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ARTICLE

NMR resonance assignments for sparsely ^{15}N labeled proteins

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Abstract For larger proteins, and proteins not amenable to expression in bacterial hosts, it is difficult to deduce structures using NMR methods based on uniform ^{13}C, ^{15}N isotopic labeling and observation of just nuclear Overhauser effects (NOEs). In these cases, sparse labeling with selected ^{15}N enriched amino acids and extraction of a wider variety of backbone-centered structural constraints is providing an alternate approach. A limitation, however, is the absence of resonance assignment strategies that work without uniform ^{15}N, ^{13}C labeling or preparation of numerous samples labeled with pairs of isotopically labeled amino acids. In this paper an approach applicable to a single sample prepared with sparse ^{15}N labeling in selected amino acids is presented. It relies on correlation of amide proton exchange rates, measured from data on the intact protein and on digested and sequenced peptides. Application is illustrated using the carbohydrate binding protein, Galectin-3. Limitations and future applications are discussed.

Keywords Amide exchange · Assignments · Mass spectrometry · Proteins · Sparse labeling

Introduction

Protein structure determination using nuclear Overhauser effect (NOE) derived distance restraints and triple resonance (^{1}H, ^{15}N, ^{13}C) assignment strategies is now well established (Wider and Wuthrich 1999; Tzakos et al. 2006; Montelione et al. 2000). However, studies of large proteins, or proteins which are difficult to express in bacterial hosts, still present problems with respect to the requirement for uniform isotopic enrichment with ^{15}N and ^{13}C. Resolving the increasing number of resonances (cross-peaks) becomes problematic for large proteins, and expression in non-bacterial hosts (insect or mammalian cells) can involve extraordinary expense when isotopes are introduced in the form of complete sets of labeled amino acids (Lustbader et al. 1996). Introduction of sparse labels from subsets of the less expensive ^{15}N labeled amino acids presents a possible alternative. The resulting ^{15}N-^{1}H pairs in amide bonds of target proteins can be detected with optimal resolution using the transverse relaxation optimized spectroscopy (TROSY) form of heteronuclear single quantum coherence (HSQC) spectroscopy (Pervushin et al. 1997; Tzakos et al. 2006), and they can be effective in returning a variety of structural information distributed over the entire backbone of the protein. One problem that remains, however, is assignment of ^{15}N–^{1}H TROSY (or HSQC) cross-peaks. Here a broadly applicable assignment method that requires no additional isotopic labeling and no prior knowledge of protein structure is presented.

Introduction of sparse ^{15}N labels, and structure determination based on data from these labels, has some precedent. Protein production methods, including cell-free expression (Klammt et al. 2004; Vinarov et al. 2004), expression in insect cells (Strauss et al. 2005), expression in yeast cells (Chen et al. 2006), and expression in mammalian cells (Eilers et al. 1999; Klein-Seetharaman et al. 2002) all allow the use of select sets of isotopically labeled amino acids. Structural applications of sparse labels have largely been to assembly of protein complexes from subunits of known structure and characterization of interdomain contacts in multi-domain proteins. In these cases

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structural information has come from ^{1}H–^{15}N centered residual dipolar couplings (RDCs), long-range paramagnetic relaxation enhancements (PREs), and pseudo-contact shifts (PCSs) (Iwahara and Clore 2006; Pintacuda et al. 2006; Bertini et al. 2004; Fischer et al. 1999). Cross-peak assignment has been accomplished by fitting observed values for RDCs, PCSs, and PREs to values back-calculated using known subunit or domain structures (Pintacuda et al. 2004). The same RDC, PCS, and PRE data can potentially be used to carry out a structure determination of an uncharacterized protein. However, the only methods applicable to assignment of cross-peaks from sparsely-labeled, structurally uncharacterized, proteins involves preparation of numerous doubly labeled samples or numerous site-specific mutants (Tzakos et al. 2006). The assignment methods described here will provide a more general method.

The method presented is based on correlating amide proton for deuteron exchange rates measured on a folded protein, with residual proton content measured on peptides derived by pepsin digestion. Rates of amide proton exchange vary enormously from site to site in a folded protein (seconds to months) (Dempsey 2001), making it possible to distinguish rates for 20 or more cross peaks even if they can be determined only to within a factor of two. The rates for the folded protein are easily determined from the time course of cross-peak intensity loss in TROSY or HSQC spectra. For measurements on peptides, aliquots of protein are removed during the exchange process, exchange is quenched by lowering the pH and temperature (2.5 and 0°C), the protein is digested with pepsin, and the peptides are fractionated by HPLC for analysis by mass spectrometry (MS) and NMR. MS provides the necessary peptide sequence information. MS could, in principle, provide information on residual proton content as well (Feng et al. 2004; Zhang and Smith 1993), however, determining content at the single amino acid level is problematic, and for the current work, peptides are returned to the NMR spectrometer for analysis by measuring amide resonance intensity in 1D, ^{15}N-edited, ^{1}H spectra.

The entire procedure outlined above is applied to a test case involving the carbohydrate recognition domain of the protein, Galectin-3 (Gal3-CRD). Gal3-CRD is a protein domain that has been a target for NMR studies in the past (Umemoto et al. 2003). It has a near-complete set of backbone resonance assignments (Umemoto and Leffler 2001) and a high quality crystal structure (Seetharaman et al. 1998). The existence of the assignments allows the validation of the new procedure. For this test case, the ^{15}N labeled Gal3-CRD will be produced by expression in an *E. coli* host in the presence of ^{15}N labeled phenylalanine. Assignment of the phenylalanines in the peptic peptide that comprises residues 158–163 of the protein (HFNPRF) will be selected to illustrate the procedure. While most peptides analyzed in sparse labeling strategies will contain just a single labeled amino acid, the pair of phenylalanines in this peptide allows illustration of an ability to deal with multiple labels, or fractions containing multiple peptides.

Materials and methods

Protein expression and preparation

A plasmid containing the coding region for full length Gal3 was obtained from Halkon Leffer at Lund University, Sweden, and the region corresponding to residues 114–250 was cloned into the expression vector pET9a. The ^{15}N phenylalanine labeled Gal3-CRD was overexpressed in *E. coli* BL21(DE3)/pRIL cells and purified using the protocol described in a previous study of galectin-3 (Iglesias et al. 1998). Following this procedure, the cells were grown in 1 l M9 media until the optical density at 600 nm reached 0.8. Scrambling of ^{15}N to other amino acids was minimized by adding unlabeled forms of the other amino acids (100 mg each) before adding ^{15}N labeled phenylalanine. 100 mg of the ^{15}N labeled form of phenylalanine was then added to the media, just before inducing the cells with 1 mM IPTG. Cells were allowed to grow for an additional 3 h, and then were then harvested and lysed by French press. The ^{15}N specific labeled Gal3-CRD was purified on a lactosyl-argarose affinity column, and appropriate fractions were concentrated and stored in 75 mM phosphate buffer (pH 7.4). The yield of purified Gal3-CRD protein was approximately 40 mg l^{-1} of initial culture. The labeling efficiency was verified by analyzing the isotopic pattern in peptide mass spectrum peaks using the program ISOTOPICA (http://coco.protein.osaka-u.ac.jp/Isotopica/).

Exchange rates on intact Gal3-CRD from Hadamard encoded HSQC

All spectra were collected on a Varian 800 MHz spectrometer equipped with a triple resonance cold probe. Based on a standard HSQC survey spectrum, eight ^{15}N excitation frequencies with a bandwidth of 50 Hz were chosen for the Hadamard data acquisition. A reference Hadamard HSQC spectrum on a fully protonated 500 µl sample (~0.2 mM in 75 mM phosphate buffer, pH 7.4) was collected at 25°C with 64 scans using a t2 spectrum width of 10,000 Hz centered at 4.76 ppm. The total acquisition time was 10 min 40 s. To observe H/D exchange, the same sample was lyophilized overnight in the NMR tube, and at time zero, 500 µl 99.9% D_2O was added to re-dissolve the protein. The tube was quickly returned to the spectrometer

with parameters set identically to those of the reference spectrum, and after locking and shimming for about 2 min a series of Hadamard spectra (approximately 10, geometrically spaced) was collected at 25°C. Spectra were processed and reconstructed using nmrPipe scripts (http://spin.niddk.nih.gov/NMRPipe/). The peaks in each Hadamard spectrum were integrated to determine the volume at each time point. The volume versus time was plotted and the data were fit to an exponential decay to extract rate constants.

Exchange rates from the deuterium content of the HFNPRF peptide

About 12 mg of Gal3-CRD labeled with ^{15}N phenylalanine were dissolved in 360 µl of the same phosphate buffer used for the HSQC spectra. The sample was dried down and redissolved in 360 µl 99.9% pure D_2O at 25°C. At time intervals of 2 min, 4 h, 18 h and 4 days, triplicate 30 µl aliquots, each containing about 1 mg partially deuterated ^{15}N F Gal3-CRD, were withdrawn and frozen in liquid nitrogen. Aliquots were then individually thawed and quickly combined with 120 µl of cold 0.1% TFA in H_2O to lower the pH to 2.5 and retain a temperature of 0°C. Cold immobilized pepsin was then added to a Gal3-CRD to pepsin mol ratio of approximately 1:1 (slurry of beads (Pierce Chemicals, Rockford, IL) washed with 0.1% TFA in H_2O) and kept at 0°C for 10 min. During this time the sample was shaken on an orbital shaker to improve the surface interaction between protein and pepsin beads. The peptic peptides were then quickly filtered using a 0.2 µ membrane disk and the filtrate again frozen in liquid nitrogen. The degree of digestion and peptic peptide identification was assessed by MALDI-TOF MS (Applied Biosystem 4700 MALDI TOF/TOF Analyzer, Foster City, CA) and estimated to have <10% fragments of mass >2000 Da.

Peptides were separated on a C_{18} reverse phase column (DELTA PAK 3.9 × 300 mm) using an Agilent 1100 HPLC binary pump system and G1314 variable wavelength UV detector (Agilent Technologies, Inc., Palo Alto, CA). The column, elution buffers, and manual sample injector were pre-cooled in an ice bath or with the use of cooling packs. 100 µl samples were injected at a rate of 1 ml/min in a loading buffer consisting of 90% buffer A (0.1% TFA in H_2O) and 10% buffer B (0.1% TFA in acetonitrile). The sample was then eluted with a gradient increasing in buffer B content from 10% to 60% over 25 min. Peptides were collected as discrete fractions in 2 ml glass vials, frozen in liquid nitrogen, and stored in a –80°C freezer.

To identify peptides, 1 µl of the MALDI matrix solution (saturated α-cyano-4-hydroxycinnamic acid (Aldrich Chemicals, Milwaukee, WI) in 50% H_2O and 50% ACN with 1% TFA) was added to 1 µl peptide fractions, and the mixture was dried on a 100-well MALDI target. Data were acquired with a laser power setting of approximately 4000 and an m/z range of 560–4000 Da. Identification of peptides was accomplished using a MASCOT database search.

A Varian Inova 800 MHz NMR spectrometer (Palo Alto, CA), equipped with a triple resonance cold probe and pulsed field gradient unit, was used for NMR analysis of deuterium content in peptides. The sequence used to collect the ^{15}N edited ^{1}H spectra was derived from a pulsed field gradient HSQC sequence (Varian Protein Biopack library, Nhsqct2h). This sequence used a selective pulse to restrict excitation to the amide region, largely suppressing H_2O and ACN peaks. Data were collected using only the first t1 time point (zero t1 evolution time).

For each aliquot the fraction containing mainly the peptide, HFNPRF, (about 500 µl) was thawed and loaded into a 5 mm NMR tube along with 100 µl deuterated acetonitrile (CD_3CN) for lock and shimming. The first time point on each sample started at approximately 16 min, and took 11 min 40 s more to finish, collecting 512 transients with a spectrum width of 8000 Hz. Additional back-exchange time points on each aliquot were collected with 2048 transients to provide better signal to noise ratios. To improve signal to noise ratios further, data from three independent samples were added to obtain final free induction decays. The data were processed and deconvoluted into individual Lorentzian lines for integration using the VNMR software. Fits of back-exchange data to an exponential decay were used to extrapolate resonance intensities in each aliquot back to the point at which a sample was placed in the NMR spectrometer (zero time). The 4 day point proved to represent complete deuteration for the HFNPRF peptide, and the apparent proton content of this sample at zero time was used to quantify back-exchange that had occurred during digestion and during HPLC separation. This amounted to a 45% correction. Injection of a fully deuterated peptide into the HPLC to mimic separation conditions indicated that approximately 30% back-exchange occurred during separation and 15% back-exchange occurred during digestion. In the future, back-exchange on the HPLC could be reduced by more efficient cooling of the column and faster separation.

Results

^{15} N phenylalanine specific labeling of Gal3-CRD

Phenylalanine was chosen as the amino acid to label based on the moderate cost of its ^{15}N-labeled form and previous success with specific amino acid labeling using this amino acid. It might be expected to display a good spread of

exchange rates among various sites in folded proteins, but this proves not to be very different from distributions found for other amino acids.(Bougault et al. 2004). Although there are some issues with scrambling of labels from one amino acid type to another via metabolic pathways in bacteria, these issues proved to be adequately addressed using feed-back suppression in the current work (Fiaux et al. 2004). They could also be addressed by generation of appropriate auxotrophs (Waugh 1996). There are eight phenylalanines in the CRD, and eight intense cross peaks were observed in the HSQC spectrum of Gal3-CRD. Mass spectrometry analysis of derived peptides showed the phenylalanines to be labeled to approximately 40% in ^{15}N. There were additional weak peaks (<25% the intensity of major peaks), the majority of which could be assigned to aspartic acids.

Amide H/D exchange of folded Gal3-CRD

For the folded protein, rates of exchange were extracted from successive HSQC style spectra acquired using Hadamard methods (Bougault et al. 2004; Kupce et al. 2003). These methods provide for efficient acquisition of data in the case of rapidly exchanging amides. They do require specification of a fixed set of ^{15}N frequencies which may not cover all peaks of interest. The seven frequencies selected for the current investigation cover 7 out of the 8 phenylalanines. Data acquisition with a second set of frequencies on a sample with uniform ^{15}N labeling provided duplicate information on several sites as well as exchange data for the one phenylalanine (F192) missed in the first set. Figure 1 shows examples of spectra collected at various times after dissolution of the Gal3-CRD in deuterated buffer. Figure 1a shows results from a fully protonated sample and serves to depict the state at time zero. The cross-peaks for a uniformly ^{15}N labeled sample have been previously assigned (Umemoto et al. 2001) and the assignments of the phenylalanine cross peaks falling in the displayed region are labeled using these assignments. After 22 min (Fig. 1b) it is clear that the cross-peak for F190 has totally disappeared making this among the most rapidly exchanging sites. After 24 h (Fig. 1c) it is clear that additional sites begin to exchange, showing a wide range of exchange rates.

Cross-peaks in spectra such as those in Fig. 1 were integrated and normalized to the peak intensities in the first spectrum (I(t)). Decay rate constants were extracted from the time courses of intensity variation by fitting to Eq. 1.

$$I(t) = I_0(\exp(-k_{ex}t)) + \text{const.} \quad (1)$$

Table 1 reports measured exchange rates, k_{ex}, ranging from $<7.7 \times 10^{-1}$ min^{-1} to $<3.66 \times 10^{-5}$ min^{-1} for Gal3-CRD at pD 7.8 (pD = pH_{read} + 0.4) and 25°C. For cases where redundant data were collected, rate constants from both sets of measurements have been included.

Residual proton content of the HFNPRF peptide

While the rates of exchange in Table 1 have been assigned to specific sites using prior data for the purpose of validation, assignment of rates to specific sites in an uncharacterized protein would rely on correlation of rates with

Table 1 Amide exchange rates of phenylalanines in ^{15}N F Gal3 measured with Hadamard spectroscopy on the folded protein and with 1D ^{15}N edited spectroscopy on the derived peptide HFNPRF (pD 7.8, 25°C)

Res.	^{1}H_ppm	^{15}N_ppm	$k_{ex}(min^{-1})_{(protein)}$	$k_{ex}(min^{-1})_{(peptide)}$
F149	9.352	127.571	1.25E–04	
F157	7.648	120.801	3.63E–05	
F157[a]	7.648	120.801	<3.66E–05	
F159[a]	8.417	129.012	<3.66E–05	
F163	8.125	120.801	1.72E–03	1.80E–03
F163[a]	8.125	120.801	2.32E–03	1.80E–03
F190[a]	8.364	127.571	>7.7E–01	
F192	5.81	113.658	1.20E–02	
F198[a]	8.772	118.419	8.70E–05	
F209[a]	8.558	114.342	<3.66E–05	

[a] Additional data were collected with a second set of Hadamard frequencies

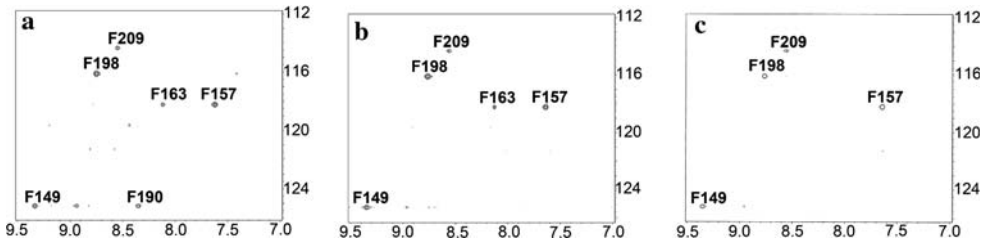

Fig. 1 Reconstructed Hadamard ^{15}N–^{1}H HSQC spectra for Gal3-CRD. (**a**) Data in H_2O collected with 64 t1 increments in 10 min 40 s. (**b**) Data after 22 min in D_2O collected with 64 scans. (**c**) Data after 24 h in D_2O collected with 2048 scans. F192 and F159 are not shown in this enlarged spectrum region

residual proton content of peptides derived from the target protein. In order to capture the proton content of amide sites at various time points during exchange, aliquots containing approximately 1 mg of Gal3-CRD were taken from a sample dissolved in deuterated buffer at time zero and quickly frozen. Later the samples were thawed and the protein digested with pepsin. The resulting peptides were then separated by HPLC (acetonitrile/0.1% trifluroacetic acid in water). Digestion and separation was done as quickly as possible at low temperature (0°C) and low pH (2.5) to minimize back-exchange of deuterons. Digestion consumed approximately 10 min, and HPLC separation consumed approximately 20 min. The HPLC profile contained more than a dozen intense peaks. Pepsin does not exhibit a high level of specificity and can produce peptides with a very broad coverage of protein sequence (Hamuro et al. 2004) Since our objective was illustrating methodology rather than achieving complete coverage, we simply selected one intense peak eluting at 12 min and containing an estimated 16 μg of peptide as the primary target of analysis. This peptide was identified as HFNPRF by correlation of MS data on a non-labeled sample with data from the MASCOT database (www.matrixscience.com). This peptide, as well as many of the other peptides, could, be identified simply on the basis of parent ion masses, but when ambiguities arise, MS/MS can be used to unambiguously identify peptides. The collected and identified fractions were frozen in liquid nitrogen and stored for subsequent NMR analysis.

Analysis of residual proton content of the peptide samples was accomplished by thawing the samples and quickly transferring them to an 800 MHz spectrometer equipped with a cryogenic probe (about 2 min). A series of spectra were then collected at 0°C with a gradient enhanced ^{15}N-edited pulse sequence. Figure 2a shows a spectrum collected in the first 16 min on the HFNPRF peptide from a protein aliquot having undergone exchange for 4 h in deuterated buffer. One intense peak is visible at 8.51 ppm and two weak peaks are visible at 7.98 and 7.73 ppm. Some peaks grow with time as protons from the solvent back-exchange into amide sites of the peptide (Fig. 2b–d). At 12 h back-exchange is nearly complete. The two peaks at 8.51 and 7.73 are large and of nearly equal intensity. They belong to the two phenylalanines in the HFNPRF peptide. The peak at 7.98 ppm proves to be an artifact from incomplete filtering of a strong water peak. The peak at 8.51 changes very little in the time course indicating either that there was no initial deuterium incorporation or that back exchange was very fast. Disappearance after dilution with a deuterated buffer indicates the latter to be the case. This unfortunately prevents assessment of the initial proton content at this site. The content at the other HFNPRF phenylalanine site at the time of aliquot sampling could, in principle, be assessed by using the back-exchange time course to extrapolate to the start of NMR data collection, as well as the point of injection into the HPLC. The latter extrapolation would be based on the similarity of separation conditions to those of NMR data collection. However, it proved difficult to control the conditions of HPLC separation, and exchange during digestion would not be accounted for in any event. Therefore, a total back-exchange correction was taken from the measured proton content of a completely deuterated, long time, aliquot (4 days) (see experimental section).

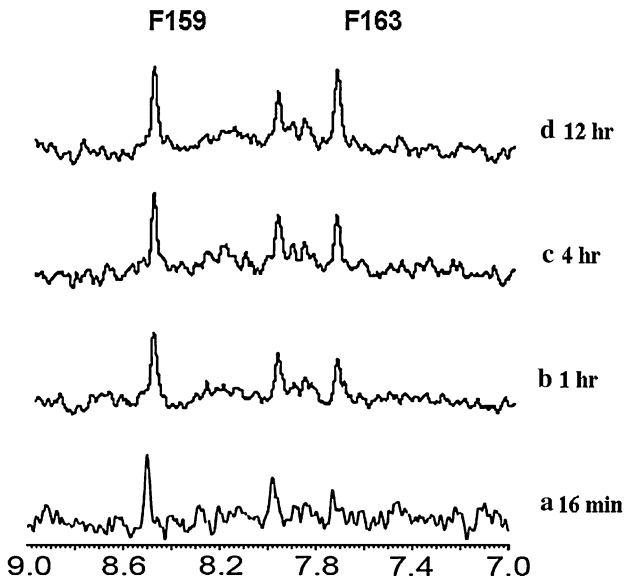

Fig. 2 H/D back exchange of peptide HFNPRF from a protein aliquot having undergone exchange in D_2O for 4 h. Data are from a ^{15}N edited, 1D ^{1}H observe, sequence

The back-exchange rates measured from the NMR data of Figure 2 do, however, prove useful in another context, namely making the assignment of the two peaks at 8.51 and 7.73 ppm to specific sites in the HFNPRF peptide. Assignments can be based on the fact that rates of amide back-exchange are predictable, given a peptide sequence, even if the solvents are mixed aqueous-organic solvents (Feng et al. 2006). The rate for F159 is predicted to be nearly two orders of magnitude faster than F163. Hence, we assign the 7.73 ppm resonance to the F163 amide and the resonance at 8.51 ppm to the F159 amide. Assignment of resonances using this strategy would be equally valuable in cases where complete separation of peptides was not achieved and resonances from more than one peptide appeared in the spectrum from a single fraction. Faster elution with less complete separation may, in fact, prove a viable means for reducing back-exchange during peptide separation in the future.

For the analysis of exchange rates from peptide data on F163, intensities of peaks from four aliquots taken during

exchange of the folded protein sample (2 min, 4 h, 18 h and 4 days), were corrected for back-exchange and plotted as a function of the time since dissolving the protonated protein in deuterated buffer (see Fig. 3). The points were fit to the same equation used for analysis of the HSQC data (Eq. 1) and a best fit rate constant of 1.8×10^{-3} min^{-1} was obtained. This exchange rate is listed in Table 1 (last column) along with rates for various cross peaks derived from HSQC data. Among the rates derived from HSQC data on the intact protein it is clear that there is only one possible match to the peptide rate, namely that for the cross peak previously assigned to F163.

Discussion

The data in Table 1 clearly illustrate an ability to assign resonances based on correlation of amide proton exchange rates measured from Hadamard-HSQC spectra on the intact protein with those measured from 1D spectra on derived peptides. There is actually quite a wide distribution of rates for different cross-peaks in the HSQC spectrum. These rates range from $>7.7 \times 10^{-1}$ min^{-1} to $<3.66 \times 10^{-5}$ min^{-1}. The exchange rate determined for the more slowly exchanging phenylalanine of the HFNPRF peptide matches that for the cross peak assigned to F163 within 25%. The next best match (F192) is a factor of seven away. Had the cross-peak of F163 not been previously assigned, we could have easily assigned it on the basis of amide proton exchange rate correlations.

For illustration purposes, just a single cross-peak assignment for Gal3-CRD, a protein of 15 kDa, has been presented. Future applications would typically be to proteins that are somewhat larger, and perhaps more difficult to produce due to non-bacterial expression requirements. Glycosylated proteins, for example, may require expression in mammalian, yeast, or insect cell cultures. This is not a small class of proteins, with as much as 50% of all mammalian proteins estimated to be glycosylated (Apweiler et al. 1999). It is clear from the dispersion of peaks in Fig. 1 that one could easily deal with 3 times as many peaks (45 kDa proteins), and hence larger proteins, whether they are glycosylated or not. Since TROSY style spectra can be used in place of the HSQC spectra used here, losses in sensitivity and resolution as size increases might be partially ameliorated. Ambiguities in assignment due to similarities in exchange rates would increase, but should be minimal providing rates can be measured to within a factor of two.

The major limitation is perhaps the labor intensive, and time consuming, nature of the procedure. However, mass spectrometric analysis of HPLC profiles prior to NMR data collection would allow targeting of just peptides containing ^{15}N labels. For a protein three times the size of the Gal3-CRD, this is still an estimated 24 peptides from each of 6 aliquots. Nevertheless, the total spectrometer time required at an average of 25 min a sample is 60 h, a time comparable to that now required for assignment using triple resonance methods. Sensitivity of NMR spectrometers, through the use of devices such as micro coil probes (Eroglu et al. 2001), HTSC (high temperature super conductor) probes (Brey et al. 2006), and DNP methods (Joo et al. 2006; Ardenkjaer-Larsen et al. 2003) is still improving, so this time can be reduced. Also, the labor intensive aspects can very likely be expedited by automation. Hence, we believe that with these advances, and parallel advances in structure determination using sparse constraints, the method for NMR cross-peak assignment presented here will find numerous applications.

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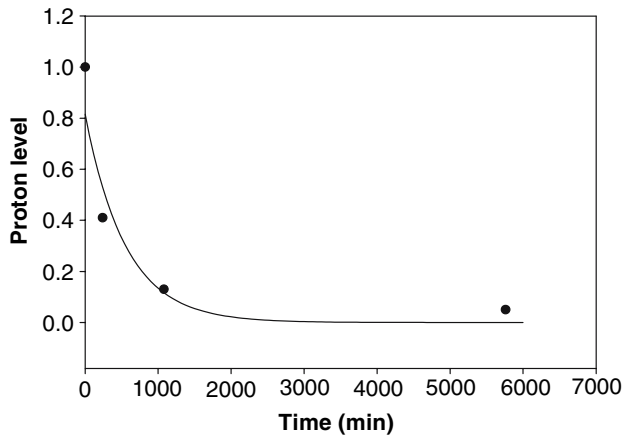

Fig. 3 Amide proton exchange for F163 from analysis of HFNPRF peptide spectra

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