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^{1}H, ^{15}N and ^{13}C backbone resonance assignments of the Kelch domain of mouse Keap1

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Abstract

Keap1 is a multi-domain protein that functions as an inhibitor of the transcription factor Nrf2 in the cellular response to oxidative stress. Under normal conditions, Keap1 binds to Nrf2 via its C-terminal Kelch domain and the interaction ultimately leads to the ubiquitin-dependent degradation of Nrf2. It has been proposed that designing molecules to selectively disrupt the Keap1-Nrf2 interaction can be a potential therapeutic approach for enhancing the expression of cytoprotective genes. Here, we reported the ^{1}H, ^{13}C, and ^{15}N backbone chemical shift assignments of the Kelch domain of mouse Keap1. Further, unlabeled Nrf2 peptide containing the Kelch-binding motif was added to the ^{15}N-labeled Kelch sample. ^{1}H-^{15}N HSQC spectra of the protein in the absence and presence of an equimolar concentration of the Nrf2 peptide were presented. A significant number of resonance signals were shifted upon addition of the peptide, confirming the protein-peptide interaction. The results here will not just facilitate the further studies of the binding between Keap1 and Nrf2, it will also be valuable for probing interactions between the Kelch domain and small molecules, as well as a growing list of protein targets that have been identified recently.

Keywords

Oxidative stress response; Keap1; Kelch domain; Nrf2; protein-protein interaction

Biological context

Keap1 (Kelch-like ECH-associated Protein 1) is a 70-kDa protein that is rich in cysteine. It functions as the repressor of Nrf2 (nuclear factor E2-related factor 2), the key transcription factor that coordinates the cellular responses to oxidative stress. Keap1 is composed of three domains (Itoh et al. 1999). The N-terminal BTB (Broad-Complex, Tramtrack and Bric-a-Brac) domain mediates the protein dimerization and is also responsible for the binding to the Cul3-dependent E3 ubiquitin ligase complex (Itoh et al. 1999). The BTB domain is linked to the C-terminal Kelch domain by the intervening region (IVR), where cysteines that are pivotal for the Keap1 functions as chemical sensor are located (Itoh et al. 1999). The C-terminal Kelch domain is responsible for target recognition. It directly interacts with the ETGE and DLG motifs present in the N-terminal Neh2 domain of Nrf2 (Baird and Dinkova-Kostova 2011; Tong et al. 2006; Zipper and Mulcahy 2002). Under unstressed conditions, Keap1 binds to Nrf2 and targets this transcription factor for degradation (Itoh et al. 2004).

The 32-kDa Kelch domain adopts a six-bladed β-propeller conformation and the structures in complex with peptides derived from Nrf2 that contain the binding motifs have been determined by X-ray crystallography (Lo et al. 2006; Padmanabhan et al. 2006; Tong et al. 2007). Due to the critical roles Keap1 and Nrf2 play in the oxidative stress response mechanism, disrupting their interaction has been proposed as a potential strategy to enhance the expression of cytoprotective genes. The backbone resonance assignments obtained here will facilitate the screening of inhibitors that can disrupt the Keap1-Nrf2 interaction. In addition to Nrf2, several novel targets of the Kelch domain of Keap1 have been identified recently. These include FAC1 (Strachan et al. 2004), PGAM5 (Lo and Hannink 2006), WTX (Major et al. 2007; Camp et al. 2012), prothymosin α (Padmanabhan et al. 2008), p62 (Komatsu et al. 2010) and PALB2 (Ma et al. 2012). The assignments reported here will also serve as important starting points for studying the interaction of Keap1 with these proteins.

Methods and experiments

Sample preparation

The mouse Keap1 cDNA (GenBank# BC055732) was purchased from the American Type Culture Collection (ATCC) and the Kelch domain (residues 324-612) was cloned into the expression vector pDEST17 (Invitrogen). The TEV cleavage recognition sequence, 'ENLYFQG', was introduced between the histidine tag and insert. This vector was transformed into *E. coli* BL21(DE3). ^{15}N, ^{13}C and ^{2}H labeled protein was produced by growing *E. coli* in deuterated M9 media. Cells adapted in 70% D_2O were used to inoculate 1L of M9 prepared in 100% D_2O. The cells were then grown at 37ºC and overexpression was induced at an OD_{600} of 0.6 with 0.5 mM IPTG. After a 60-hour induction, the cells were centrifuged and the pellets were frozen. The N-terminally His-tagged protein was purified by affinity chromatography using Ni Sepharose™ 6 Fast Flow beads (Amersham Biosciences). The tag was cleaved by incubation with His-tagged tobacco etch virus (TEV) protease overnight at 25 ºC and the protein product was purified by passing the mixture through Ni Sepharose™ 6 Fast Flow beads (Amersham Biosciences). For NMR experiments, the purified protein was dialyzed against 50 mM sodium phosphate at pH 7

containing 50 mM NaCl and 1 mM DTT. The samples were then concentrated to ~0.3 mM. All samples contained 10% D_2O and 1 mM 2,2-dimethyl-2-sila-pentane-5-sulfonic acid (DSS) as ^{1}H and ^{13}C chemical shift references.

Sequential assignment experiments

NMR experiments for the backbone resonance assignment were conducted at 25ºC on Varian INOVA 800 MHz (NANUC) and Bruker Avance 800 MHz (Singapore) spectrometers equipped with cryogenic probes. Sequential assignments were obtained from ^{1}H-^{15}N TROSY-HSQC, HNCA, HN(CO)CA, HNCACB, HN(CO)CACB and ^{15}N-NOESY-HSQC spectra. The data was processed with NMRPipe (Delaglio et al. 1995) and analyzed using CARA (Keller 2004).

Ligand binding experiment

To demonstrate the binding of the Kelch domain to a mouse Nrf2 peptide (Ac-^{72}AQFQLDEETGEFLP^{85}-NH_2; ordered from NEOpeptide), ^{1}H-^{15}N HSQC spectra of ^{15}N-labeled Kelch domain (~ 200 μM) were collected on a Varian Inova 600 MHz spectrometer with cryogenic probe (UWO Biomolecular NMR Facility) at 25ºC in the absence and presence of equimolar concentration of the Nrf2 peptide.

Assignments and data deposition

The ^{1}H-^{15}N TROSY-HSQC of the Kelch domain of Keap1 (Figure 1a) had well dispersed peaks for a protein of this size. We were able to assign 91.3% of the $^{1}H^{N}$ and ^{15}N resonances of non-proline residues, 90.7% of all $^{13}C\alpha$ and 90% all $^{13}C\beta$ resonances. Repetitive sequences of the β-propeller structure (Figure 1b) made it difficult to obtain a higher percentage of resonance assignments. The ^{1}H, ^{15}N and ^{13}C α/β chemical shifts of the backbone resonances have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu), under BMRB accession number 18353.

Figure 2 shows the residue-specific secondary structure propensity (SSP) scores determined using the program SSP on the basis of the assigned ^{13}C α/β chemical shifts (Marsh et al. 2006). The result strongly indicates that the protein has an all-β fold (Figure 2). This is in good agreement with the crystal structure of the Kelch domain (PDB id: 1X2J), which shows that the protein adopts a six-bladed β-propeller conformation (Padmanabhan et al. 2006). The SSP scores we obtained here are also consistent with the DSSP analysis (Kabsch and Sander 1983) and the secondary structure plot (Laskowski 2009) of the crystal structure (Padmanabhan et al. 2006).

The overlay of the ^{1}H-^{15}N HSQC spectra of the Kelch domain in the absence and presence of an equimolar concentration of the 14-mer Nrf2 peptide is shown in Figure 3a. A significant number of resonance signals are shifted upon addition of the peptide (Figure 3a). To quantify the magnitude of peak shifts, combined chemical shift changes ($\Delta\omega = |\Delta^{15}N| + |\Delta^{1}H^{N}|$) were calculated, where $|\Delta^{15}N| + |\Delta^{1}H^{N}|$ are the absolute values of resonance frequency change (in Hz) in the ^{15}N and ^{1}H dimensions, respectively. Figure 3b shows that many of the traceable assigned residues with $\Delta\omega > 50$ Hz (in descending order: G378, N504, G364, G477, G600, G570, G571, G417, G524, G379 and S431) are residues that

comprise, or are in close proximity, to the binding interface as identified in the crystal structure (Padmanabhan et al. 2006).

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Figure Legends

Fig. 1 a ^{1}H-^{15}N TROSY-HSQC spectrum and backbone resonance assignment of the $^{2}H/^{13}C/^{15}N$ labeled Kelch domain of mouse Keap1. The figure was generated using Sparky (Goddard and Kneller). **b** Protein sequence of the Kelch domain with unassigned resonances colored red and regions with high sequence identities boxed. The starting 'G' was a non-native residue from the TEV recognition sequence that remained after cleavage.

Fig. 2 Secondary structure propensity (SSP) scores and DSSP analysis of the mouse Kelch domain of Keap1. SSP scores were calculated based upon the ^{13}C α/β chemical shifts (Marsh et al. 2006). The crystal structure of the mouse Kelch domain (PDB id: 1X2J) (Padmanabhan et al. 2006) was used for the DSSP analysis (Kabsch and Sander 1983) and generation of the secondary structure cartoon diagram (Laskowski 2009).

Fig. 3 a Overlay of ^{1}H-^{15}N HSQC spectra in the absence (black) and presence (pink) of an equimolar concentration of the Nrf2 peptide (Ac-^{72}AQFQLDEETGEFLP^{85}-NH_2). **b** Crystal structure of the mouse Kelch domain of Keap1 (grey) in complex with an Nrf2 peptide (red) (PDB id: 1X2R) (Padmanabhan et al. 2006). Residues with traceable assigned resonances are colored based on their combined absolute proton and nitrogen resonance frequency changes (Hz) upon ligand binding (yellow <25 Hz, orange 25-50 Hz and pink >50 Hz).

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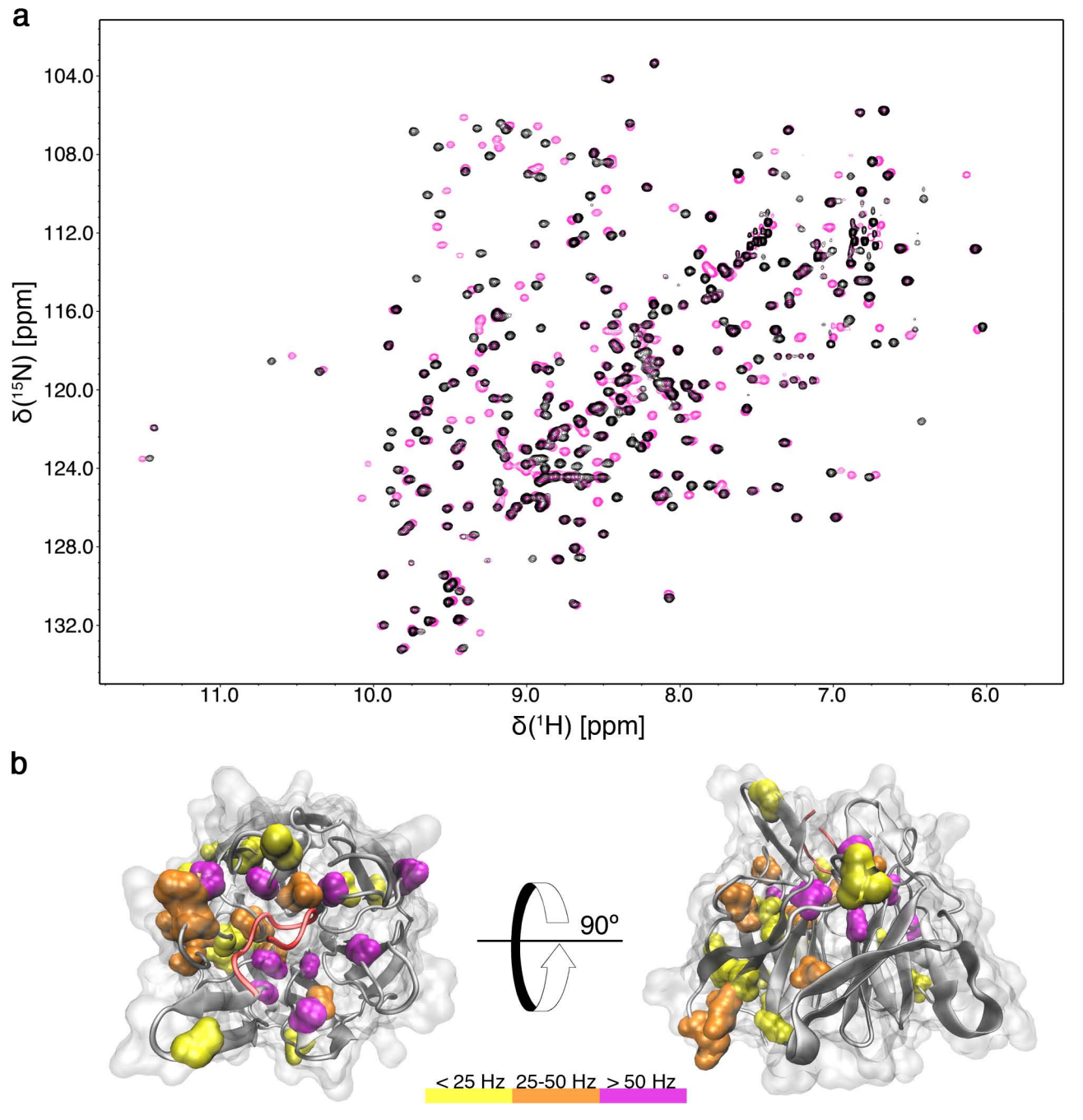
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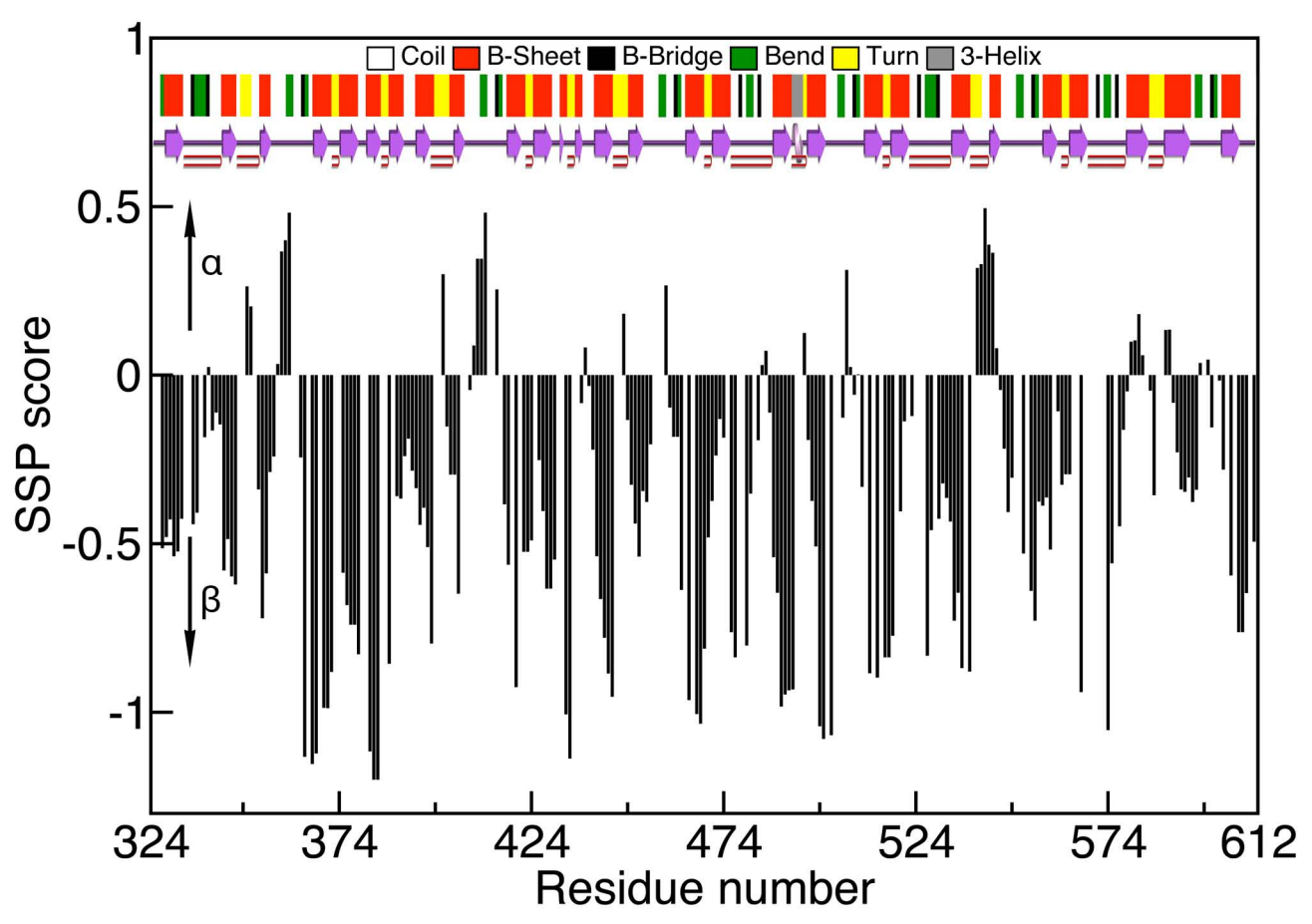
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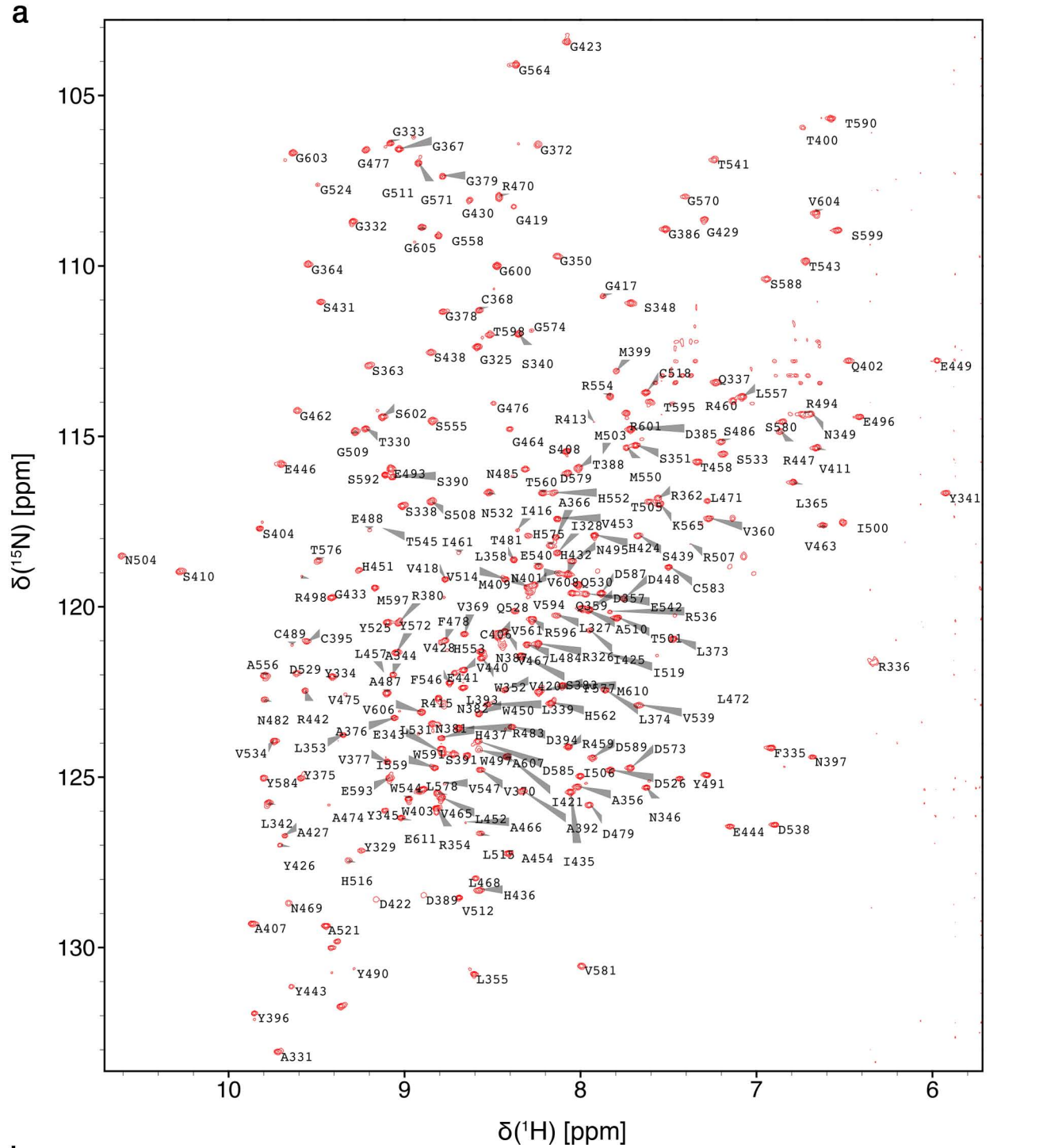

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 440                 460                 480
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      500                 520                 540
WRMITPMNTIRSGAGVCVLHNCIYAAGGYDGQDQLNSVERYDVETETWTFVAPMRHHR
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