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ARTICLE

Spectral editing of intra- and inter-chain methyl–methyl NOEs in protein complexes

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Abstract

Specific isotopic labeling of methyl groups in a perdeuterated protein background enables the detection of long range NOEs in proteins or high molecular weight complexes. We introduce here an approach, combining an optimized isotopic labeling scheme with a specifically tailored NMR pulse sequence, to distinguish between intramolecular and intermolecular NOE connectivities. In hetero-oligomeric complexes, this strategy enables sign encoding of intra-subunit and inter-subunit NOEs. For homo-oligomeric assemblies, our strategy allows the specific detection of intra-chain NOEs in high resolution 3D NOESY spectra. The general principles, possibilities and limitations of this approach are presented. Applications of this approach for the detection of intermolecular NOEs in a hetero-hexamer, and the assignment of methyl ^{1}H and ^{13}C resonances in a homo-tetrameric protein complex are shown.

Keywords NMR · Large protein · Protein complex · Long range NOE · Isotopic labeling · Methyl groups · Assignment · Structure determination

Introduction

Specific labeling of methyl groups (Ruschak et al. 2010; Rosenzweig and Kay 2014; Kerfah et al. 2015a), combined with optimized NMR spectroscopy (Tugarinov et al. 2003; Amero et al. 2009), provides a powerful tool to study structure, dynamics, interaction and mechanism of large protein assemblies (Sprangers and Kay 2007; Rosenzweig et al. 2013; Macek et al. 2017; Mas et al. 2018). Protonation of methyl probes in a perdeuterated protein background enables the detection of long range NOEs (nuclear overhauser effects) between methyl groups up to 12 Å apart in high molecular weight proteins (Sounier et al 2007; Kerfah et al. 2015b; Chi et al. 2018; Ayala et al. submitted). These distance restraints are particularly useful to determine the structure of large proteins and protein complexes (Tugarinov et al. 2005a; Gauto et al. 2019). If the tertiary structure of a protein is previously known, the network of NOE connectivities can also be exploited for automated NMR assignment of methyl resonances (Xu and Matthews 2013; Chao et al. 2014; Pritišanac et al. 2017; Monneau et al. 2017; Pritišanac et al. 2019). In protein complexes, special care should be taken to separate the large number of intra-chain NOEs from the few inter-chain signals, as any erroneous interpretation could significantly impact NMR assignment or structure determination (Werner et al. 1997). Separation of intra-chain and inter-chain NOEs is particularly challenging in homo-oligomeric complexes.

Standard approaches to identify inter-chain NOEs in protein complexes use asymmetric isotope labeling of the different subunits with either ^{15} N or ^{13}C in order to specifically filter/edit inter-chain NOEs (Handel and Domaille 1996; Zwahlen et al. 1997; Caffrey et al. 1998; Anglister et al. 2016). Such strategies have proven to be very efficient for unambiguous extraction of inter-molecular distance restraints, but they suffer from fast proton transverse relaxation, and are therefore limited to complexes with a molecular weight of less than about 35–40 kDa. Perdeuteration combined with asymmetric isotope labeling of methyl groups has also been proposed to identify intermolecular NOEs. Veglia and co-workers have used the fact that isoleucine-δ_1, and

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leucine-δ/valine-γ methyl groups can be labeled separately, by supplementing culture media with different keto-acid precursors (Gardner and Kay 1997; Goto et al. 1999; Tugarinov et al. 2006), to introduce an asymmetry between protomers (Traaseth et al. 2008). By reconstituting a homo-pentamer from a mix of protomers labeled either on isoleucine-δ_1 or leucine-δ/valine-γ methyl groups, the authors were able to unambiguously identify inter-chain NOEs between I and V/L methyl groups. While this approach allows to extract NOEs between distant methyl groups in large protein complexes, only inter-chain NOEs between I–V and I–L pairs can be identified among the 36 possible pairs of methyl containing residues, reducing significantly the number of structurally meaningful inter-molecular distance restraints.

Herein, we introduce an alternative approach that allows to distinguish intra-chain NOEs from intermolecular connectivities. The method is based on the labeling of the different subunits with either ^{12}C–$^{13}CH_3$ or ^{13}C–$^{13}CH_3$ isotopomers, and the acquisition of 3D-NOESY experiments, in which both methyl ^{13}C frequencies are edited in constant time mode. In hetero-oligomeric complexes, this strategy enables simultaneous editing of both intra-chain and inter-chain NOEs, with an opposite sign. This allows immediate and unambiguous identification of the 'precious' inter-molecular distance restraints between methyl groups of alanine, isoleucine, leucine, methionine, threonine and valine. For the even more challenging case of large homo-oligomeric assemblies, we demonstrate that our strategy allows to suppress signals of inter-chain NOEs in a high-resolution 3D NOESY spectrum, in order to detect exclusively intra-chain NOEs between methyl groups of alanine, valine, leucine and isoleucine. Principles, limits and possibilities of this approach are presented together with applications to the 87 kDa hetero-hexameric prefoldin complex and the 53 kDa homo-tetramer formed by prefoldin β-subunits.

Material and methods

Protein expression and purification

E. coli BL21 (DE3) cells transformed with pET23c plasmids encoding either for the α- or β-subunit of Prefoldin (denoted here PFD but also known as co-chaperonin) from *Pyrococcus horikoshii* (denoted *Ph*), were progressively adapted to M9/^2H_2O media in three stages over 24 h (M9 in H_2O, M9 in 50% H_2O + 50% ^2H_2O, M9 in ^2H_2O). In the final culture the bacteria were grown at 37 °C in M9 media prepared with 99.85% ^2H_2O (Eurisotop), 2 g/L of deuterated D-glucose and 1 g/L $^{15}NH_4Cl$ (CIL).

– For production of the U-[^{2}H], A-[$^{13}CH_3$]$^{\beta}$, I-[$^{13}CH_3$]$^{\delta1}$, L-[$^{13}CH_3$]$^{\delta1}$, V-[$^{13}CH_3$]$^{\gamma1}$ PFD-β subunit, 2 g/L of D-glucose-d_7 (Sigma Aldrich) was used as carbon source. When the O.D. at 600 nm reached 0.7, a solution containing 2-[$^2H_3$]methyl-2,4-[$^{13}C_2$]-acetolactate (NMR-Bio) was added for the stereoselective labeling of *proR* Leu$^{\delta1}$ and Val$^{\gamma1}$ (Mas et al. 2013) methyl groups (240 mg/L). 40 min later 3-[^{13}C]-2-[^{2}H]-L-Alanine and (S)-2-hydroxy-2-(2′-[^{13}C],1′-[$^2H_2$])ethyl-3-oxo-4-[$^2H_3$]-butanoic acid (NMR-Bio) were added to a final concentration of 250 mg/L and 100 mg/L respectively (Kerfah et al. 2015b) for the simultaneous labeling of Ile$^{\delta1}$ and Ala$^{\beta}$ methyl groups, and 20 min afterwards protein production was induced by IPTG. U-[^{2}H], A-[$^{13}CH_3$]$^{\beta}$, I-[$^{13}CH_3$]$^{\gamma1}$, L-[$^{13}CH_3$]$^{\delta2}$, V-[$^{13}CH_3$]$^{\gamma2}$, T-[$^{13}CH_3$]$^{\gamma2}$ PFD-β subunit sample was produced using a similar protocol with 2-[^{13}C]methyl-4-[$^2H_3$]-acetolactate (NMR-Bio) for the stereoselective labeling of *proS* (Leu$^{\delta2}$ and Val$^{\gamma2}$; Gans et al. 2010) and addition of 50 mg/L of 2,3-(^{2}H) 4-(^{13}C)-L-Threonine (Ayala et al. submitted) together with the other precursors 20 min before induction.
– For production of the U-[^{2}H, ^{13}C], A-[$^{13}CH_3$]$^{\beta}$, I-[$^{13}CH_3$]$^{\delta1}$, L-[$^{13}CH_3$]$^{\delta1}$, V-[$^{13}CH_3$]$^{\gamma1}$ PFD-β and PFD-α subunits, 2 g/L of U-[^{2}H,^{13}C]-glucose was used as carbon source (CIL). When the O.D. at 600 nm reached 0.7, a solution containing 2-[$^2H_3$]methyl-1,2,3,4-[$^{13}C_4$]-acetolactate (NMR-Bio) was added (240 mg/L), and 40 min later U-[^{13}C]-2-(^{2}H)-L-Alanine and (S)-2-hydroxy-2-(1′,1′-[$^2H_2$],1′,2′-[$^{13}C_2$])ethyl-3-oxo-1,2,3-[$^{13}C_3$]-4,4,4-[$^2H_3$]butanoic acid were added to a final concentration of 250 mg/L and 100 mg/L, respectively (Kerfah et al. 2015b). 20 min afterwards protein production was induced by IPTG.

The expression was performed at 37 °C for 3 h, then cells were collected by centrifugation at 7000×*g* for 15 min at 4 °C. The purification protocol of prefoldin subunits was adapted from (Okochi et al. 2002). The cells, corresponding to 1 L of culture, were resuspended in 50 ml of buffer A containing 50 mM Tris pH 8.0, 0.1 mM EDTA, and complemented with 0.025 mg/mL RNAse (Eurome-dex), 0.025 mg/mL DNAse (Sigma Aldrich) and 1 anti-protease tablet (cOmplete™), and disrupted by sonication on ice. After removal of cell debris by centrifugation (40,000×*g*, 30 min, 4 °C), the supernatant was heated to 80 °C for 30 min and precipitated proteins were removed by centrifugation. Then, the supernatant was loaded on a Q Sepharose—XK 26/20 column (Sigma-Aldrich), pre-equilibrated with buffer A. With a linear gradient the protein was eluted at 20% of buffer B (50 mM Tris pH 8.0, 0.1 mM EDTA, 500 mM NaCl) and the combined fractions were loaded on a Superdex 200 gel filtration column (Sigma Aldrich) equilibrated with the NMR buffer (50 mM Tris, 100 mM NaCl, pH 8.5).

- For preparation of U-[^{2}H], A-[$^{13}CH_3$]$^{\beta}$, I-[$^{13}CH_3$]$^{\gamma1}$, L-[$^{13}CH_3$]$^{\delta1}$, V-[$^{13}CH_3$]$^{\gamma1}$ PFD-β_4 sample (denoted β_4-$^{13}CH_3$), the gel filtration fractions containing PFD subunits were concentrated in NMR buffer using Amicon® Centrifugal Filter Units (Merck) to 200 µM (concentration of the β_4 oligomer). A 200 µL aliquot was lyophilized and re-suspended in 200 µL of ultra-pure D_2O before loading in a 4 mm shigemi tube.
- For preparation of the β_4 mixed-labeled sample (denoted β_4-mix), the β-subunits with the two type of labeling (i.e. U-[^{2}H], A-[$^{13}CH_3$]$^{\beta}$, I-[$^{13}CH_3$]$^{\delta1}$, L-[$^{13}CH_3$]$^{\delta1}$, V-[$^{13}CH_3$]$^{\gamma1}$ and U-[^{2}H,^{13}C], A-[$^{13}CH_3$]$^{\beta}$, I-[$^{13}CH_3$]$^{\delta1}$, L-[$^{13}CH_3$]$^{\delta1}$, V-[$^{13}CH_3$]$^{\gamma1}$) were combined in a 1:1 ratio, concentrated to 200 µM (total concentration of the β_4 oligomer), and incubated at 70 °C for 3 days to ensure mixing, and therefore a statistic distribution of both types of labeling schemes in the resulting β_4 oligomers. This sample was lyophilized and re-suspended in ultra-pure D_2O before loading in a 4 mm shigemi-tube.
- For preparation of PFD-$\alpha_2\beta_4$ sample, purified U-[^{2}H, ^{13}C], A-[$^{13}CH_3$]$^{\beta}$, I-[$^{13}CH_3$]$^{\delta1}$, L-[$^{13}CH_3$]$^{\delta1}$, V-[$^{13}CH_3$]$^{\gamma1}$ α-subunits and purified U-[^{2}H], A-[$^{13}CH_3$]$^{\beta}$, I-[$^{13}CH_3$]$^{\delta1}$, L-[$^{13}CH_3$]$^{\delta2}$, V-[$^{13}CH_3$]$^{\gamma2}$, T-[$^{13}CH_3$]$^{\gamma}$β-subunits were combined in a ratio of 1:1 and incubated at 80 °C for 30 min. Excess α_2 subunits were removed by gel-filtration on a Superdex200 column, the fractions containing PFD ($\alpha_2\beta_4$) were combined, concentrated, lyophilized and re-suspended in ultra-pure D_2O before loading in a 4 mm shigemi tube. The final concentration of PFD ($\alpha_2\beta_4$) was 225 µM.
- U-[^{2}H], A-[$^{13}CH_3$]$^{\beta}$, I-[$^{13}CH_3$]$^{\delta1}$, M-[$^{13}CH_3$]$^{\varepsilon}$ Malate Synthase G (denoted MSG) sample was produced and purified as described previously (Kerfah et al. 2015b). For the specific labeling of methyl groups, deuterated L-methionine specifically $^{13}CH_3$-labeled on ε position, 3-[^{13}C]-2-[^{2}H]-L-Alanine and (S)-2-hydroxy-2-(2′-[^{13}C],1′-[$^2H_2$]) ethyl-3-oxo-4-[$^2H_3$]-butanoic acid (NMR-Bio) were added 1 h prior to IPTG induction. The MSG protein was concentrated to 0.5 mM in 100% ^2H_2O buffer containing 25 mM MES (pH 7.0 uncorrected), 20 mM $MgCl_2$, 5 mM DTT. 200 µL were loaded in a 4 mm shigemi tube.

NMR spectroscopy

All the 3D ^{13}C edited-NOESY experiments were acquired on a Bruker Avance III HD spectrometer operating at a ^{1}H frequency of 950 MHz and equipped with a cryogenic probe. For all experiments, the interscan delay was set to 1.25 s, the heteronuclear ^{1}H- > ^{13}C transfer delay was set to 4 ms ($0.5/^1J_{HC}$) and the acquisition times in ^{13}C indirect dimension were fixed to 23.5 ms (t_{1max} and t_{2max}) and in the ^{1}H detection dimension (t_{3max}) to 60 ms. The 3D HMQC-NOESY-HMQC experiment (Tugarinov et al. 2005b) was implemented using EBURP2 (Geen and Freeman 1991) as 90° excitation pulse and time reverse EBURB2t was used to flip-back ^{1}H magnetization on the z-axis before the NOE mixing period (Lescop et al. 2010). The NOE mixing period was set to 600 ms to detect a maximum number of long range NOEs. ^{1}H chemical shift evolution during the HMQC was refocused using a pair of high power band-selective BIP-720-50-20 ^{1}H pulses (Kupce and Freeman 1994; Amero et al. 2009). The 3D CT^2-HMQC-NOESY-HMQC pulse scheme (Fig. 1a) was derived by adding a pair of 180° ^{13}C hard pulses during the constant time ^{13}C editing period (Powers et al. 1991) fixed to 25 ms. The constant time editing allows to selectively invert the sign of $^{13}CH_3$ multiquantum coherences depending on the nature of the covalently bound carbon (^{12}C or ^{13}C). The acquisition time for 3D NOESY experiments varied from 2 to 3 days. All the NMR data were acquired at 70 °C for PFD samples from hyperthermophilic *P. horikoshii* or 37 °C for MSG sample.

Data processing and analysis

All data were processed and analyzed using nmrPipe/nmrDraw (Delaglio et al. 1995) and CcpNMR (Vranken et al. 2005). For all 3D spectra, the number of points in both ^{13}C indirect dimensions were doubled using forward–backward linear prediction. A $\cos^2$ apodization function was applied on all FIDs, before zero-filling and Fourier transform. For the identification of NOEs in all the 3D NOESY experiments, a S/N threshold of 3 was used.

Automated methyl assignment was performed using MAGIC software (Monneau et al. 2017) with the 3D structure of the PFD-β_4 complex, built by homology modeling using MODELLER (Šali and Blundell 1993) implemented in the NMRBox server (Maciejewski et al. 2017). The homology model is based on the X-ray crystallography structure of the PFD-β_4 complex from *Thermococcus* sp. strain KS-1 (Kida et al. 2008, PDB:2ZQM) whose β-subunit is 83.4% identical and 94.9% similar to the β-subunit of *Ph. PFD*. Input NOE lists for MAGIC were created with CcpNMR. MAGIC was run with a score threshold factor of 1 and distance thresholds of 7–10 Å using all inter methyl NOEs detected.

Results

In large perdeuterated proteins, selectively labeled on methyl groups, long-range methyl–methyl NOEs are optimally detected using 3D or 4D HMQC-NOESY-HMQC-type experiments (Tugarinov et al. 2005b; Kerfah et al. 2015b) preserving the methyl-TROSY effect (Tugarinov et al. 2003). The 3D (^{13}C, ^{13}C, ^{1}H)-edited CT^2-HMQC-NOESY-HMQC experiment used here is shown in Fig. 1a. The pulse

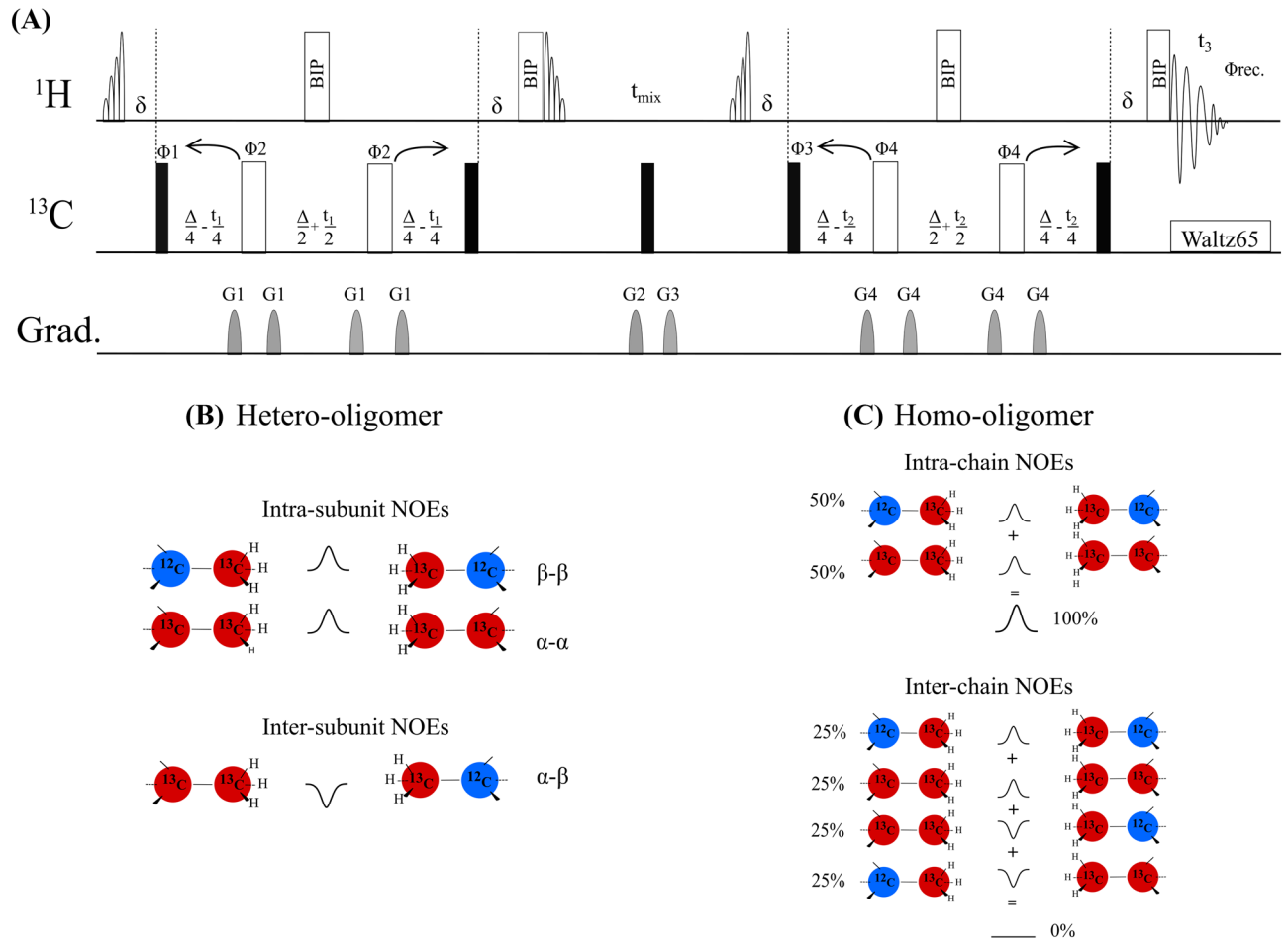

Fig. 1 Specific editing of intra vs. inter-chain methyl–methyl NOEs. **a** 3D CT^2-HMQC-NOESY-HMQC pulse sequence. Excitation of proton magnetization was achieved using 1.2 ms selective 90° EBURP2 pulses covering a bandwidth of 4 ppm (Geen and Freeman 1991) and was followed by a delay δ = 4 ms for heteronuclear magnetization transfer. Methyl carbon frequencies (F_1 and F_2) were encoded in constant time mode during the two multi-quantum evolution periods (Δ = 25 ms) flanking the NOE mixing period (t_{mix} = 600 ms). ^{1}H chemical shift evolution during each HMQC element was refocused using a pair of high power band-selective BIP-720-50-20 ^{1}H pulses (Kupce and Freeman 1994; Amero et al. 2009). 1.2 ms time reverse EBURB2 pulse (Lescop et al. 2010) was used to flip-back ^{1}H magnetization on the z-axis before the NOE mixing period. Narrow and wide bars indicate nonselective 90° and 180° ^{13}C pulses. ^{13}C decoupling during ^{1}H signal detection (F_3) was achieved using waltz65 decoupling pulse sequence (Zhou et al. 2007). All the pulses were applied along x axis unless indicated otherwise on the figure. Phase cycling was performed as follows: Φ1 = x, − x; Φ2 = x, y; Φ3 = x, x, − x, − x; Φ4 = x, x, y, y; and receiver phase was cycled with Φrec = x, − x, − x, x. Recycling delay was set to 1.25 s and 200 ms gradients were applied on z-axis with magnitude G1 = 19 G/cm; G2 = 7 G/cm; G3 = 13 G/cm and G4 = 11 G/cm. **b** Schematic representation of inter-subunit NOE sign encoding principles. This application is restricted to hetero-oligomeric complexes and is enabled by reconstituting the complex from the two different subunits labeled either with a ^{13}C–^{13}CH$_3$ isotopomer (α-subunits) or with ^{12}C–^{13}CH$_3$ groups for the β-subunits. Such a sample was used to acquire a 3D ^{13}C-edited NOESY experiment with the pulse scheme presented on Fig. 1a. NOEs between two methyl groups of α-subunits or two methyls of β-subunits are edited as positive signals, while inter-subunit NOEs between methyl groups of α- and β-subunits have a negative sign due to the evolution of the $^1J_{CC}$ coupling during only one of the two CT-HMQC elements. **c** Schematic representation of the principles of specific editing of intra-chain NOEs. This application is restricted to homo-oligomeric complexes and is enabled by reconstituting the complex from a mix in a ratio 1:1 of the same subunit fully enriched with either ^{13}C–^{13}CH$_3$ or ^{12}C–^{13}CH$_3$ isotopomers. NOEs between two methyl groups of the same chain are positive whatever is the labeling scheme of the chain. Inter-chain NOEs between two methyl groups are either positive or negative due to $^1J_{CC}$ evolution and cancel each other, leading to the suppression of inter-chain NOEs

sequence uses selective ^{1}H pulses covering only methyl resonances (Amero et al. 2009) together with constant time (CT) editing of methyl ^{13}C frequencies (Powers et al. 1991) before and after the NOE mixing period. The CT period Δ was set to 25 ms in order to invert the sign of ^{13}C-bound methyl groups (i.e. ^{13}C–^{13}CH$_3$ isotopomers) with respect to ^{12}C-bound methyl (i.e. ^{12}C–^{13}CH$_3$ isotopomers). As the ^{13}C-frequencies are edited twice in this 3D experiment,

all diagonal and NOE cross-peaks between methyl groups labeled with the same isotopomer are positive (Fig. 1b), while negative NOE cross peaks are detected between methyl groups that are labeled with different methyl isotopomers (one bound to ^{12}C, the other one to ^{13}C).

Sign encoding of intra- and inter-chain NOEs in hetero-oligomeric complexes

First, we have used the above described 3D CT^2-HMQC-NOESY-HMQC experiment to identify inter-chain NOEs in *Pyrococcus horikoshii* (*Ph*) prefoldin (PFD), a co-chaperonin forming a hetero-hexameric assembly of 87 kDa with 2α and 4β subunits arranged in a ring-like structure (Fig. 2a). The two α-subunits are structurally and spectroscopically equivalent, both preceded and followed by β-subunits. The four β-subunits have the same sequence, but are not structurally equivalent, as they can either be preceded or followed by an α-subunit in the assembly, and therefore give rise to two different sets of NMR signals (denoted β and β'). Combinatorial labeling of several methyl containing amino acids represents an obvious route for increasing the number of NMR probes in the sample. To date, only alanine, isoleucine, leucine and valine are available in the ^{13}C–$^{13}CH_3$ isotopomeric form (Gans et al. 2010; Mas et al. 2013; Miyanoiri et al. 2013; Kerfah et al. 2015a). Therefore, we have labeled the methyl groups of alanine (Ala-β), isoleucine (Ile-δ_1), leucine (Leu-δ_1) and valine (Val-γ_1) in the α-subunits with ^{13}C–$^{13}CH_3$ isotopomers, while in the β-subunits alanine (Ala-β), isoleucine (Ile-δ_1), leucine (Leu-δ_2), valine (Val-γ_2) and threonine (Thr-γ) were enriched with ^{12}C–$^{13}CH_3$ isotopomers. Stereo-selective isotope labeling of methyl groups

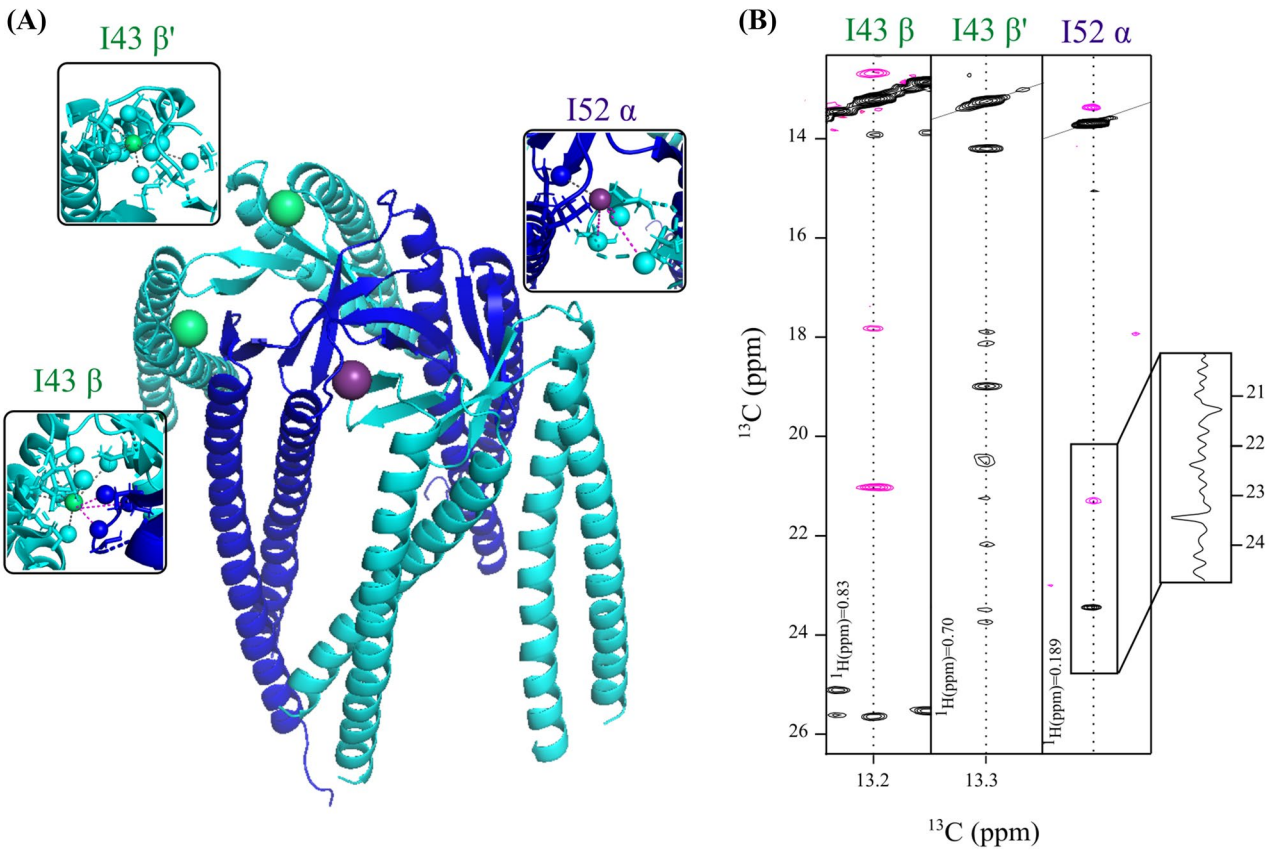

Fig. 2 Sign encoding of inter-subunit NOEs in prefoldin. **a** Structure of prefoldin from *Pyrococcus hirokoshii* (Ohtaki et al. 2008), an 87 kDa heterohexameric co-chaperonin. The two α-subunits (dark blue) are structurally and spectroscopically equivalent, whereas the β-subunits (cyan) adopt a different structure depending on their position in the ring-like assembly, resulting in a different set of NOE peaks, here named β and β'. Zooms around the δ1-methyl groups from Ile-43 (β and β') and Ile-52 (β), are showing nearby methyl groups. **b** Examples of 2D extracts from the 3D CT^2-HMQC-NOESY-HMQC experiment (Fig. 1a) acquired using a NOE mixing time t_{mix} = 600 ms on a perdeuterated sample of prefoldin in which ^{13}C–$^{13}CH_3$ isotopomers were introduced in Ala, Ile, Leu, and Val side chains of the α-subunits and ^{12}C–$^{13}CH_3$ isotopomers were introduced in Ala, Ile, Leu, Thr and Val side chains of the β-subunits (Fig. 1b). 2D planes extracted at the ^1H frequencies of δ1-methyl from Ile-52 (α and Ile-43(β), both located at the interface between of the α- and β-subunits, show both positive and negative NOEs cross-peaks, corresponding to intra- and inter-subunit NOEs respectively. Only positive NOEs are detected in the 2D slice corresponding to δ1-methyl from Ile-43 (β'), which is surrounded by methyls from β and β' subunits

in valine, isoleucine, and leucine provided improved spectral resolution, and enabled the detection of long-range NOEs (Gans et al. 2010; Kerfah et al. 2015a, b).

Using the above described labeling scheme, a high-resolution 3D CT^2-HMQC-NOESY-HMQC spectrum was acquired on the PFD-$\alpha_2\beta_4$ sample at 950 MHz ^{1}H frequency in an experimental time of 2 days. Examples of 2D slices extracted for methyl residues located either in the core of the subunits or at the α/β interface are presented in Fig. 2. It can be seen that I43-δ_1 in the β' subunit only gives rise to positive NOEs. Because this residue is far from the α/β interface, only NOEs with methyl groups in β'-type subunits are observed. I43-δ_1 in the β-subunit however is located in sufficient proximity to ^{13}C–$^{13}CH_3$ labeled methyl groups in the α-subunit to give also rise to negative inter-chain NOEs. Another example showing intra-chain (positive) and inter-chain (negative) NOEs is I52-δ_1 in the α-subunit. Complete analysis of the 3D NOESY spectrum allowed the extraction of 237 NOEs, 20 of them corresponding to negative cross peaks. These structurally meaningful inter-chains NOEs can be distinguished easily from the large number of intra-chain connectivities without requiring a tedious analysis of the 3D NOESY connectivity matrix. Such a set of NOE-derived distance restraints is particularly useful to characterize the structure of protein complexes using solution NMR.

As the sign inversion of inter-chain NOEs with respect to intra-chain signals relies on the possibility to label different subunits with distinct isotope labeling schemes, only NOEs between the α and β chains are selectively edited as negative signals, while NOEs between two homologous chains (two α or two β/β' chains) remain positive and cannot be easily distinguished from intra-chain signals.

Selective intra-chain NOE detection in homo-oligomeric complexes

In order to selectively detect intra-chain NOEs in homo-oligomeric complexes, we have again exploited the possibility of modulating the sign of NOE cross peaks with the CT^2-HMQC-NOESY-HMQC experiment using an appropriate labeling scheme. For this purpose, a ^{13}C–$^{13}CH_3$ labeled sample was mixed in a 1:1 ratio with a ^{12}C–$^{13}CH_3$ labeled sample, both in an otherwise deuterated background. The reconstituted homo-oligomeric complex contains a statistic distribution of each type of isotopomer. Figure 1c shows the expected outcome of a CT^2-HMQC-NOESY-HMQC performed on such a specifically labeled homo-oligomer. Positive intra-chain NOEs are detected whatever the labeling of the interacting methyl groups. For inter-chain connectivities, the NOE can be either positive if the two subunits are enriched with the same isotopomer, or negative if the two subunits are enriched with different isotopomers. Therefore, in a perfect 1:1 mixture, positive and negative signals cancel each other, and no inter-chain NOEs are detected in the final spectrum. In order to increase the amount of detected NOE connectivities, a maximum number of methyl-containing amino acids should be labeled, while stereospecific labeling of isoleucine, leucine and valine is used again to reduce spectral overlap and favor the detection of long range NOEs between remote amino acids (Gans et al. 2010; Kerfah et al. 2015b).

We have applied this strategy to the 53 kDa homo-tetrameric prefoldin $_4$-complex. In absence of α-subunits, the β-subunits of archaeal prefoldin form a symmetric homo-tetramer as shown for the β_4-complex from *Thermococcus* KS1 (Kida et al. 2008). Due to 94.9% sequence similarity, the *P. horikoshii* β_4-PFD is expected to behave similarly, and its structure (Fig. 3a) was calculated by homology modeling using Thermococcus KS1 PFD-β_4 as a reference. For this application, we have chosen to label the 40 Ala-β, Ile-δ_1, Leu-δ_1 and Val-γ_1 methyl positions, as these residues can be labeled simultaneously with both type of isotopomers (Gans et al. 2010; Mas et al. 2013; Miyanoiri et al. 2013; Kerfah et al. 2015a). In a first sample $^{13}CH_3$ groups were incorporated at the 40 methyl positions in a ^{12}C, ^2H background (β_4-$^{13}CH_3$ regular methyl labeled sample). The second β_4-PFD sample was reconstituted by mixing an equimolar ratio of two types of β-subunits labeled on Ala-β, Ile-δ_1, Leu-δ_1 and Val-γ_1 positions with either ^{12}C–$^{13}CH_3$ or ^{13}C–$^{13}CH_3$ isotopomer (β_4-mixed labeled sample).

3D spectra were acquired for both samples using the CT^2-HMQC-NOESY-HMQC pulse sequence of Fig. 1a) on a spectrometer operating at a ^{1}H frequency of 950 MHz. 2D strips extracted from these 3D spectra for I46-δ_1 are shown in Fig. 3c. I46-δ_1 is located at the interface between the two β-subunits. This methyl group is part of a methyl cluster involving 3 methyl containing residues belonging to a different subunit (V59-γ_1, V64-γ_1 and L62-δ_1) distant by less than 8 Å from the I46-δ_1 methyl group (Fig. 3b). A total of 8 NOEs were detected between I46-δ_1 and other methyl groups using standard β_4-$^{13}CH_3$ labeled PFD sample. As expected, the 3 inter-chain NOEs are missing in the 3D spectrum acquired on the β_4-mix labeled sample (Fig. 3c). Full analysis of the 3D CT^2-HMQC-NOESY-HMQC spectra allowed the identification of 99 NOE cross peaks, corresponding to 60 different pairs of methyl groups, for the β_4-$^{13}CH_3$ sample, while 27 of these NOEs, corresponding to 14 inter-chain methyl pairs, are missing in the spectrum recorded for the β_4-mixed labeled sample. Interestingly, inspection of the spectrum acquired using the β_4-mixed labeled sample at very low contour level does not reveal any residual signal at the positions of the missing inter-chain NOEs (Fig. S1), demonstrating the efficiency of the proposed approach to suppress selectively inter-chain NOEs.

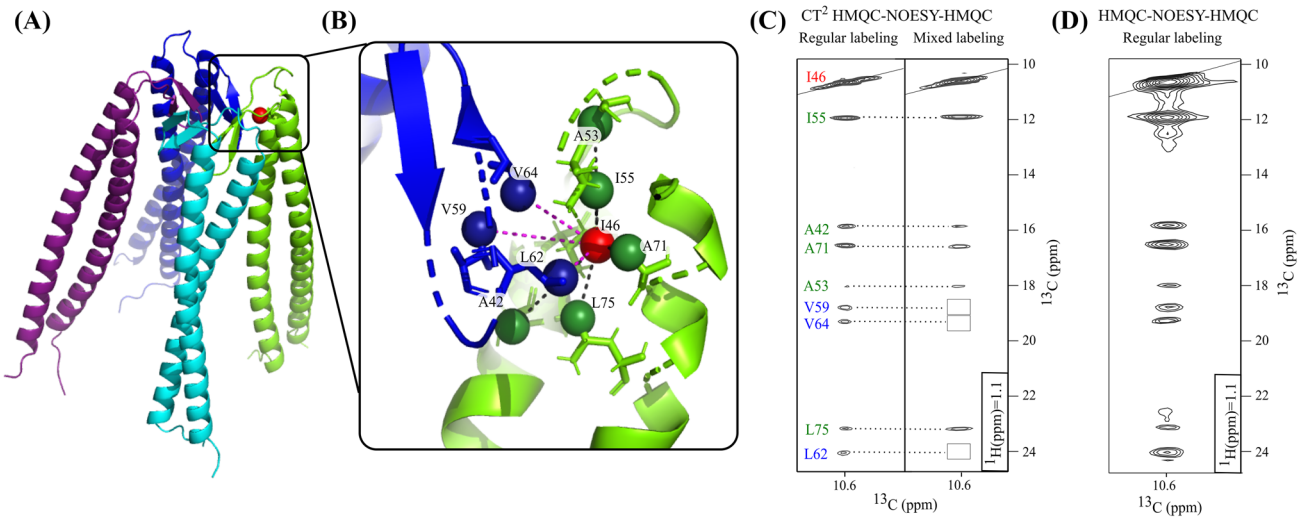

Fig. 3 Selective editing of intra-chain NOEs in the 53 kDa PFD-β_4 homotetramer. **a** Homology model of the tetramer formed by only four β-subunits of PFD from *Pyrococcus horikoshii*. Each one of the four β-subunit copies are represented by different colors. **b** Zoom around the δ1-methyl from Ile-46, showing nearby methyl groups belonging to the same chain or a different β-subunit chain. **c** 2D planes extracted at the ^{1}H frequency of δ1-methyl from Ile-46 from a 3D CT^{2}-HMQC-NOESY-HMQC experiment (Fig. 1a) acquired with a NOE mixing period t_{mix} = 600 ms on either regular methyl labeled (left), or mixed-labeled PFD-β_4 samples (right). **d** 2D plane extracted at the ^{1}H frequency of Ile-46 δ_1-methyl from a 3D HMQC-NOESY-HMQC experiment acquired with a NOE mixing time t_{mix} = 600 ms on the regular methyl labeled sample

Filter efficiency for homo-oligomeric complexes

The selective cancellation of inter-chain NOEs in homo-oligomeric complexes relies on the inversion of 50% of the NMR signal corresponding to particular methyl isotopomers (Fig. 1c). Several factors may affect the efficiency of this filter: (i) J-mismatch. The theoretical CT period Δ for a perfect sign inversion of ^{13}C-bound methyl group signals is $\Delta = 1/^{1}J_{CC} = 28.6$ ms, with $^{1}J_{CC} \approx 35$ Hz for the methyl groups. As shown in Fig. 4a, the filter efficiency to suppress signals corresponding to inter-chain NOEs does not significantly vary with the value of Δ, and a variation of the constant time period by 5 ms still allows to reduce the intensity of inter-chain NOEs by more than two orders of magnitude. Intensities of intra-chain NOEs depend more strongly on Δ and should be optimized taking into account relaxation losses. Calculation indicates that the optimal CT period Δ is on the order of 24–25 ms (Fig. S2) for methyl groups characterized by average transversal relaxation rates of 25 Hz (corresponding to the expected average methyl relaxation rates of a protein with a τ_C of 40 ns). Therefore, for applications to large proteins, we propose to use a constant time delay Δ of 25 ms, preserving signal intensities of selected intra-chain NOEs in large proteins and providing a good suppression of inter-chain connectivities. (ii) Imperfect sample preparation. This can be due to an imbalance between the two labeling schemes, or an incomplete incorporation of precursors. While an imbalance in the two differently labeled (^{12}C–^{13}CH$_3$ or ^{13}C–^{13}CH$_3$) protein chains does not affect the intensity of intra-chain NOEs (Fig. 4b), incomplete labeling of methyl groups will affect the intensity of both, intra-chain and inter-chain NOEs (Fig. 4c). Figure 4b, c shows the calculated residual intensity of inter-chain NOEs due to imperfect isotope labeling. In the extreme case of an isotope incorporation level of only 66%, or a 1:2 imbalance of the two isotopomers in the complex, the intensities of inter-chain NOEs are still reduced by a factor of 10, demonstrating the robustness of this filter with respect to errors in sample preparation. In case of doubts, the acquisition of a conventional 3D HMQC-NOESY-HMQC on the same sample, should allow to discriminate if a weak signal corresponds to the residual intensity of a strong inter-chain or a weak intra-chain NOE.

NOE-based methyl NMR assignment of the prefoldin β_4 complex

A particularly interesting application of methyl NOEs is their use, together with an available 3D structure, for NMR assignment of methyl resonances, a prerequisite for functional, interaction and dynamic studies of a target protein. Here, we have used the measured long-range NOEs (Fig. 5a) together with the 3D structure of prefoldin-β4 (Fig. 3a) to assign Ala-β, Ile-δ_1, Leu-δ_1 and Val-γ_1 methyl groups of the tetrameric complex aided by the software MAGIC (Monneau et al. 2017). 39 NMR signals are detected in a 2D methyl-TROSY spectrum (Tugarinov et al. 2003; Amero et al. 2009) of PFD-β_4, indicating that only one methyl group

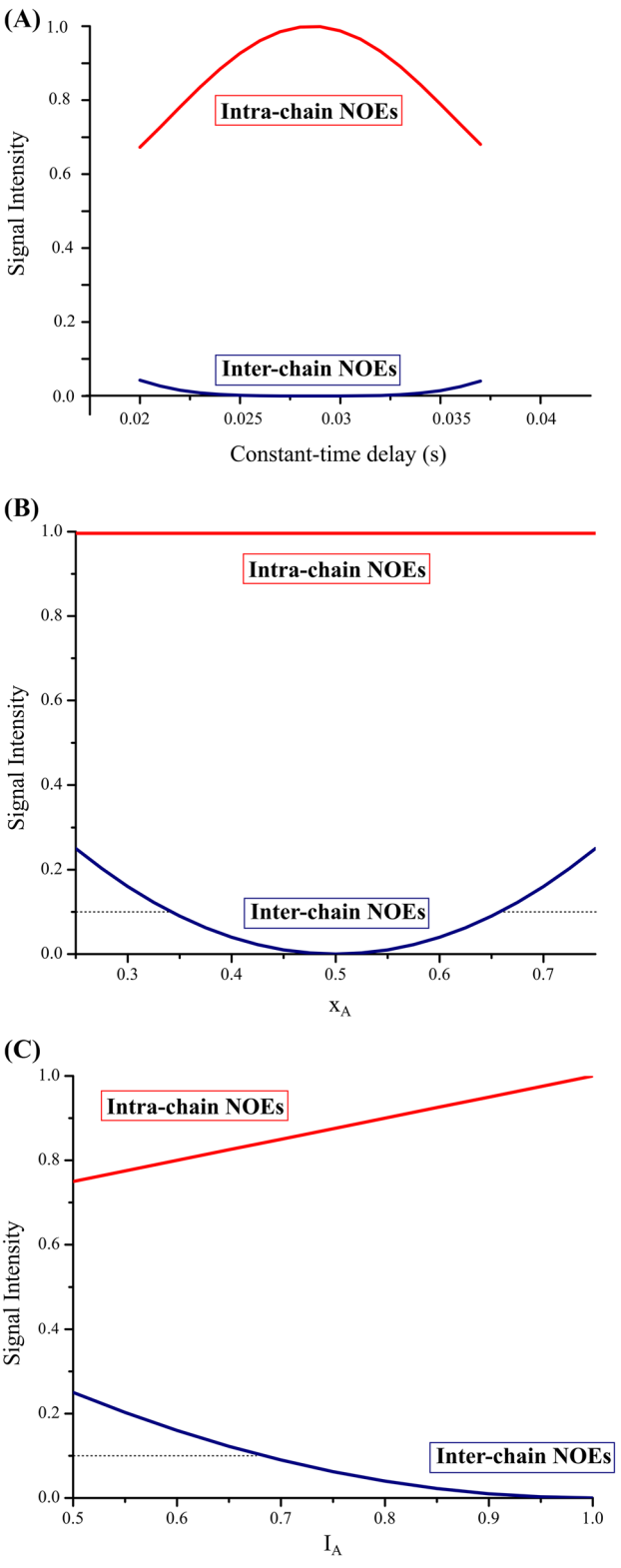

Fig. 4 Filter efficiency. **a** Effect of constant time delays (Δ) on the efficiency of the 3D CT^2-HMQC-NOESY-HMQC experiment to preserve signals of intra-chain NOEs and to suppress signals of inter-chain NOEs. **b** Effect of an imbalance in the ratio of the two labeled chains. The blue curve represents the efficiency of the suppression of inter-chain NOEs as function of x_A: the fraction of chain labeled with $^{12}C–^{13}CH_3$ isotopomers ($1-x_A$ being the fraction of chain enriched with $^{13}C–^{13}CH_3$ isotopomers). The red curve depicts the effect on intra-chain NOEs. **c** Effect of imperfect isotope labeling. A low incorporation of $^{12}C–^{13}CH_3$ or $^{13}C–^{13}CH_3$ isotopomers (I_A) in one type of labeled chains will not only lead to lower intra-chain NOE intensities (red curve), but also to partial suppression of inter-chain NOEs (blue curve)

is not detected or not visible due to peak overlap. In a first step, we identified the amino-acid-type of each methyl group giving rise to a NMR signal using a 3D HCC experiment (Ayala et al. 2009, 2012). While the isoleucine-δ_1 methyl groups can be unambiguously identified from their particular chemical shifts (Fig. 5b), alanine-β, valine-γ and Leucine-δ methyl groups were identified based on the characteristic frequencies of the neighboring Ala-Cα, Val-Cβ and Leu-Cγ carbon frequencies (Fig. S3). With this residue-type information in hand, the MAGIC input was created from the full set of detected (inter- and intra-chain) NOEs and the β_4 structure. A particular strength of the MAGIC algorithm is to provide information about the ambiguity of the calculated assignment and to propose multiple assignments in case of insufficient information. In our case, only 15% of the methyl resonances could be unambiguously assigned by the software, five were left without assignment (three of those due to missing NOE cross-peaks), and the remaining ones were given multiple assignments. Taking the software-generated assignment as a starting point, the assignment was completed manually. At this point, the information regarding inter-chain and intra-chain connectivities was introduced. In a first step, it was used to distinguish residues at the interface of the different β-chains from residues further away from the interface. Furthermore, this additional information was particularly useful to resolve assignment ambiguities at the interface region, the upper part of the coiled coil helices and the β-barrel. For instance, some pairs of residues, such as Val-72 and Val-59-γ_1, or Leu-62-δ_1 and Leu-75-δ_1, are characterized by a similar NOE connectivity network. These ambiguities could be sorted out, as one of the methyl groups is only connected by NOEs identified as intra-chain, whereas for the other one the respective connections were identified as inter-chain NOEs. Starting from unambiguously assigned methyl groups, the assignment was iteratively extended to other connected methyl groups, resulting in the unambiguous assignment of 36 out of the 39 observable methyl resonances (Fig. 5b–e). Finally, two of the three methyl resonances, for which no NOE cross-peaks were observed, Ile63-δ_1 and Leu24-δ_1, were assigned by exclusion principle, as only one possibility remained after all other peaks of the same methyl type were assigned. The last unassigned alanine resonance can correspond either to Ala-10 or Ala-116. Taking into consideration that this alanine methyl group gives rise to an intense signal and is connected in the 3D

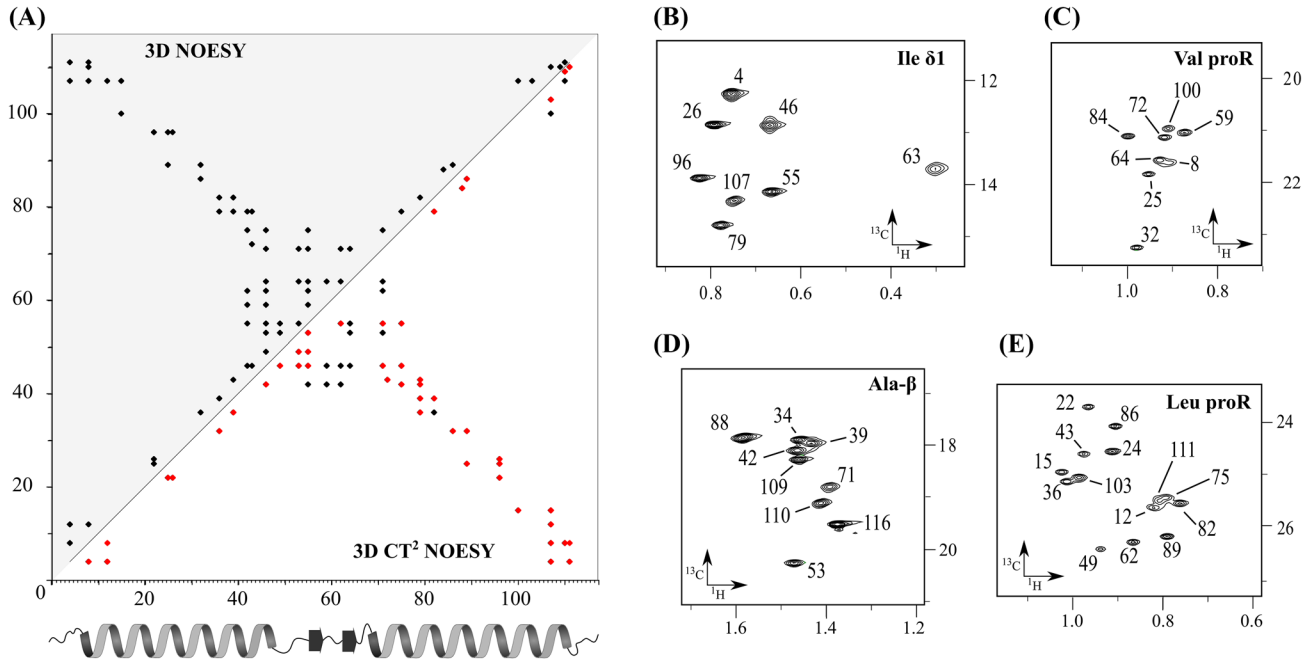

Fig. 5 NOEs based assignment of the methyl resonances in β_4-prefoldin. **a** 2D representation of the PFD-β_4 residue pairs for which inter-methyls NOEs are observed. The secondary structure elements of the protein are presented below the matrix. Top left part of the matrix corresponds to the 67 methyl pairs for which NOEs (113 cross-peaks) were detected using the standard 3D HMQC-NOESY-HMQC experiment acquired with a regular methyl labeled PFD-β4 sample. Bottom right part of the matrix presents in red the intra-chain NOEs (71 cross-peaks, 47 pairs) detected using 3D CT^2-HMQC-NOESY-HMQC with regular and mixed labeled PFD-β_4 sample. Inter-chain NOEs in black (28 cross-peaks, 14 pairs) are only detected when the 3D CT^2-HMQC-NOESY-HMQC experiment is acquired with the regular sample. **b–d** 2D SOFAST-methyl-TROSY sub-spectra of PFD-β_4 corresponding to Ile-δ1 (**b**), Val-γ1 (**c**), Ala-β (**d**) and Leu-δ1 (**e**) resonances. Assignment was obtained using MAGIC software (Monneau et al. 2017) based on the 3D structure of PFD-β_4 and experimental methyl-methyl NOEs (**a**)

HCC spectra to a single C_α signal with a chemical shift characteristic of a random coil structure, this signal has been tentatively assigned to Ala-116 in the C-terminal unfolded segment of the protein.

Discussion

Spectral editing of intra- and inter-chain NOEs by the CT^2-HMQC-NOESY-HMQC experiments relies on the use of two CT ^{13}C editing periods. While this allows the recording of highly resolved, isotopomer-edited NOESY spectra for both, homo- and hetero-oligomeric complexes, the additional CT delays of about 25 ms will result in relaxation-induced signal loss. In order to quantify this signal loss for large proteins, we have used a sample of deuterated, methyl-labeled Malate Synthase G, an 82 kDa monomeric protein extensively studied by NMR (Tugarinov and Kay 2005a) and characterized by a rotational correlation time of $\tau_C = 39.2$ ns at 37 °C in D_2O buffer (Tugarinov and Kay 2005b). We have previously shown that methyl–methyl long range NOEs between probes up to 12 Å apart are detectable in such a protein, suitably labeled using the standard 3D-HMQC-NOESY-HMQC experiment (Kerfah et al. 2015b; Ayala et al. submitted). Using the same MSG sample and comparable acquisition and processing parameters, we acquired 3D CT^2-HMQC-NOESY-HMQC (Fig. 1) and standard 3D-HMQC-NOESY-HMQC (Tugarinov et al. 2005b; Kerfah et al. 2015b) spectra. Comparing the signal intensities measured in these two spectra, we observed an average reduction of 65% of the signal intensities (Fig. S4A), in agreement with an estimated average MQ relaxation rate of 24 Hz (Fig. S4B). Taking into account that the NOE intensity decreases with the sixth power of the inter-methyl distance, we estimate that the two extra CT-periods in the 3D CT^2-HMQC-NOESY-HMQC experiment will reduce the maximum distance for which a NOE cross peak can be detected by 15% for this protein (with a $\tau_c = 40$ ns). Therefore, inter-methyl NOEs between methyl probes separated by up to 10 Å should be detectable with the 3D CT^2-HMQC-NOESY-HMQC pulse scheme within 3 days on a modern high field NMR spectrometer (equipped with a cryogenic probe) using a 1 mM sample of well behaving protein such as the 82 kDa Malate Synthase G. Lower protein solubility, or presence of conformational exchange in the micro- to millisecond time scale, as observed for

prefoldin samples (data not shown), affect the quality of the NMR spectra and reduces the theoretical distance for which inter-methyl NOEs can be detected. Full analysis of the 3D CT^2-HMQC-NOESY-HMQC spectrum acquired with PFD-β_4 sample indicates that NOEs can still be detected between methyl probes distant by up to 8 Å (Fig. S5). However, to extract distance restraints, the contribution of spin diffusion cannot be neglected as a relatively long mixing period (600 ms) was used to detect a maximum number of long range NOEs in these highly deuterated proteins. Full relaxation-matrix analysis is required to extract accurate distance restraints from such 3D NOESY spectra (Sounier et al. 2007; Vögeli 2014; Nichols et al. 2018).

For the applications presented here, we have selectively labeled methyl groups of Alanine, Isoleucine, Leucine and Valine, representing on average *ca.* 80% of methyl containing amino-acids in proteins. Current protocols only allow to selectively label Threonine with a $^{13}CH_3$ group in an otherwise U-[^{12}C, ^{2}H]-labeled amino-acid (Velyvis et al. 2012; Ayala et al. submitted), but the approaches presented here could be further extended to Threonine residues when suitably labeled Threonine precursors will become available. Commercially available deuterated Methionine and Threonine, specifically enriched with $^{13}CH_3$ groups, can be incorporated together with Alanine, Isoleucine, Leucine and Valine in protein enriched with ^{12}C–$^{13}CH_3$ isotopomers, in order to increase the number of intra-chain and inter-chain NOEs (of opposite sign) in hetero-oligomeric complexes. It has been previously reported that the mixing of two proteins, labeled on different types of methyl-containing residues can be used to selectively detect inter-chain methyl-methyl NOEs between Isoleucine-δ_1 and Valine-γ, as well as between Isoleucine-δ_1 and Leucine-δ methyl groups (Traaseth et al. 2008). The approach proposed in this article allows to distinguish intra-chain and inter-chain NOEs simultaneously between all the methyl groups of Alanine, Isoleucine, Leucine and Valine, thus significantly increasing the number of both intramolecular and intermolecular distance restraints between remote methyl probes.

The selective detection of intra-chain NOEs in a homo-oligomeric complex requires the preparation of a mixed labeled sample with half of the subunits perdeuterated and labeled with ^{13}C–$^{13}CH_3$ isotopomers and the remaining perdeuterated subunits labeled only with $^{13}CH_3$ groups. Production of such a mixed labeled sample, with a statistic distribution of both labeling schemes in the complex, requires that the subunits are in exchange with a transient soluble monomer or between two distinct oligomers. Otherwise, a more demanding sample preparation, including unfolding of the native complex before mixing of both labeling schemes in a 1:1 ratio and re-oligomerization, need to be implemented (Macek et al. 2017). Such a sample can be used with the CT^2-HMQC-NOESY-HMQC experiment (Fig. 1) to detect exclusively 'precious' intra-chain NOEs, enabling assignment of methyl resonances based on a previously known structure of symmetric oligomers (Xu and Matthews 2013; Chao et al. 2014; Pritišanac et al. 2017; Monneau et al. 2017; Pritišanac et al. 2019).

In homo-oligomeric protein complexes, the identification of inter-chain NOEs was obtained by comparing two 3D CT^2-HMQC-NOESY-HMQC spectra acquired on mixed methyl labeled and regular methyl labeled samples prepared at the same concentration (Figs. 3, 5a). Alternatively, in order to work with a single sample, a standard 3D ^{13}C-edited NOESY experiment (Tugarinov et al. 2005b; Kerfah et al. 2015b) and a 3D CT^2-HMQC-NOESY-HMQC spectra, both acquired on the mixed labeled sample, can be used to identify both intra-chain and inter-chain NOEs in homo-oligomeric complexes. The conventional 3D NOESY (i.e. non-constant-time) should be recorded with a maximum acquisition time of *c.a.* 10 ms, in both ^{13}C dimensions, in order to limit peak shape distortion due to the presence of a $^{1}J_{CC}$ coupling for 50% of the signal. A comparison of the NOE pattern allows to identify unambiguously intra-chain NOEs in the CT^2-HMQC-NOESY-HMQC experiment, and potential inter-chain NOEs as the supplementary signals present only in the conventional 3D ^{13}C-edited NOESY experiment.

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References

Amero C, Schanda P, Dura A, Ayala I, Marion D, Franzetti B, Brutscher B, Boisbouvier J (2009) Fast two dimensional NMR spectroscopy of high molecular weight protein assemblies. J Am Chem Soc 131:3448–3449.

Anglister J, Srivastava G, Naider F (2016) Detection of intermolecular NOE interactions in large protein complexes. Prog Nucl Magn Reson Spectrosc 97:40–56

Ayala I, Chiari L, Kerfah R, Boisbouvier J, Gans P, Hamelin O (Submitted) Chemical synthesis of methyl specifically labelled L-threonine and application to the NMR studies of high molecular weight proteins

Ayala I, Sounier R, Use N, Gans P, Boisbouvier J (2009) An efficient protocol for the complete incorporation of methyl-protonated alanine in perdeuterated protein. J Biomol NMR 43:111–119

Ayala I, Hamelin O, Amero C, Pessey O, Plevin MJ, Gans P, Boisbouvier J (2012) An optimized isotopic labelling strategy of isoleucine-gamma2 methyl groups for solution NMR studies of high molecular weight proteins. Chem Commun 48:1434–1436

Caffrey M, Cai M, Kaufman J, Stahl SJ, Wingfield PT, Covell DG, Gronenborn AM, Clore GM (1998) Three-dimensional solution structure of the 44 kDa ectodomain of SIV gp41. EMBO J 17:4572–4584

Chao FA, Kim J, Xia Y, Milligan M, Rowe N, Veglia G (2014) FLAMEnGO 2.0: an enhanced fuzzy logic algorithm for structure-based assignment of methyl group resonances. J Magn Reson 245:17–23

Chi CN, Strotz D, Riek R, Vögeli B (2018) NOE-derived methyl distances from a 360 kDa proteasome complex. Chemistry 24:2270–2276

Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J Biomol NMR 6:277–293

Gans P, Hamelin O, Sounier R, Ayala I, Dura MA, Amero CD, Noirclerc-Savoye M, Franzetti B, Plevin MJ, Boisbouvier J (2010) Stereospecific isotopic labeling of methyl groups for NMR spectroscopic studies of high-molecular-weight proteins. Angew Chem Int Ed Engl 49:1958–1962

Gardner KH, Kay LE (1997) Production and incorporation of 15N, 13C, 2H (1H-δ1 methyl) isoleucine into proteins for multidimensional NMR studies. J Am Chem Soc 119:7599–7600

Gauto DF, Estrozi LF, Schwieters CD, Effantin G, Macek P, Sounier R, Sivertsen AC, Schmidt E, Kerfah R, Mas G, Colletier JP, Güntert P, Favier A, Schoehn G, Schanda P, Boisbouvier J (2019) Integrated NMR and cryo-EM atomic-resolution structure determination of a half-megadalton enzyme complex. Nat Commun 10:2697. https://doi.org/10.1038/s41467-019-10490-9

Geen H, Freeman R (1991) Band-selective radiofrequency pulses. J Magn Reson 93:93–141.

Goto N, Gardner K, Mueller G, Willis R, Kay L (1999) A robust and cost-effective method for the production of Val, Leu, Ile (δ1) methyl-protonated 15N-, 13C-, 2H-labeled proteins. J Biomol NMR 13:369–374

Handle TM, Domaille PJ (1996) Heteronuclear (H-1, C-13, N-15) NMR assignments and solution structure of the monocyte chemoattractant protein-1 (MCP-1) dimer. Biochemistry 35:6569–6584

Kerfah R, Plevin MJ, Sounier R, Gans P, Boisbouvier J (2015a) Methyl-specific isotopic labeling: a molecular tool box for solution NMR studies of large proteins. Curr Opin Struct Biol 32:113–122

Kerfah R, Plevin MJ, Pessey O, Hamelin O, Gans P, Boisbouvier J (2015b) Scrambling free combinatorial labeling of alanine-β, isoleucine-δ1, leucine-proS and valine-proS methyl groups for the detection of long range NOEs. J Biomol NMR 61:73–82

Kida H, Sugano Y, Iizuka R, Fujihashi M, Yohda M, Miki K (2008) Structural and molecular characterization of the prefoldin beta subunit from Thermococcus strain KS-1. J Mol Biol 383:465–474

Kupce E, Freeman R (1994) Wideband excitation with polychromatic pulses. J Magn Reson 108:268–273

Lescop E, Kern T, Brutscher B (2010) Guidelines for the use of band-selective radiofrequency pulses in hetero-nuclear NMR: example of longitudinal-relaxation-enhanced BEST-type 1H–15N correlation experiments. J Magn Reson 203:190–198

Macek P, Kerfah R, Boeri Erba E, Crublet E, Moriscot C, Schoehn G, Amero C, Boisbouvier J (2017) Unraveling self-assembly pathways of the 468 kDa proteolytic machine TET2. Sci Adv 3:e1601601

Maciejewski MW, Schuyler AD, Gryk MR, Moraru II, Romero PR, Ulrich EL, Eghbalnia HR, Livny M, Delaglio F, Hoch JC (2017) NMRbox: a resource for biomolecular NMR computation. Biophys J 112:1529–1534

Mas G, Crublet E, Hamelin O, Gans P, Boisbouvier J (2013) Specific labeling and assignment strategies of valine methyl groups for NMR studies of high molecular weight proteins. J Biomol NMR 57:251–262

Mas G, Guan J-Y, Crublet E, ColasDebled E, Moriscot C, Gans P, Schoehn G, Macek P, Schanda P, Boisbouvier J (2018) Structural investigation of a chaperonin in action reveals how nucleotide binding regulates the functional cycle. Sci Adv 4:eaau4196

Miyanoiri Y, Takeda M, Okuma K, Ono AM, Terauchi T, Kainosho M (2013) Differential isotope-labeling for Leu and Val residues in a protein by *E. coli* cellular expression using stereo-specifically methyl labeled amino acids. J Biomol NMR 57:237–249

Monneau YR, Rossi P, Bhaumik A, Huang C, Jiang Y, Saleh T, Xie T, Xing Q, Kalodimos CG (2017) Automatic methyl assignment in large proteins by the MAGIC algorithm. J Biomol NMR 69:215–227

Nichols PJ, Born A, Henen MA, Strotz D, Celestine CN, Güntert P, Vögeli B (2018) Extending the applicability of exact nuclear overhauser enhancements to large proteins and RNA. ChemBioChem. https://doi.org/10.1002/cbic.201800237

Ohtaki A, Kida H, Miyata Y, Ide N, Yonezawa A, Arakawa T, Iizuka R, Noguchi K, Kita A, Odaka M, Miki K, Yohda M (2008) Structure and molecular dynamics simulation of archaeal prefoldin: the molecular mechanism for binding and recognition of nonnative substrate proteins. J Mol Biol 376:1130–1141

Okochi M, Yoshida T, Maruyama T, Kawarabayasi Y, Kikuchi H, Yohda M (2002) Pyrococcus prefoldin stabilizes protein-folding intermediates and transfers them to chaperonins for correct folding. Biochem Biophys Res Commun 291:769–774

Powers R, Gronenborn AM, Clore GM, Bax A (1991) Three-dimensional triple resonance NMR of 13C/15N enriched proteins using contant-time evouliton. J Magn Reson 94:209–213

Pritišanac I, Degiacomi MT, Alderson TR, Carneiro MG, Ab E, Siegal G, Baldwin AJ (2017) Automatic assignment of methyl-NMR spectra of supramolecular machines using graph theory. J Am Chem Soc 139:9523–9533

Pritišanac I, Würz JM, Alderson TR, Güntert P (2019) Automatic structure-based NMR methyl resonance assignment in large proteins. bioRxiv. https://doi.org/10.1101/538272

Rosenzweig R, Kay LE (2014) Bringing dynamic molecular machines into focus by methyl-TROSY NMR. Annu Rev Biochem 83:291–315

Rosenzweig R, Moradi S, Zarrine-Afsar A, Glover JR, Kay LE (2013) Unraveling the mechanism of protein disaggregation through a ClpB-DnaK interaction. Science 339:1080–1083

Ruschak AM, Religa TL, Breuer S, Witt S, Kay LE (2010) The proteasome antechamber maintains substrates in an unfolded state. Nature 467:868–871

Sali A, Blundell TL (1993) Comparative protein modelling by satisfaction of spatial restraints. J Mol Biol 234:779–815

Sounier R, Blanchard L, Wu Z, Boisbouvier J (2007) High-accuracy distance measurement between remote methyls in specifically protonated proteins. J Am Chem Soc 129:472–473

Sprangers R, Kay LE (2007) Quantitative dynamics and binding studies of the 20S proteasome by NMR. Nature 445:618–622

Traaseth NJ, Verardi R, Veglia G (2008) Asymmetric methyl group labeling as a probe of membrane protein homo-oligomers by NMR spectroscopy. J Am Chem Soc 130:2400–2401

Tugarinov V, Kay LE (2005a) Methyl groups as probes of structure and dynamics in NMR studies of high-molecular-weight proteins. ChemBioChem 6:1567–1577

Tugarinov V, Kay LE (2005b) Quantitative 13C and 2H NMR relaxation studies of the 723-residue enzyme malate synthase G reveal a dynamic binding interface. Biochemistry 44:15970–15977

Tugarinov V, Hwang PM, Ollerenshaw JE, Kay LE (2003) Cross-correlated relaxation enhanced 1H–13C NMR spectroscopy of methyl groups in very high molecular weight proteins and protein complexes. J Am Chem Soc 125:10420–10428

Tugarinov V, Choy W-Y, Orekhov VY, Kay LE (2005a) Solution NMR-derived global fold of a monomeric 82-kDa enzyme. Proc Natl Acad Sci USA 102:622–627

Tugarinov V, Kay LE, Ibraghimov I, Orekhov VY (2005b) High-resolution four-dimensional 1H–13C NOE spectroscopy using methyl-TROSY, sparse data acquisition, and multidimensional decomposition. J Am Chem Soc 127:2767–2775

Tugarinov V, Kanelis V, Kay LE (2006) Isotope labeling strategies for the study of high-molecular-weight proteins by solution NMR spectroscopy. Nat Protoc 1:749–754

Velyvis A, Ruschak AM, Kay LE (2012) An economical method for production of 2H, 13CH3-threonine for solution NMR studies of large protein complexes: application to the 670 kDa proteasome. PLoS ONE 7:e43725

Vögeli B (2014) The nuclear Overhauser effect from a quantitative perspective. Prog Nucl Magn Reson Spectrosc 78:1–46

Vranken WF, Boucher W, Stevens TJ, Fogh RH, Pajon A, Llinas M, Ulrich EL, Markley JL, Ionides J, Laue ED (2005) The CCPN data model for NMR spectroscopy: development of a software pipeline. Proteins 59:687–769

Werner MH, Clore GM, Fisher CL, Fisher RJ, Trinh L, Shiloach J, Gronenborn AM (1997) Correction of the NMR structure of the ETS1/DNA complex. J Biomol NMR 10:317–328

Xu Y, Matthews S (2013) MAP-XSII: an improved program for the automatic assignment of methyl resonances in large proteins. J Biomol NMR 55:179–187

Zhou Z, Kümmerle R, Qiu X, Redwine D, Cong R, Taha A, Baugh D, Winniford B (2007) A new decoupling method for accurate quantification of polyethylene copolymer composition and triad sequence distribution with ^{13}C NMR. J Magn Reson 187:225–233

Zwahlen C, Gardner KH, Sarma SP, Horita DA, Byrd RA, Kay LE (1997) An NMR experiment for measuring methyl−methyl NOEs in ^{13}C-labeled proteins with high resolution. J Am Chem Soc 120:7617–7625