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Isotopically Discriminated NMR Spectroscopy: A Tool for Investigating Complex Protein Interactions in Vitro

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Abstract: A new NMR approach is presented for observing in vitro multicomponent protein−protein−ligand(s) interactions, which should help to understand how cellular networks of protein interactions operate on a molecular level and how they can be controlled with drugs. The method uniquely allows at least two polypeptide components of the mixture to be simultaneously closely monitored in a single sample, without increased signal overlap, and can be used to study complex (e.g., sequential, competitive, cooperative, allosteric, induced, etc.) binding events, witnessed by two polypeptides independently. One polypeptide is uniformly labeled with ^{15}N and another with ^{15}N and ^{13}C. The ^{1}H−^{15}N correlation spectra are recorded for each of these molecules separately, discriminated on the basis of the type of ^{13}C′/^{12}C′ atom attached to the amide group nitrogen. Any changes to the state of the two differently isotopically labeled molecules will be reported individually by fingerprint signals from amide groups, e.g., as unlabeled ligands are added. To our knowledge, no other technique currently exists which can monitor complex binding events in similar detail. The proposed method can be combined easily with traditional protein NMR techniques and incorporated in a variety of applications.

Protein−protein interactions play an enormous role in virtually all aspects of biological processes. Within a cell, many proteins participate in multiprotein complexes, either transiently or stably, resulting in complex protein interaction networks.[1−4] The molecular mechanisms behind the precise ordering of binding and dissociation events in multiprotein complexes remain unclear, but provide a topic of utmost interest and importance.[5−7] Interest in the field is heightened by the possibility of developing new specific drugs to target key protein−protein interactions.[6,8,9] Significant attention, therefore, is being paid to the development of new methods by which to detect, probe, and characterize complex multiprotein interactions reconstructed in vitro and to extend these methods to drug screening.[10]

The atomic-resolution structure of multiprotein complexes provides a static snapshot of a particular stage of complex assembly or function cycle and can reveal the role of different molecular elements. Obtaining such detailed structure is however laborious and is subject to difficulties, e.g., with crystallization or NMR signal analysis. Furthermore, since many complexes use transient interactions or interactions via flexible domains,[11] they cannot be adequately described by a single snapshot. Therefore, the role and involvement of different molecular fragments in protein interactions, as well as effects of ligand binding, are often studied by more general biochemical and biophysical nonstructural methods.[6,10] Since these methods usually monitor a limited number of net signals or measurable parameters characteristic for the whole complex, it remains difficult to detect and characterize allosteric effects, competitive binding, conformational changes, or changes in stoichiometry[6,10] or even to attribute such effects to a particular component.

Probably the most detailed information about protein behavior in complexes can be obtained by NMR spectroscopy.[6,12,13] The protein is usually labeled isotopically with ^{15}N or ^{13}C and ^{15}N and mixed with unlabeled ligands.[13−15] (Several isotopically labeled proteins usually are not mixed together as this increases signal overlap and makes it difficult to tell which signal originates from which protein.) Typically, the changes in the fingerprint ^{1}H−^{15}N correlation spectra caused by additions of

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(1) Zhu, H.; Bilgin, M.; Bangham, R.; Hall, D.; Casamayor, A.; Bertone, P.; Lan, N.; Jansen, R.; Bidlingmaier, S.; Houfek, T.; Mitchell, T.; Miller, P.; Dean, R. A.; Gerstein, M.; Snyder, M. *Science* **2001**, *293*, 2101−2105.
(2) Ito, T.; Chiba, T.; Ozawa, R.; Yoshida, M.; Hattori, M.; Sakaki, Y. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 4569−4574.
(3) Gavin, A. C.; et al. *Nature* **2002**, *415*, 141−147.
(4) Krogan, N. J.; et al. *Nature* **2006**, *440*, 637−643.
(5) Nooren, I. M. A.; Thornton, J. M. *EMBO J.* **2003**, *22*, 3486−3492.
(6) Arkin, M. R.; Wells, J. A. *Nat. Rev. Drug Discovery* **2004**, *3*, 301−317.
(7) Sprinzak, E.; Altuvia, Y.; Margalit, H. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 14718−14723.
(8) Pommier, Y.; Cherfils, J. *Trends Pharmacol. Sci.* **2005**, *26*, 138−145.
(9) Chène, P. *Chem. Med. Chem.* **2006**, *1*, 400−411.
(10) Droit, A.; Poirier, G. G.; Hunter, J. M. *J. Mol. Endocrinol.* **2005**, *34*, 263−280.
(11) Vaynberg, J.; Qin, J. *Trends Biotechnol.* **2006**, *24*, 22−27.
(12) Bonvin, A.; Boelens, R.; Kaptein, R. *Curr. Opin. Chem. Biol.* **2005**, *9*, 501−508.
(13) Kay, L. E. *J. Magn. Reson.* **2005**, *173*, 193−207.
(14) Breeze, A. L. *Prog. NMR Spectrosc.* **2000**, *36*, 323−372.
(15) Zuiderweg, E. R. P. *Biochemistry* **2002**, *41*, 1−7.

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unlabeled ligand are then interpreted to deduce information about the complex and map binding sites.[6,12,15−18] This approach suffers from the serious disadvantage that, to see what happens with unlabeled partners in a protein mixture, the complex needs to be recreated again, with a different combination of labeled−unlabeled components. This necessity to prepare multiple samples makes it impossible to run all the experiments required to study a particular system under identical conditions. Moreover, the correlated changes, happening to several polypeptide components as further ligands are added, cannot be detected, again making it difficult to study cooperative, competitive, and allosteric binding events.

Ideally, to effectively monitor steps in complex assembly and functional transitions, simultaneous observation of fingerprint signals originating from each component is required. The resulting individual "witness reports" from several participants of the complex would enable a general picture of how a complex assembles, dissociates, and functions to be recreated. Here we propose a new NMR-based approach, which allows the simultaneous but separate observation of the individual fingerprint $^{1}H-^{15}N$ correlation spectra of two different polypeptide molecules in a single mixture of two or more components, by using a simple isotope-labeling strategy and new isotope-discriminated NMR experiments. This approach dramatically increases the information that can be obtained from a single NMR sample, allowing monitoring of complex binding events in multicomponent mixtures.

Results

Observing Spectra of Two Polypeptide Chains Separately in One Sample. The 2D $^{1}H-^{15}N$ correlation, where each amino acid residue is normally represented by one signal from its amide group, is probably the most often used type of spectra in protein NMR, and it forms the basis for numerous families of other experiments.[13,15,19,20] Here, we propose to select and observe fingerprint $^{1}H-^{15}N$ correlation subspectra of either of two polypeptide components from a single NMR sample. Both components are isotopically labeled (one with ^{15}N, another with ^{15}N and ^{13}C), with an isotope filter selecting or discriminating against $^{12}C'$ vs $^{13}C'$ atoms connected to ^{15}N. As a result, two normal-looking $^{1}H-^{15}N$ correlation subspectra are obtained in a single experiment for the two differently labeled components, one for $^{1}H-^{15}N(^{12}C')$ and another for $^{1}H-^{15}N(^{13}C')$, thus allowing each polypeptide to be monitored separately and independently. To highlight the fact that the NMR experiment can observe the same type of correlation spectra for two different polypeptide molecules and discriminate between them in a mixture, we introduce a new term for this approach: isotopically discriminated (IDIS) NMR. This term also distinguishes from the currently accepted nomenclature for isotopically "edited" and "filtered" experiments used to select or discriminate against protons connected to different isotopes of C and N atoms.[14]

In the first instance, we have created and tested two types of IDIS $^{1}H-^{15}N$ correlation experiments, one based on HSQC[19] and another based on the TROSY[21] principle. The approach and associated new pulse sequences (see the Methods and Supporting Information Figure S1 for details) were tested on a model triple-component polypeptide system. In this system, the first component is a uniformly ^{15}N-labeled construct containing a pair of WW domains, domains 3 and 4 (WW3−4), from a *Drosophila* Nedd4 family protein called Suppressor of deltex (Su(dx)).[22] The second component is a uniformly $^{15}N,^{13}C$-labeled peptide fragment (N3) of the Notch receptor, a cell-surface receptor of fundamental importance for multiple cell fate decisions.[23,24] Su(dx) is a negative regulator of Notch receptor signaling, influencing the receptor's endocytic trafficking.[25] The N3 peptide was shown recently to bind preferentially to WW domain 4 (WW4) of Su(dx), with somewhat weaker affinity to WW domain 3 (WW3).[26] A third component of the multiple polypeptide system is an unlabeled proline-rich peptide (P1), which was shown previously[22] to bind to WW3, but not to WW4. Thus, competitive binding of P1 and N3 to WW3−4 domain 3 was anticipated. The sample solubility problem[22] for mixing these polypeptides at high concentrations was solved by using 50 mM L-Arg and L-Glu additives to the buffer,[27] which do not interfere with peptide binding.[26] This relatively small and simple model system possesses all the hallmarks of multicomponent protein complexes, such as multiple binding sites and interdependent binding events, competitive binding, and changing stoichiometry. All these processes are difficult to monitor directly with current techniques.

Figure 1A shows the new $^{1}H-^{15}N$ IDIS-HSQC and IDIS-TROSY spectra collected for the 1:1 mixture of $[^{15}N]$WW3−4 and $[^{15}N,^{13}C]$N3 in the presence of a 3-fold excess of peptide P1. The conventional HSQC and TROSY spectra of the same sample displayed below contain signals from both WW3−4 and N3, which clearly increases the signal crowding. The use of IDIS-HSQC and IDIS-TROSY separates the signals originating from the two polypeptides and allows their individual observation and analysis within a single experiment, as $^{1}H-^{15}N(^{12}C')$ and $^{1}H-^{15}N(^{13}C')$ correlation subspectra. It should be noted that IDIS-type experiments implemented here largely remove signals from the side chains of Gln and Asn, further simplifying the appearance of the spectra. It should also be noted that the signals from indole groups of tryptophans and guanidinium groups of arginines (if present) of doubly labeled protein are visible in *both* $^{1}H-^{15}N(^{13}C')$ and $^{1}H-^{15}N(^{12}C')$ subspectra (data not shown). Signals from these groups belonging to ^{15}N-labeled protein are visible only in $^{1}H-^{15}N(^{12}C')$ subspectra.

The ability to discriminate between differently labeled molecules clearly hinges on the ability to incorporate ^{13}C in one polypeptide with efficiency close to 100%. We have not observed any residual signals from the "wrong" component in the spectra of an equimolar mixture of $[^{15}N]$WW3−4 and $[^{15}N,^{13}C]$N3 (Figure 1A). However, closer inspection of IDIS spectra of these and other proteins (e.g., Figure S2 in the

(16) Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. *Science* **1996**, *274*, 1531−1534.
(17) D'Silva, L.; Ozdowy, P.; Krajewski, M.; Rothweiler, U.; Singh, M.; Holak, T. A. *J. Am. Chem. Soc.* **2005**, *127*, 13220−13226.
(18) Shimada, I. *Methods Enzymol.* **2005**, *394*, 483−506.
(19) Bodenhausen, G.; Ruben, D. J. *Chem. Phys. Lett.* **1980**, *69*, 185−189.
(20) Grzesiek, S.; Bax, A. *J. Magn. Reson.* **1992**, *96*, 432−440.
(21) Pervushin, K.; Riek, R.; Wider, G.; Wuthrich, K. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 12366−12371.
(22) Fedoroff, O. Y.; Townson, S. A.; Golovanov, A. P.; Baron, M.; Avis, J. M. *J. Biol. Chem.* **2004**, *279*, 34991−35000.
(23) Wilkin, M. B.; Baron, M. *Mol. Membr. Biol.* **2005**, *22*, 279−289.
(24) Bray, S. J. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 678−689.
(25) Wilkin, M. B.; Carbery, A. M.; Fostier, M.; Aslam, H.; Mazaleyrat, S. L.; Higgs, J.; Myat, A.; Evans, D. A. P.; Cornell, M.; Baron, M. *Curr. Biol.* **2004**, *14*, 2237−2244.
(26) Jennings, M. D.; Blankley, R. T.; Baron, M.; Golovanov, A. P.; Avis, J. M. Submitted for publication.
(27) Golovanov, A. P.; Hautbergue, G. M.; Wilson, S. A.; Lian, L. Y. *J. Am. Chem. Soc.* **2004**, *126*, 8933−8939.

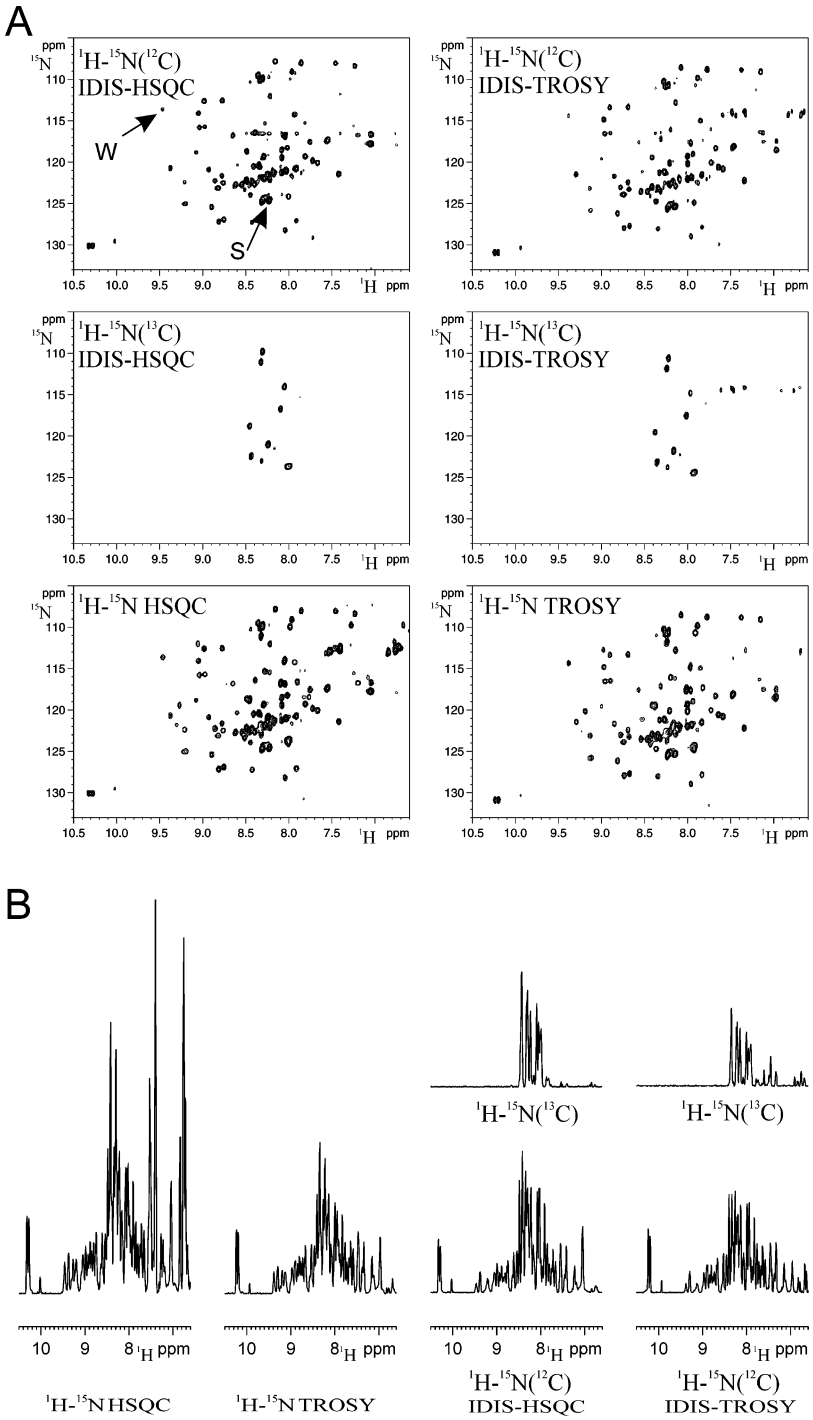

Figure 1. Comparison of ^{1}H−^{15}N(^{12}C) and ^{1}H−^{15}N(^{13}C) components of IDIS-HSQC and IDIS-TROSY with the conventional ^{1}H−^{15}N HSQC and TROSY spectra of the same sample. The sample contains a 1:1:3 mixture of uniformly ^{15}N-labeled protein WW3−4, ^{15}N,^{13}C-labeled peptide N3 (0.25 mM concentration), and unlabeled peptide P1, respectively. (A) Two-dimensional ^{1}H−^{15}N correlation spectra acquired using new IDIS-HSQC and IDIS-TROSY, in which ^{1}H−^{15}N(^{12}C) and ^{1}H−^{15}N(^{13}C) components are observed separately (upper two panel rows), compared to traditional HSQC and TROSY spectra (lower panels). Positions of representative signals are marked which were used to assess the relative sensitivity of the experiments. S = "strong" signal originating from the flexible part of the system, and W = "weak" signal originating from the folded part. (B) Comparison of ^{1}H projections of 2D ^{1}H−^{15}N correlation spectra showing the relative intensities of the signals.

Supporting Information) revealed that doubly labeled polypeptide can show traces of its fingerprint amide signals in ^{1}H−^{15}N(^{12}C) subspectra. This happens in the case of incomplete ^{13}C-labeling, when a fraction of the [^{15}N,^{13}C]polypeptide appears only ^{15}N-labeled. The false peaks due to incomplete labeling are likely to cause interference only if the doubly labeled component is going to be used in large excess over the ^{15}N-labeled one. Generally, special measures should be taken to reduce traces of residual ^{12}C present in doubly labeled protein, e.g., by cell pregrowth in labeled media and their transfer to fresh labeled media before inducing expression.

The comparison of the ^{1}H projections of the traditional ^{1}H−^{15}N HSQC and TROSY spectra with those for IDIS-HSQC and IDIS-TROSY, acquired similarly and within the same experimental time, is shown in Figure 1B. IDIS-HSQC is 2-fold less sensitive than HSQC for typical signals from the folded part of

Table 1. Comparison of the Sensitivity of the New $^{1}H-^{15}N$ IDIS-HSQC and IDIS-TROSY Experiments with That of Parent Pulse Programs

	S/N[a]			decrease in S/N[b]		
experiment	S	av	W	S	av	W
HSQC	159.2	48.9 ± 7.2	36.0			
IDIS-HSQC	102.6	24.1 ± 4.5	9.63	1.55	2.05 ± 0.13	3.74
TROSY	117.4	35.8 ± 6.2	26.7			
IDIS-TROSY	100.0	26.9 ± 6.5	11.7	1.17	1.35 ± 0.13	2.28

[a] The signal-to-noise ratio (S/N) was calculated for four typical well-resolved signals from the folded domain and averaged and for two extreme cases: signal S (strong signal from the flexible part of the complex) and signal W (weak signal from the folded domain which displays exchange broadening). [b] The decrease in S/N was calculated relative to that of the parent experiment for each signal.

the protein, the loss lower for strong signals from the flexible region (1.55-fold) and higher for broad signals with short relaxation times (3.74-fold, Table 1). As expected, for IDIS-TROSY, which selects the slowest relaxing component of the signal, the loss in sensitivity compared to normal TROSY is lower: only 1.35 times on average, with 2.3-fold loss for the broad signal. IDIS-TROSY is thus more suitable for folded or larger systems which display faster transverse relaxation.

Monitoring Simultaneous Spectral Changes to Different Components by IDIS-NMR. The ability to observe separately the spectra of two differently labeled components in a single mixture allows changes occurring to both of them to be monitored, e.g., changes caused by the addition of unlabeled ligand(s). Figure 2 shows the changes caused by the addition of unlabeled peptide P1 to a 1:2.5 mixture of [^{15}N]WW3−4 and [^{15}N,^{13}C]N3. For the initial mixture without P1, the number of signals in the spectra is less than expected, likely due to exchange broadening caused by the interaction between both domains of WW3−4 and N3; the spectra of free WW3−4 and N3 (Supporting Information Figure S3) are very different from those of the complex. As P1 is added, which is known to interact only with WW3,[22] both IDIS-TROSY subspectra reflect changes in multicomponent interactions. In the WW3−4 subspectrum, one indole signal disappears, while several signals, which can be traced to WW domain 3,[22] appear. (The same set of signals from WW domain 3 is affected directly by P1 binding to WW3−4[22]). For the N3 subspectrum, addition of P1 causes the appearance of signals characteristic of unbound N3 (see the spectrum of free N3 in Supporting Information Figure S3). In the N3 subspectra (Figure 2B), the addition of P1 peptide causes the appearance and growth of signals III and IV corresponding to the unbound form of N3, whereas the intensity of signals I and II, reflecting the total amount of N3 present in the sample, does not change. These changes in N3 subspectra are consistent with the partial release of N3 from WW domain 3 in response to competitive P1 binding, and changes in WW3−4 subspectra are consistent with binding of P1 instead of N3 to WW domain 3. Thus, having identified characteristic signals in individual IDIS-NMR subspectra for two polypeptide components in different states, the experiment then allows us to monitor independently correlated changes occurring to both polypeptide components upon addition of a further unlabeled ligand(s).

The titration in this test system was also done in reverse order: a 3-fold excess of nonlabeled P1 was initially added to [^{15}N]WW3−4, and IDIS-TROSY spectra were recorded as increasing amounts of [^{15}N,^{13}C]N3 were further added, up to a 1:1 molar ratio for the labeled compounds (Figure 3). Addition of N3 causes the broadening and disappearance of a number of signals in the subspectra of WW3−4, all of which belong to domain 4. Similarly, signals from the N3 region involved in binding are broadened beyond detection in the N3 subspectrum. The N3 subspectrum shows an increase in signal intensities, in proportion to amounts of added N3. The sequence-specific assignment of observable signals was achieved by running the standard set of triple-resonance experiments using the same sample, revealing that peptide region 1−11 remains unbound, whereas region 12−21 participates in binding. When the concentration of N3 is less than or equal to that of WW3−4, all N3 molecules exist in bound form as no signals characteristic of the unbound form are visible. The intensities of the N3 and WW3−4 signals from flexible residues observed in IDIS subspectra for a 1:1 complex are very similar (see the projections above each panel, Figure 3). Peptide P1, known to bind specifically to WW3, occupies one of the two potential binding sites for N3 on WW3−4;[26] therefore, its presence should alter the N3:WW3−4 binding stoichiometry from 2:1 to 1:1. Indeed, IDIS spectra reveal that further addition of N3 beyond a 1:1 molar ratio causes the appearance of signals corresponding to the unbound form of N3 (data not shown). Hence, direct monitoring of the binding stoichiometry in a multicomponent mixture is achieved.

In summary, the IDIS-NMR experiments enabled *correlated* spectral changes for two labeled polypeptides to be monitored in a multicomponent mixture, in response to addition of further, unlabeled, ligand. The proposed approach is thus distinct in the type of information it provides to traditional experiments, where only one protein at a time is isotopically labeled in a mixture. Additionally, use of IDIS-NMR achieves chemical shift mapping for binary or multicomponent complexes much more efficiently (fewer samples and titrations), while also ensuring spectral changes are recorded under identical experimental conditions.

Discussion

Traditionally, isotope filtering in NMR has been used to distinguish between protons linked to particular isotope atoms (^{14}N/^{15}N or ^{12}C/^{13}C).[14] On the basis of the type (^{12}C vs ^{13}C) of atom connected, a selection of particular homonuclear (namely, $^{1}H-^{1}H$) correlation subspectra have also been described previously.[28−31] Our strategy is a significant extension of these old ideas: here we discriminate heteronuclear ^{1}H,^{15}N correlation subspectra of different molecules on the basis of the type (^{12}C′ vs ^{13}C′) of atom connected to the HN moiety. The value of $^{15}N-^{13}C'$ one-bond coupling is uniform throughout the protein and does not depend on the conformation, making the discrimination extremely robust. Use of heteronuclear correlation enables an increase in the spectral resolution and provides useful, readily interpretable fingerprint $^{1}H-^{15}N$ spectra that reflect a protein state in solution. Importantly, the additional layer of isotope editing provided by heteronuclear correlation spectroscopy

(28) Otting, G.; Senn, H.; Wagner, G.; Wuthrich, K. *J. Magn. Reson.* **1986**, *70*, 500−505.
(29) Fesik, S. W.; Gampe, R. T.; Rockway, T. W. *J. Magn. Reson.* **1987**, *74*, 366−371.
(30) Otting, G.; Wuthrich, K. *J. Magn. Reson.* **1989**, *85*, 586−594.
(31) Andersson, P.; Otting, G. *J. Magn. Reson.* **2000**, *144*, 168−170.

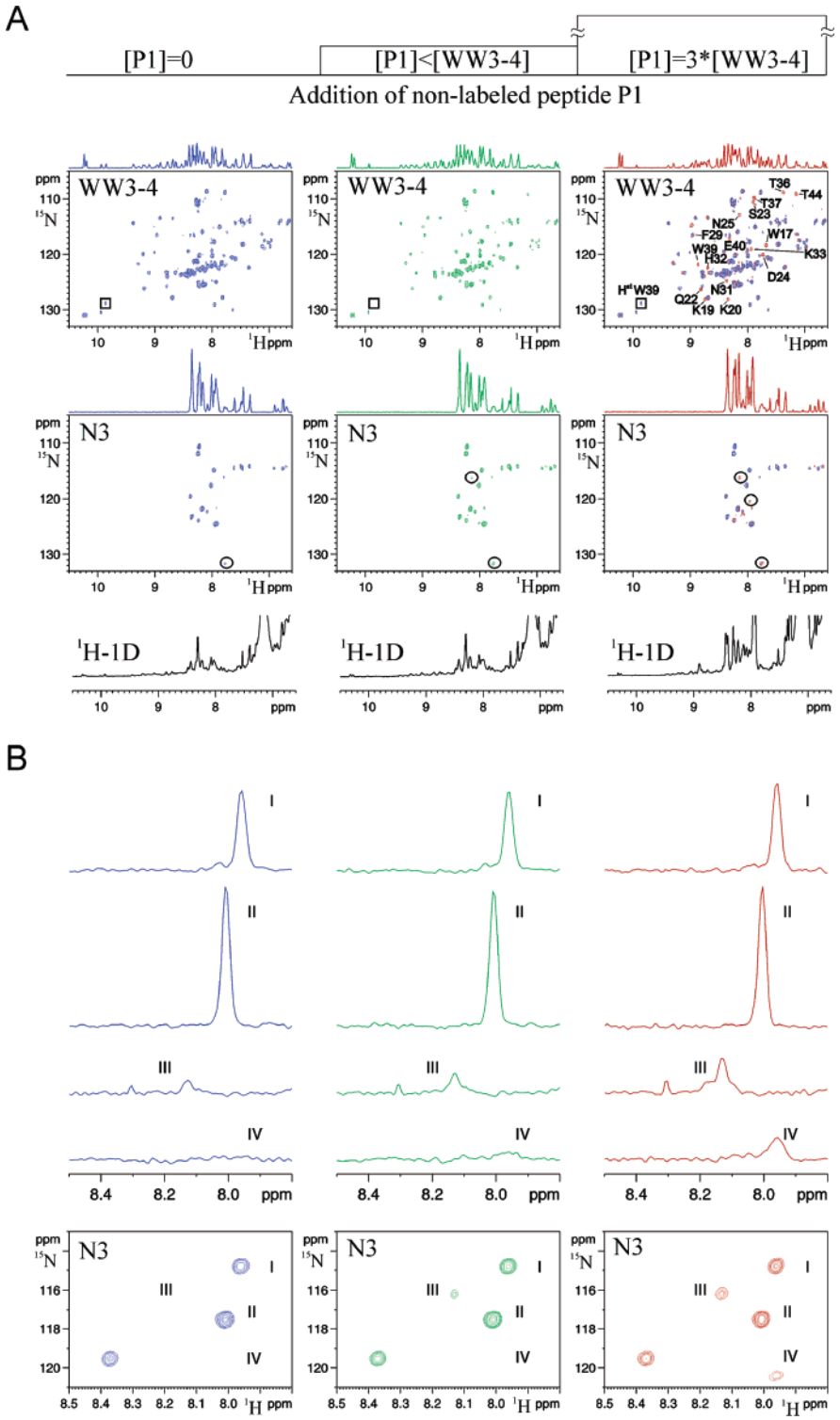

Figure 2. Monitoring changes in IDIS subspectra of a multicomponent mixture as unlabeled ligand is added. The bars at the top represent amounts of unlabeled P1 peptide present in the sample and relate to all the spectra shown below them. (A) $^{1}H-^{15}N(^{12}C)$ and $^{1}H-^{15}N(^{13}C)$ IDIS-TROSY subspectra for the two polypeptide components (WW3−4 and N3, respectively), observed separately. The sample initially contained a 1:2.5 mixture of ^{15}N-labeled WW3−4 (0.1 mM) and $^{15}N,^{13}C$-labeled peptide N3 (left column, signals shown in blue). The addition of substoichiometric amounts of nonlabeled peptide P1 caused changes in the subspectra of both WW3−4 and N3 (middle column, signals in green), with the changes becoming more pronounced when a 3-fold excess of P1 was added (right column, signals in red). The subspectra of WW3−4 and N3 without P1 (blue) are overlaid with their subspectra in triple complex (red) to highlight the spectral changes. The sequence-specific assignments are shown for the affected signals in the WW3−4 subspectrum, all of which belong to WW domain 3. The square marks an indole signal from WW3−4, disappearing when P1 is added. Circles mark signals characteristic of unbound N3, which originate from region 12−21 (see Figure 3 for assignment of visible signals from N3). The ^{1}H projection is shown above each 2D spectrum to reflect changes in the signal intensities. The black 1D proton spectrum at the bottom reflects the overall composition of each mixture and shows the increasing amounts of nonlabeled P1. (B) Observing the release of N3 peptide from the complex using IDIS-TROSY. The expanded fragments of N3 $^{1}H-^{15}N(^{13}C)$ IDIS-TROSY subspectra are shown. Signals marked I and II originate from flexible residues not involved in intermolecular interactions. Their intensities reflect the total amount of N3 peptide in the sample, which remains constant. Signals III and IV, reflecting the amount of the unbound form of N3, grow as P1 is added. The spectra above each panel represent horizontal slices through the respective signals.

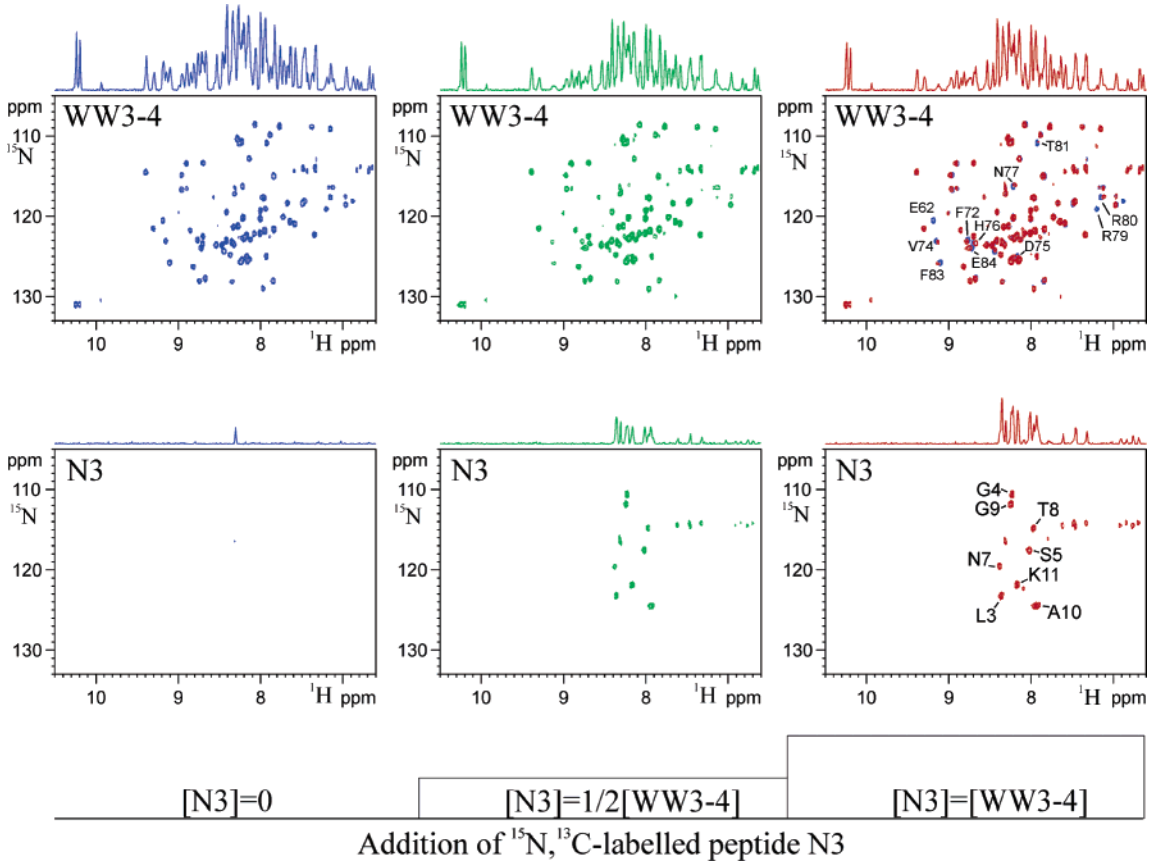

Figure 3. Monitoring changes in the subspectra of a multicomponent mixture as the isotopically labeled component is added. The sample initially contained (blue spectra) 0.25 mM ^{15}N-labeled WW3−4 in the presence of a 3-fold excess of unlabeled peptide P1. The ^{1}H−^{15}N(^{13}C) IDIS-TROSY subspectrum is empty (except the spike signal in the middle). A substoichiometric amount of ^{15}N,^{13}C-labeled peptide N3 was then added (green spectra), causing the broadening of a number of signals in the ^{1}H−^{15}N(^{12}C) IDIS-TROSY spectrum of WW3−4. Red spectra were obtained with a 1:1 ratio of N3 to WW3−4 in the presence of the same excess of P1. The blue spectrum is overlaid with the red one to highlight the signals of WW3−4 which disappear when an equimolar amount of N3 is added. The sequence-specific assignments are shown for the affected signals, all of which belong to WW domain 4. In the N3 subspectra, the sequence-specific assignment of signals from the nonbound peptide fragment is shown. Signals from residues 12−21 of the peptide are broadened beyond detection, thus mapping the interaction site with WW3−4. Further additions of N3 beyond a 1:1 ratio cause the appearance of signals characteristic of unbound N3 (data not shown). The ^{1}H horizontal projection is shown on the top of each 2D spectrum to reflect the overall signal intensities. The bars at the bottom represent the relative concentration of N3 peptide.

makes nonlabeled ligands, contaminants, and buffer additives invisible. To our knowledge, separate recording of fingerprint ^{1}H−^{15}N correlation spectra for a mixture of ^{15}N- and ^{15}N,^{13}C-labeled proteins to monitor them independently has not been reported previously.

It is important to stress the advantages the new IDIS-NMR method offers compared to traditional approaches where only one protein at a time is labeled isotopically. Conveniently, it makes titrations for chemical shift mapping more efficient. For a binary complex, mapping can be done for both polypeptides simultaneously in one titration under identical sample conditions. For multicomponent complexes, it reduces the number of samples required for chemical shift mapping of all its individual components and makes it easier to ensure that the solvent conditions and component concentrations used are similar. Most importantly, however, the IDIS approach provides unique information not offered by traditional methods: it enables *the correlated* changes happening *simultaneously* to two different labeled molecules to be observed and interpreted quantitatively in a multicomponent environment. Spectral changes can evolve in time or may be induced by addition of ligands or denaturing agents, changing temperature or pressure, etc. Labeling of just one protein at a time in a multicomponent environment makes it impossible to see what happens to the unlabeled components while changes to the system are induced. Monitoring correlated changes by IDIS-NMR might be useful, for example, for investigation of cooperative folding or unfolding of two polypeptides. Also, it can be used in ligand-binding screens, where ligands are proposed to affect interprotein interactions either antagonistically or inductively. Any change in protein−protein interactions should necessarily affect the fingerprint spectra of both proteins simultaneously, which is easily monitored by IDIS-NMR, but is difficult using methods where only one polypeptide component is labeled and observed.[17,32] The IDIS approach would constitute a useful screen for ligands against polypeptide complexes, rather than single polypeptides.[16] Additionally, by observing signals from two labeled components, reflecting their quantities and state in solution, IDIS can provide direct information about the stoichiometry of binding, whereas, using traditional methods, binding partners are unlabeled and therefore “invisible”. Thus, for multicomponent interactions, IDIS-NMR offers numerous advantages over the existing approaches.

(32) Krajewski, M.; Ozdowy, P.; D’Silva, L.; Rothweiler, U.; Holak, T. A. *Nat. Med.* **2005**, *11*, 1135−1136.

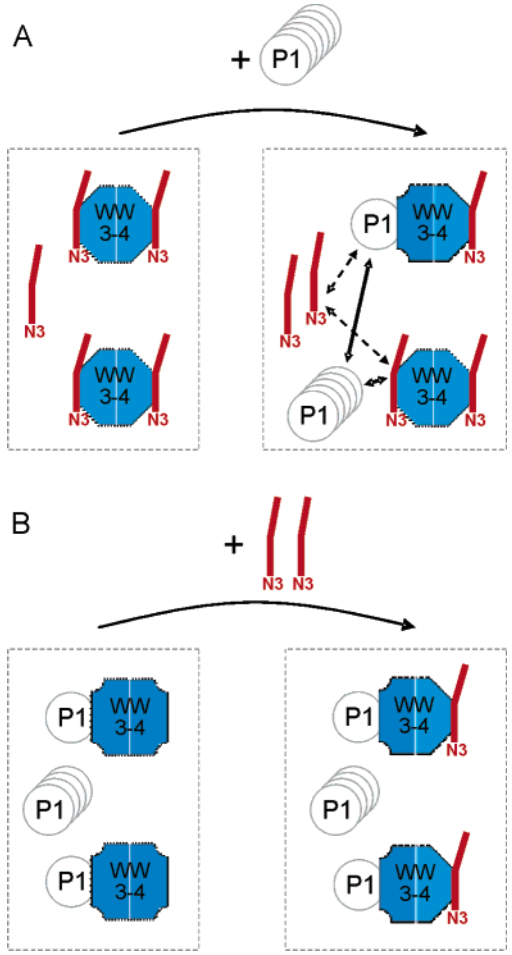

Figure 4. Model for the interactions observed during the titration experiments. The colored shapes correspond to isotope-labeled polypeptides with fingerprint signals observable by IDIS-TROSY. The gray shape corresponds to unlabeled P1 peptide. (A) Displacement of bound N3 from WW3−4 by the added P1 peptide. The amount of unbound N3 in the solution is increased, as indicated by the increase of the representative signal intensities. P1 is added in excess; therefore, the equilibrium is shifted toward P1 binding. Simultaneously, the P1 interaction region on WW domain 3 is mapped, and the WW3−4 interaction region 12−21 on N3 is also mapped. (B) Addition of labeled N3 peptide to the system. WW3−4 is preloaded with P1 peptide. The added N3 becomes bound to a spare binding site on WW3−4, up to equimolar levels. Simultaneously, N3 interaction is mapped to WW domain 4, and the WW3−4 binding region of N3 is mapped to residues 12−21.

The IDIS approach may also be useful for single-chain proteins with site-specific[33] or segmental isotope labeling.[34] IDIS-NMR, applied to selectively labeled systems, where site-specific or segmental labeling of some fragments is performed with ^{15}N and that of other fragments with ^{15}N and ^{13}C, would enhance the information content of experiments obtained from a single sample, enabling simultaneous yet independent observation of spectra from different parts of the same protein.

The IDIS-HSQC or IDIS-TROSY elements, instead of normal HSQC[19] or TROSY,[21] can be plugged into many existing NMR pulse sequences, allowing this principle of discrimination between differently isotopically labeled polypeptides in a single mixture to be extended to a variety of applications.[13] IDIS-NMR analysis then can be performed for both polypeptides in parallel. The traditional triple-resonance experiments for sequence-specific signal assignment of doubly ^{15}N,^{13}C-labeled polypeptide[20] can be run for the sample prepared for IDIS experiments, providing cost efficiency and maximizing information obtained from the single sample.

The sensitivity of the IDIS-TROSY experiment compared to the IDIS-HSQC experiment is significantly improved for the signals from the structured parts of the protein. In the current pulse sequence implementation, the ^{15}N magnetization resides for approximately 66 ms in the transverse plane, which is slightly longer than the typical 54 ms required for CT-HNCO,[20] causing additional magnetization losses due to transverse relaxation. The practical size limits for protein complexes amenable for studies using the IDIS approach are still to be determined experimentally; however, it is anticipated that use of the TROSY version of the experiment and protein deuteration, combined with the use of higher field NMR spectrometers, will be beneficial for larger systems.[21,35] The increased information content of IDIS-based experiments, with spectra of two separate polypeptides obtained in a single experiment, partly offsets the loss in sensitivity and opens new possibilities in studies of multicomponent polypeptide complexes in vitro.

Conclusion

It is generally acknowledged that fingerprint ^{1}H−^{15}N correlation spectra reflect the quantity and state of a protein in solution and can be used for monitoring and mapping protein interactions. Here we demonstrate for the first time that it is possible to observe separately fingerprint ^{1}H−^{15}N correlation spectra from two different polypeptide molecules in a mixture. The system used for the proof-of-principle demonstration includes ^{15}N,^{13}C-labeled peptide N3, ^{15}N-labeled protein WW3−4, and unlabeled peptide P1, for which detailed interaction studies are described elsewhere.[26] The IDIS-NMR experiments enabled independent *simultaneous* chemical shift mapping for two polypeptide components and have allowed direct observation of the N3:WW3−4 binding stoichiometry and its change upon addition of a further unlabeled ligand (summarized in Figure 4). Given the versatility of the new isotope-discriminating filtering technique and the associated advantages afforded, we would envisage its application to a wide range of multicomponent systems in the future.

Methods

Pulse Programs. A detailed description of the new IDIS-HSQC and IDIS-TROSY pulse sequences is included in the Supporting Information. The IDIS-HSQC and IDIS-TROSY pulse programs for Bruker spectrometers are available from us.

Sample Preparation. Both the WW3−4 and N3 constructs were cloned as GST fusions into pGEX-6P-1 (GE Healthcare). To prepare ^{15}N-labeled WW3−4 and ^{15}N,^{13}C-labeled N3, *Escherichia coli* BL21 cells were grown in M9 minimal medium containing [U-^{15}N, >98%]-ammonium chloride and either normal glucose or [U-$^{13}C_6$, 99%]glucose (CIL), accordingly. Polypeptides were affinity purified using glutathione−Sepharose 4B beads (GE Healthcare), cleaved using PreScission Protease (GE Healthcare), and further purified by reversed-phase chromatography (PepRPC HR 10/10, Pharmacia) with a gradient of 0−40% acetonitrile in 0.1% trifluoroacetic acid before lyophilization and resuspension in NMR buffer (20 mM sodium phosphate, pH 6.75,

(33) Yabuki, T.; Kigawa, T.; Dohmae, N.; Takio, K.; Terada, T.; Ito, Y.; Laue, E. D.; Cooper, J. A.; Kainosho, M.; Yokoyama, S. *J. Biomol. NMR* **1998**, *11*, 295−306.
(34) Yamazaki, T.; Otomo, T.; Oda, N.; Kyogoku, Y.; Uegaki, K.; Ito, N.; Ishino, Y.; Nakamura, H. *J. Am. Chem. Soc.* **1998**, *120*, 5591−5592.
(35) Pervushin, K. *Q. Rev. Biophys.* **2000**, *33*, 161−197.

50 mM NaCl, 50 mM L-Arg, 50 mM L-Glu,[27] 1 mM EDTA, 5% D_2O). The synthetic nonlabeled peptide P1 (GPPPPPYPG)[22] was purchased from Pepceuticals (Nottingham, U.K.). The sequence of the WW3−4 construct was described previously.[22] The N3 peptide[26] included the Notch protein fragment, residues 2318−2333, with resultant sequence *GPLGS*PNTGAKQPPSYEDCIK (the italicized part of the sequence is plasmid-derived).

NMR Experiments. The NMR experiments were performed at 20 °C on a Bruker 600 MHz Avance DRX spectrometer equipped with TXI Cryoprobe, using XWINNMR3.5. The sample concentrations of labeled polypeptides for NMR experiments were in the range 0.1−0.25 mM. The 2D data matrices contained typically 2048 points in the ^1H acquisition dimension and 128 points in the indirect ^{15}N dimension, with a sweep width of 8 and 2 kHz, respectively. Experiments used for sensitivity comparison were collected under identical conditions in 1.5 h each, with 32 scans per point in the indirect dimension.

The sequence-specific assignment of N3 signals visible in complex with WW3−4 was achieved using triple-resonance 3D HNCO, HNCA, HNCOCA, CBCACONH, and HBHACONH experiments, for the sample containing an equimolar mixture of [^{15}N,^{13}C]N3 and [^{15}N]-WW3−4 with a 3-fold excess of unlabeled P1 present. The signal assignments of WW3−4 in the presence of P1 have been reported previously.[22]

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Supporting Information Available: Complete refs 3 and 4, detailed description of IDIS-HSQC and -TROSY pulse sequences with diagrams, illustration of the effect of incomplete incorporation of ^{13}C label in doubly labeled protein on IDIS spectra, and reference spectra of unbound [^{15}N,^{13}C]N3 and [^{15}N]-WW3−4. This material is available free of charge via the Internet at http://pubs.acs.org.

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