 time increments (NOESY) or 96 scans per 200 f1 time increments (GaussNOESY) were employed, giving equivalent total accumulation times (12 h); each spectrum was zero-filled to a total matrix of 1024 × 1024 points and transformed after application of shifted squared sine-bell window functions in both dimensions. The solvent OH signal was suppressed either with a DANTE (Morris and Freeman, 1978) sequence (NOESY) or with continuous irradiation during the relaxation delay and mixing time (GaussNOESY). Neither spectrum was baseline corrected, nor processed to suppress t_1 noise. The amide cross-peak assignments have been published (Bazzo et al., 1988).

In addition to the suppression of lipid signals, the use of semiselective pulses in 2D experiments has the advantage of reducing the ratio of spectral collection time/f1 digital resolution under conditions where the signal/noise ratio is adequate (Cavanagh et al., 1987; Oschkinat et al., 1988). Figure 2 illustrates the enhanced f1 digital resolution obtainable in a phase-sensitive semiselective NOESY (GaussNOESY) experiment of a sample similar to that of Fig. 1 (Fig. 2B) compared with a nonselective phase-sensitive NOESY spectrum of melittin alone (Fig. 2A), resulting largely from a fourfold decrease in the f1 frequency dimension (1250 Hz vs. 5000 Hz), even in the presence of a 50-fold molar excess of cosolubilized membrane phospholipids. The comparison also illustrates that the amide chemical shifts of methanol-solubilized melittin are little affected by the presence of a large molar excess of cosolubilized lipids, even though the enhanced solubility in methanol of melittin in the presence of lipids indicates some interaction with the lipids (Dempsey and Butler, 1992). We have generally found that polypeptides in a helical conformation have very similar amide and CH^{α} chemical shifts, irrespective of the presence or absence of codissolved membrane lipids; this usually allows straightforward assignment of the 2D subspectra (unpublished results). The excellent selectivity and phase properties of the GaussNOESY experiment for acquiring amide-selective subspectra in the presence of large lipid signals can be seen by inspection of the first f2 acquisition of the experiment (Fig. 3), which shows suppression of strong lipid signals by around 10^5-fold.

The contraction of the f1 frequency dimension allows 2D spectra to be obtained rapidly, while maintaining acceptable f1 digital resolution and enough scans per f1 increment for adequate

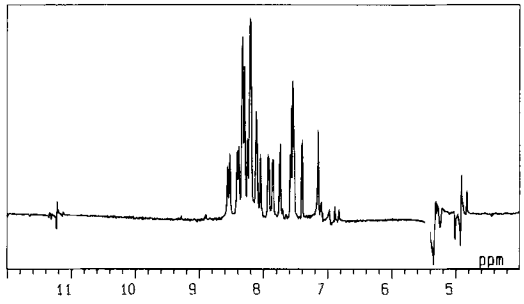

Fig. 3. First increment of a GaussNOESY experiment, illustrating the suppression of signals outside the amide region and the good phase characteristics of the experiment (P1 ≈ 180°). Signals above 10 ppm are residual lipid signals, 'folded' from the upfield regions of the spectrum. The peptide and lipid concentrations of the samples of Figs. 3–5 are the same as in the legend to Fig. 1.

phase cycling. Figure 4 illustrates semiselective NOESY experiments of methanol-solubilized melittin-reconstituted vesicles trapped after 0, 3 and 8 h of hydrogen–deuterium exchange in acetate buffer in D_2O at pH* 4.0. Reduction of the spectral acquisition time to 3 h (compared to 12 h for the spectra of Fig. 2) was achieved by halving both the number of f1 increments and the number of scans per f1 increment; this length of time (3 h) is short compared to the exchange rates of most of the melittin amides in deuteromethanol at the pH* of the measurement (Dempsey, 1992). A zero exchange time point ensures that any artefactual exchange that might occur after exchange trapping, or of poorly protected amides that exchange rapidly in methanol, is accounted for; i.e., the only difference between the zero and subsequent time-point samples is the time of

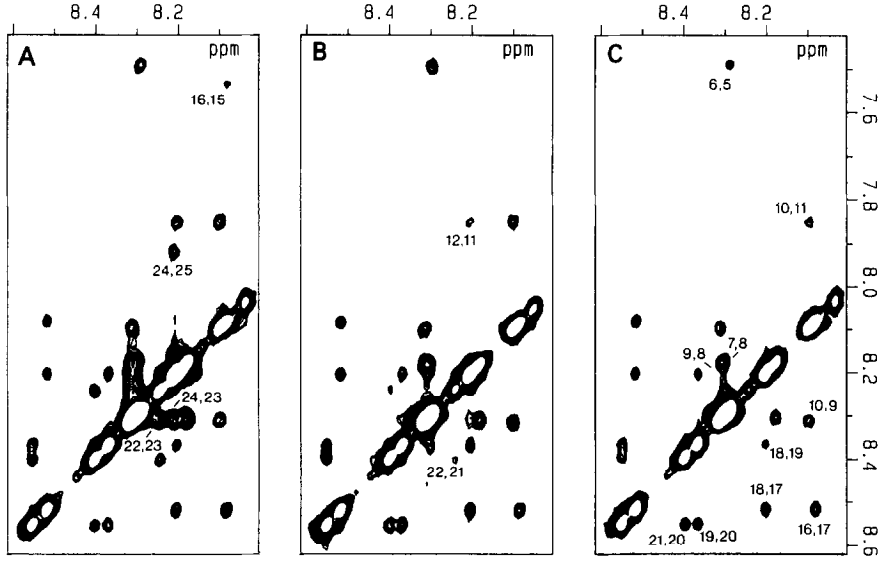

Fig. 4. GaussNOESY spectra, acquired in 3 h, of melittin/lipid complexes dissolved in CD_3OD, trapped after 0, 3 and 8 h (A, B and C, respectively) of hydrogen–deuterium exchange in the membrane-reconstituted state (reconstituted vesicles diluted 10-fold into 20 mM sodium acetate buffer, pH* 4.0, made in D_2O). The NH,NH cross peak is annotated in the spectrum in which the peak is last observed.

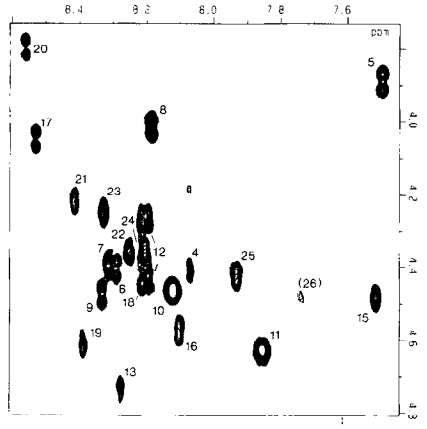

Fig. 5. PingCOSY spectra, acquired in 2 h, of dried melittin/lipid complexes dissolved in CD_3OD, trapped after 5 min of hydrogen–deuterium exchange in the membrane-bound state. $NHCH^{\alpha}$ correlation peaks are identified by amino acid residue number. f2 and f1 frequency dimensions were 4000 and 1000 Hz, respectively, and successive Ping pulse pairs in the COSY sequence (Xu et al., 1992) employed semiselective 180° Gaussian pulses of 1.8 ms, centred on 4.3 ppm (CH^{α}) and 7.9 ppm (NH) to generate a fingerprint-selective subspectrum.

amide exchange of the peptide reconstituted in vesicles in D_2O exchange buffer. Exchange rates of some of the poorly protected amides may also be determined by 1D NMR spectroscopy (Dempsey and Butler, 1992).

Because the amide–amide cross-peak intensity is proportional to the product of the residual intensities of the amides, the amide–amide NOESY cannot generally be used for direct determination of amide exchange rates and these spectra are best used together with 1D spectra to resolve ambiguities in assignment. We expect this experiment to be useful for following amide exchange from small membrane proteins containing transmembrane helices that might be defined by the retention of sequential stretches of slowly exchanging amides. For example, although melittin lies on the membrane surface under the conditions employed for amide exchange in vesicles (Dempsey and Butler, 1992), two relatively stable segments are apparent in the exchange protection of two stretches of sequential amides (residues 5–11 and 16–21; Fig. 4C) on either side of the Pro^{14} residue. Together with the knowledge that melittin adopts an α-helical conformation when bound to membranes (Dawson et al., 1979; Vogel, 1981), these experiments define the extent of stable helix in the membrane-bound state.

A second semiselective 2D experiment that is useful for following amide exchange from membrane-bound melittin using amide exchange trapping is the 'PingCOSY' experiment of Xu et al. (1992). The 1D 'Ping' experiment uses alternate 90_x(hard)-180_x(soft)-acquire(+), 90_x(hard)-180_y(soft)-acquire(−) pulse pairs, resulting in subtraction of frequency-dependent phase errors and good selectivity. An off-diagonal selective COSY subspectrum of the $NHCH^{\alpha}$ fingerprint region can be obtained by replacing the first hard 90° pulse of the COSY sequence with the PING pair centred on the CH^{α} spectral region (4.0 ppm) and the second hard 90° pulse with the PING pair centred on the NH spectral region (Xu et al., 1992). We have used a non-phase-sensitive version of this experiment which, while neglecting the excellent phase characteristics of the PING pulse pair, allows rapid spectral acquisition. Figure 5 shows the fingerprint region of methanol-solubilized freeze-dried bilayers obtained in a 2-h PingCOSY experiment. Virtually all the

$NHCH^{\alpha}$ correlation peaks (except Ile^{2}, Gly^{3} and Gln^{26}) are observed in the spectrum, making them candidates for extracting exchange rates from time-dependent changes in signal intensities in spectra obtained after increasing periods of amide exchange in the membrane (not shown).

These experiments demonstrate that exchange trapping, together with semiselective 2D NMR experiments, may be used to monitor amide exchange from membrane-bound polypeptides. We have obtained similar data using exchange trapping and cosolubilization for membrane-bound peptides soluble in chloroform–methanol mixtures, trifluoroethanol or dimethylsulfoxide, all of which can dissolve many membrane-forming phospholipids. The study of small membrane proteins using these methods is, of course, limited by the inherent size limitations in protein NMR, and the use of semiselective pulses, while effective in suppressing lipid signals, does not always offer the advantage of enhanced f1 digital resolution because of the rapid transverse relaxation in larger proteins. It is likely that improvements in these experiments can be made, particularly if methods for biosynthetic expression of small membrane proteins become generally available to allow isotopic labelling and spectral filtering for suppressing lipid signals and enhancing spectral resolution.

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