Isotopically discriminated NMR spectroscopy: a tool for investigating complex protein interactions *in vitro*.

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Supporting Information

Full list of authors for references 3 and 4 in the main text:

- Ref. 3:** Gavin, A. C.; Bosche, M.; Krause, R.; Grandi, P.; Marzioch, M.; Bauer, A.; Schultz, J.; Rick, J. M.; Michon, A. M.; Cruciat, C. M.; Remor, M.; Hofert, C.; Schelder, M.; Brajenovic, M.; Ruffner, H.; Merino, A.; Klein, K.; Hudak, M.; Dickson, D.; Rudi, T.; Gnau, V.; Bauch, A.; Bastuck, S.; Huhse, B.; Leutwein, C.; Heurtier, M. A.; Copley, R. R.; Edelmann, A.; Querfurth, E.; Rybin, V.; Drewes, G.; Raida, M.; Bouwmeester, T.; Bork, P.; Seraphin, B.; Kuster, B.; Neubauer, G.; Superti-Furga, G. *Nature* **2002**, *415*, 141-147.
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Supporting Methods

Description of pulse programs. Previously, the correlation spectra of labeled vs unlabeled components were recorded using filtered ¹⁻⁷ experiments where protons were discriminated on the basis of whether they are bound to a NMR active (¹⁵N/¹³C) or inactive (¹⁴N/¹²C) nucleus⁸. In our approach, the ¹⁵N atoms are discriminated on the basis of whether they are connected to ¹²C' or ¹³C' nucleus, adding additional layer of ¹⁵N-separation for HN protons and enabling to discriminate further well-resolved protein fingerprint ¹H-¹⁵N correlation spectra. Conceptually the experiment described in this paper is a 2D version of a HNCO^{9, 10}, in which the CO chemical shift evolution time is omitted and the FIDs obtained for different phase settings of the first CO 90° pulse are stored separately¹¹. The experiment is also closely related to half-filter experiments ^{1, 3, 4, 7}. In order to avoid unnecessary pulses, a somewhat simplified scheme is described here. In this scheme no transverse CO magnetisation is created, which has the additional advantage of avoiding CO relaxation due to CSA, in particular for larger proteins and/or higher fields.

The isotope-filtering building block described here can be inserted in either HSQC¹² or TROSY¹³ based sequences. Since both HSQC and TROSY are discussed in the literature in detail, the following description will focus on the actual filtering/editing element. For the sake of simplicity, the ¹⁵N/¹H coupling will be ignored, as it does not contribute to the filtering/editing step. At time point *a* (Fig. S1a,b), transverse ¹⁵N magnetisation (-N_y) has been created as usually done in either HSQC or TROSY. In the presence of the hatched pulses the NC' coupling will evolve during a period of 4T:

$$-N_y \rightarrow 2N_x C_z \sin(\pi 4T J) - N_y \cos(\pi 4T J)$$

With 4T being adjusted to 1/(¹J(NC')) this leads to +N_y at time point b in the case of a ¹⁵N/¹³C moiety whilst, for molecules containing only ¹⁵N, there is no coupling evolution and -N_y is

retained. Hence, the signals for the two types of molecules will differ in sign. In the absence of the hatched pulses, the coupling evolution is refocused due to the ^{15}N 180° pulses, and the signals for both types of molecules will have the same sign. As a result, the sum of the two spectra, acquired with and without hatched pulses, yields only signals from the molecule containing ^{15}N , ^{13}C , while in the difference spectra only signals from the molecule with both ^{15}N and ^{13}C labels are present. After point **b**, the ^{15}N magnetisation is transferred back to ^1H using the normal HSQC or TROSY scheme. ^1H magnetisation is finally detected. In the case of a HSQC scheme, ^{15}N is decoupled during the acquisition time. The duration of the $4T$ period is determined by the value of $^1J(\text{NC}')$ coupling constant (15 Hz) which is uniform across a protein and is not dependent on protein conformation, providing a robust filtering for different sites. The labeling of ^{15}N chemical shifts is done in a semi-constant time fashion¹⁴⁻¹⁶, thus posing no limitation on the achievable digital resolution in the indirect dimension. At the same time the chemical shift evolution is time-shared with the NC' scalar coupling evolution to improve sensitivity, similar to a previously proposed strategy⁷. For water flip-back, a Sinc1 shaped pulse of 1000 μs length and 424 Hz amplitude was used. Q3 shapes were used as refocusing on resonance CO and off resonance $\text{C}\alpha$ pulses, with a length of 256 μs and a field strength of 12.905 kHz. The adiabatic pulse was a smoothed chirp of 60kHz sweepwidth, 500 μs length and 20% smoothing on either side¹⁷. Apodization using a QSIN functions (SSB parameters 2.5 and 2.2 in F2 and F1, respectively) and zero filling to 2048 and 512 complex points in F2 and F1, respectively, were applied prior to Fourier transform.

Isotopically-discriminated NMR

Figure S1a. Pulse sequence for the HSQC-based¹² IDIS-HSQC experiment. Narrow and wide bars represent 90° and 180° pulses respectively. Solid shapes are selective pulses applied to the water magnetization. The hatched shapes are Q3 pulses applied on resonance to the CO spins. When the hatched pulses are present, the open shape marked with asterisk in the middle of the 4T constant-time period is a Q3 pulse¹⁸ applied off resonance to the Cα spins to refocus the NCα coupling. In the absence of the hatched pulses it is an adiabatic smoothed chirp pulse¹⁷ centered at 100ppm, refocusing the NCα coupling as well as removing signal modulation by the NCO coupling in t₁. The ¹H decoupling⁹ (DIPSI2¹⁹) is flanked by 90° pulses given at the same power level. The delays are: τ = 2.3 ms (1/(4¹J(NH)) reduced to partially compensate for relaxation losses), τ' = τ reduced by the length of the selective pulse, Δ = 5.5 ms (1/(2¹J(NH))), constant time delay T = 16.4 ms (4T = 1/(¹J(NC'))), T' = T reduced by the initial value of k t₁/4 and additionally reduced to compensate for the contribution of chemical shift evolution during the 90° ¹⁵N pulses. Two interleaved experiments are recorded with/without the hatched pulses and stored separately¹¹. Adding and subtracting the two experiments yields the desired sub-spectra of differently-labeled polypeptide components⁷. Garp decoupling²⁰ is used during acquisition. All pulses are along x except otherwise noted. The phase cycles are φ₁ = x, x, x, x, -x, -x, -x, -x; φ₂ = x, x, -x, -x; φ₃ = x, -x; φ(receiver) = x, -x, x, -x, -x, x, -x, x. The gradients are sine shaped and have an amplitude of 27, 32 and 16 G/cm respectively. Phase sensitive spectra are obtained using the States-TPPI method²¹ by decrementing φ₃ after pairs of experiments. By virtue of the choice of the length of the delay Δ (as 1/(2¹J(NH))) a multiplicity selection in favour of NH (over NH₂) is obtained. Therefore the signals from side-chain amines are largely removed from the spectra. Water suppression is achieved by using a soft pulse watergate scheme^{22, 23}. The separate spectra of individual polypeptide components, ¹⁵N, ¹²C or ¹⁵N, ¹³C-labeled, can also be obtained by directly co-adding experiments with/without the hatched pulses, while either leaving the receiver phase or inverting it at the same time. This allows to avoid the necessity to split and pre-process the acquired spectra: only one of the isotopically-discriminated component sub-spectra will be obtained per experiment in this case.

Figure S1b. Pulse sequence for the TROSY-based^{13, 24-33} IDIS-TROSY experiment. Narrow and wide bars represent 90° and 180° pulses respectively. Solid shapes are selective pulses applied to the water magnetization. The hatched shapes are Q3 pulses applied on resonance to the CO spins. When the hatched pulses are present, the open shape marked with asterisk in the middle of the 4T constant-time period is a Q3 pulse¹⁸ applied off resonance to the Cα spins to refocus the NCα coupling. In the absence of the hatched pulses it is an adiabatic smoothed chirp pulse¹⁷ centered at 100ppm, refocusing the NCα coupling as well as removing signal modulation by the NCO coupling in t₁. The delays are: τ = 2.3 ms (1/(4¹J(NH))) reduced to partially compensate for relaxation losses), τ' = τ reduced by the length of the selective pulse, constant time delay T = 16.4 ms (4T = 1/(¹J(NC'))), T' = T reduced by the initial value of k t₁/4 and additionally reduced to compensate for the contribution of chemical shift evolution during the 90° ¹⁵N pulses. Two interleaved experiments are recorded with/without the hatched pulses and stored separately ¹¹. Adding and subtracting the two experiments yields the desired sub-spectra of differently-labeled polypeptide components⁷. All pulses are along x except otherwise noted. The phase cycles are φ₁ = y, -y, x, -x or y, -y, -x, x for consecutive pairs of experiments; φ₂ = -y; φ(receiver) = y, -y, -x, x. The gradients are sine shaped and have an amplitude of 16, 10 and 24 G/cm respectively. Phase sensitive spectra are obtained according to the TROSY method²⁴⁻³⁰ by using a different setting for φ₁ and inverting φ₂ after pairs of experiments. By virtue of the back transfer in the TROSY a multiplicity selection in favour of NH (over NH₂) is obtained. Therefore the signals from side-chain amines are largely removed from the spectra. Water suppression is achieved by using a soft pulse watergate scheme^{22, 23}. The separate spectra of individual polypeptide components, ¹⁵N, ¹²C or ¹⁵N, ¹³C-labeled, can also be obtained by directly co-adding experiments with/without the hatched pulses, while either leaving the receiver phase or inverting it at the same time. This allows to avoid the necessity to split and pre-process the acquired spectra: only one of the isotopically-discriminated component sub-spectra will be obtained per experiment in this case.

Figure S2. Incomplete incorporation of ^{13}C -label causes appearance of the “wrong” H-N correlation signal pattern of the doubly-labeled protein in $^1\text{H}-^{15}\text{N}(^{12}\text{C})$ subspectrum. The ^{15}N , ^{13}C -labeled 1.5 mM ubiquitin has been obtained using the typical expression protocol, but in reality appears only 90% ^{13}C -labeled. (a) The horizontal (^1H) projections of 2D $^1\text{H}-^{15}\text{N}(^{13}\text{C})$ (black) and $^1\text{H}-^{15}\text{N}(^{12}\text{C})$ (red) IDIS TROSY sub-spectra are shown for ^{15}N , ^{13}C -labeled ubiquitin. Signals from the residual “wrong” component shown in red constitute ca. 10% of intensity of the correct component. (b) One-dimensional ^1H spectra were acquired for the same sample with and without ^{13}C decoupling. Two well-resolved representative signals (left from $\text{H}\alpha$, and right from methyl H) show splitting due to coupling with ^{13}C ; the splitting is removed when ^{13}C is decoupled. The signals marked with asterisks originate from the presence of ^{12}C -labeled species in the sample (estimated to be ca. 10% based on the analysis of signal intensities in undecoupled spectrum). The ubiquitin used for this illustration was a generous gift of Dr Igor Barsukov.

Figure S3. The ^1H - ^{15}N HSQC spectra of unbound ^{15}N , ^{13}C - N3 and ^{15}N - WW3-4. Signals labeled I and II originate from N3 amino acid residues which are not involved in interaction with WW3-4. Circled signals are characteristic to the free form of N3; two of these signals are labeled as III and IV. Same signal labels are used on Figure 2 in the main part of the manuscript.

Supplemental References

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