NMR Backbone and Methyl Resonance Assignments of an inhibitory G-alpha subunit in complex with GDP

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Abstract:

G-proteins are essential switch points at the cell membrane that control downstream signaling by their ability to adopt an inactive, GDP-bound or an active, GTP-bound state. Among other exchange factors, G-protein coupled receptors (GPCRs) induce exchange of GDP to GTP and thus promote the active state of the G-protein. The nucleotide-binding α subunit of the G-protein undergoes major conformational changes upon nucleotide binding. Thus, an NMR analysis of the two distinct nucleotide-bound states is essential for a more detailed understanding of associated structural changes. Here, we provide an NMR backbone as well as methyl group resonance assignment of an inhibitory G-alpha subunit subtype 1 ($G\alpha_{i,1}$) in the GDP-bound form and show that, in contrast to the GTP-bound form, large parts of the protein are mobile, presumably caused by a loose arrangement of the two subdomains in Gα that tightly interact with each other only in the GTP-bound state. As the GDP-bound form represents the GPCR-binding-competent state, the presented NMR data will be essential for further studies on G-protein-GPCR interactions and dynamics in solution for receptor systems that couple to G-proteins containing an inhibitory Gα,1 subunit.

Keywords GPCR, G-protein, nucleotide binding, NMR, signal transduction

Biological context

Heterotrimeric G proteins are a main player in mediating signal transduction from the cell membrane to the interior of the cell (Gao et al. 1987). G-proteins have two distinct functional units, the α subunit (Gα) and the βγ subunit complex. Within Gα, two structural domains, the Ras-like domain which mediates binding to guanine nucleotides, and a helical domain that can close the nucleotide binding cleft to trap the bound nucleotide, are of functional importance. Heterotrimeric G proteins transfer signals from activated G-protein-coupled receptors (GPCRs) to intracellular effectors. This is facilitated by stimulation of nucleotide exchange from the inactive GDP-bound to the GTP-bound state by an activated GPCR, presumably by facilitating sub-domain opening within Gα (Rasmussen et al. 2011) that allows for dissociation of GDP and subsequent binding of GTP. The GTP-bound conformation of Gα induces dissociation of the complex of the G-protein from the GPCR and dissociation of the heterotrimeric G-protein into the α subunit and the βγ heterodimer, with both parts acting as mediators of downstream cellular signaling (Gilman 1987). Subsequently, the intrinsic GTP hydrolysis activity of the Gα subunit returns the protein back to the GDP-bound state. In the GDP-bound state, the Gα subunit assembles with the βγ heterodimer and this complex is then again ready for binding to an agonist-bound, activated GPCR. The details of this mechanism have been investigated by biophysical methods (Oldham et al. 2006; Oldham et al. 2007; Van Eps et al. 2006) and X-ray crystallography (Coleman et al. 1994; Coleman and Sprang 1998; Lambright et al. 1994; Lambright et al. 1996; Wall et al. 1995) and recently by NMR (Goricanec et al. 2016; Toyama et al. 2017).

Here, we provide NMR backbone and methyl group resonance assignments of an inhibitory Gα subunit subtype 1 in complex with GDP and show that this complex is dynamic in the ms to μs time scale as monitored by severe line broadening within the protein backbone and side chain methyl resonances. As GPCRs preferably interact with Gα:GDP rather than Gα:GTP, the herein reported chemical shift assignments will be essential to probe this interaction with NMR methods and extract dynamical information linked to functionality.

Methods and experiments

The human $G\alpha_{i,1}$ gene (GenBank accession no. BC026326, residues 1-354, molecular weight 40.4kDa) was cloned into an in-house-modified pQE30 vector (Qiagen) using PCR methods. The obtained DNA construct encodes for an N-terminal His_6-tag followed by GB1 (B1 domain of protein G), a tobacco-etch-virus (TEV) protease cleavage site, and for $G\alpha_{i,1}$ protein. This construct was used to generate another construct, $G\alpha_{i,1}\Delta31$, where the first 31 amino acids were deleted using PCR methods. Gαi1Δ31 was generated for NMR optimization and was shown to have similar nucleotide binding behavior. The final plasmid was used to transform *E. coli* NEB*express* cells (NEB) for protein expression. The bacteria were grown at 37 °C until an OD_{600} of 0.6 was reached, then the temperature was lowered to 20°C before the cells were induced with 100 μM IPTG for protein production. After

additional 20 h of expression at 20 °C, the cells were harvested via centrifugation at 6,000 × g for 15 minutes at 10 °C and stored at -80 °C until further usage.

For the production of isotope labeled $G\alpha_{i,1}\Delta31$ for triple-resonance assignment experiments, bacteria were grown in D_2O M9 minimal medium (Green and Sambrook 2012) containing 1 g/L ^{15}N-ammonium chloride ($^{15}NH_4Cl$) as well as 2g/L *U*-^{2}H,^{13}C glucose as sole carbon source, as described previously (Goricanec et al. 2016). For stereospecific pro-*S* Leu/Val labeling, 300 mg/L ethyl 2-hydroxy 2-^{13}C-methyl 3-oxobutanoate (Gans et al. 2010; Plevin et al. 2011), for Ile-δ1 labeling, 80 mg/L α -ketobutyrate (Goto et al. 1999) and for Alanine methyl group labeling, 0.8 g/L 3-[$^{13}CH_3$]-2-D-Ala together with 2.5 g/L d_4-succinate (Ayala et al. 2009) was added one hour before induction of protein production in M9 medium consisting of 99% D_2O and supplemented with 2 g/L ^{2}H,^{12}C Glucose and 1 g/L$^{15}NH_4Cl$. This recipe yielded ^{2}H,^{15}N $G\alpha_{i,1}\Delta31$ that is specifically $^{13}CH_3$-labeled at Ile-δ1, Val-γ2, Leu-δ2 and Ala-β in a fully deuterated and ^{12}C background. All stable isotopes were obtained from Sigma-Aldrich or Eurisotop (Saarbrücken, Germany).

Purification was done according to previous protocols (Greentree and Linder 2004) that have been modified for $G\alpha_{i,1}$ recently (Goricanec et al. 2016). After thawing the frozen pellets, the harvested cells were homogenized in 50 mM NaPi, pH 8.0, 200 mM NaCl, 10 mM β-mercaptoethanol, 1% Triton X-100, and 10% glycerol (buffer A) plus EDTA-free protease inhibitor (Roche). Following the homogenization, the cells were lysed by lysozyme treatment and sonication. After the cells were broken, the lysate was treated with DN*ase* and then centrifuged at 50,000 × g at 4 °C for 45 min. The obtained cell lysate was then applied to a Ni-NTA column equilibrated with buffer A. Subsequently, the Ni-NTA column was washed with buffer A supplemented with 0.5% Triton X-100 and then with buffer A containing 10 mM imidazole without Triton X-100. Finally, the protein was eluted from the Ni-NTA column with buffer B (50 mM NaPi, pH 8.0, 200 mM NaCl, 10 mM β-mercaptoethanol, 10% glycerol, 300 mM imidazole, 20 μM GDP, 40 μM $MgCl_2$). The elution fraction was dialyzed against 20 mM NaPi, pH 7.5, 50 mM NaCl, 3 mM DTT, 0.5 mM EDTA, 10% glycerol over night at 4 °C in a 6-8 kD MWCO dialysis tube (SpectraPor). After dialysis, the protein was digested with TEV protease (1 A_{280} TEV for 100 A_{280} protein) for 36 h. Following TEV digestion, buffer was exchanged to 20 mM NaPi, pH 7.8, 200 mM NaCl, 5 mM β-mercaptoethanol, 5% glycerol by dialysis over night at 4 °C. This solution was then applied to a Ni-NTA column. The desired digested product is lacking a His_6-tag and is now in the flow-through and wash fractions. These fractions were pooled and concentrated with a 30 kDa MWCO centrifugal device (Millipore), and applied to a S75 Hi-Load 16/600 size-exclusion column (GE Healthcare) equilibrated with NMR buffer (20 mM NaPi pH 7.0, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT). $G\alpha_{i,1}\Delta31$ typically elutes from the column at a volume of 70 mL.

TRACT NMR experiments (Lee et al. 2006) have been used to estimate the rotational correlation time of Gαi1 constructs and in complex with different nucleotides using T2 relaxation times of up to 56 ms for the anti-TROSY and 96 ms for the TROSY line, respectively.

For NMR backbone resonance assignment, we used a *U*-^{2}H,^{13}C,^{15}N-labeled protein sample of 400µM G$\alpha_{i,1}\Delta$31 in NMR buffer (see above) supplemented with 5 mM GDP, 5% (v/v) D_2O and 0.01 % (w/v) NaN_3. NMR experiments were performed at 25 °C on a Bruker AvanceIII spectrometer operating at 600 or 750 MHz proton frequency and equipped with cryogenic probes.
In addition to a 2D-[^{15}N,^{1}H]-TROSY experiment, we recorded a series of 3D triple resonance experiments (Salzmann et al. 1998), consisting of HNCO, HN(CA)CO, HNCA, HN(CO)CA and HNCACB, as well as a 3D-^{15}N-edited-[^{1}H-^{1}H]-NOESY (200 ms mixing time) in a non-uniformly sampled (NUS) manner using 15-20% sampling density. NUS setup was facilitated by the recently published NUS schedule generator (Hyberts et al. 2012) that utilizes Poisson-gap sampling (Hyberts et al. 2010). For rapid spectra reconstruction we employed iterative soft thresholding (IST) (Hyberts et al. 2012).

Methyl group resonance assignment of Ile and Val/Leu was done by HCCACB experiments with a *U*-^{2}H,^{13}C,^{15}N-labeled sample containing specific protonation at Ile δ1, Val γ and Leu δ methyl sites, where the labeled methyl group can be correlated with the chemical shifts of the intraresidual Cα and Cβ atoms. Hβ chemical shifts of alanine residues were assigned with a ^{15}N-edited-[^{1}H-^{1}H]-NOESY experiment, where a strong NOE between the backbone amide and the methyl protons can be observed.

All NUS 3D spectra were processed with NMRpipe (Delaglio et al. 1995). All other spectra were processed with Topspin3.5 (Bruker Biospin). Data analysis was done with Sparky (Goddard and Kneller, UCSF).

Results

Sample optimization for NMR resonance assignment

In order to optimize our G-protein samples for subsequent multidimensional NMR experiments, we used TRACT experiments (Lee et al. 2006), where the relaxation rates for the TROSY (R_β) and the anti-TROSY (R_α) components are obtained and used for calculation of the rotational correlation time and the apparent molecular weight of the particle. These experiments have been run in a pseudo-2D manner where a series of 1D ^{1}H,^{15}N-filtered spectra are recorded with increasing relaxation time. The integral of a certain spectral region of the amide spectrum is then plotted against the relaxation time for curve fitting. The width of the integral region can be adapted to capture all signals, i.e. folded and unfolded residues (6.5-12 ppm) or only the well-dispersed folded spectral region (8.5-11 ppm) (**Fig. 1**). In the case of the larger spectral window the rotational correlation time is smaller due to a pronounced contribution of the unfolded residues that tumble much faster than the overall rotation of the protein. By comparing the calculated weight of different samples, one can estimate whether unfolded regions are present in the protein (**Fig. 1**). With this approach, we first investigated ^{15}N-labeled full-length apo G$\alpha_{i,1}$ and in complex with GDP or the non-hydrolyzable GTP analogue GMPPNP. These experiments show that Gαi1 has a fairly large content of unfolded

residues in each ligand-bound state or in the apo form. We used this information together with a crystal structure of Gαi1 in complex with GMPPNP (Coleman and Sprang 1999), where the first 31 amino acids could not be resolved, and constructed a truncated version of Gαi1 that lacks the first 31 amino acids ($G\alpha_{i,1}\Delta31$). This sample was investigated in the same manner as full length $G\alpha_{i,1}$ (**Fig. 1**). In this case, the calculated molecular weights are almost identical for apo $G\alpha_{i,1}\Delta31$ or in complex with GDP or GMPPNP, indicating that the first 31 amino acids are unfolded in solution. Thus, we chose to used truncated $G\alpha_{i,1}$ for our NMR resonance assignment experiments. We previously reported the assignments of $G\alpha_{i,1}\Delta31$ in the GTPγS-bound state (Goricanec et al. 2016). However, due to large chemical shift changes upon nucleotide exchange, a simple transfer of resonance assignments was not possible for backbone resonances. Also, an NMR assignment of $G\alpha_{i,3}$ (Mase et al. 2014) did not provide sufficient spectral similarity for a reliable transfer of resonance assignments. Thus, we here present the *de novo* backbone resonance assignments of the GDP-bound state.

NMR backbone resonance assignment of Gαi1Δ31 in complex with GDP

Using standard TROSY-based triple resonance experiments, we were able to assign 73% of all backbone amide resonances of non-proline residues in the protein (**Fig. 2a**). Carbon resonances (Cα, Cβ, CO) could be assigned to a similar degree. The chemical shift information for Cα and Cβ resonances could be used to estimate the secondary structure of $G\alpha_{i,1}\Delta31$ bound to GDP. A plot of the secondary chemical shift, where positive and negative values are indicative of α-helical or β-sheet secondary structure, respectively could be calculated from the assigned Cα and Cβ chemical shifts (**Fig. 2b**). As a comparison, the secondary structure elements as extracted from the crystal structure of $G\alpha_{i,1}$:GDP (Coleman & Sprang, 1998) are plotted as grey lines in Figure 2b. Despite regions where chemical shift information could not be obtained, we generally find a very good correlation between of the secondary structure in the crystal and derived from chemical shifts obtained by NMR. As described previously (Goricanec et al. 2016), in contrast to the complex with GTP, the intrinsic dynamics within $G\alpha_{i,1}$ in complex with GDP is markedly enhanced leading to line broadening and reduced signal intensity. These features lead to the disappearance of a large number of NMR signals in a 2D-[^{15}N,^{1}H]-TROSY spectrum (**Fig. 2a**). Some parts of structural elements that are located close to the nucleotide binding site are also not resolved in a crystal structure, highlighting the high level of dynamics in this state. Our NMR data provide a thorough picture of mobile sites in Gα that might be relevant for nucleotide exchange and/or binding to a GPCR. Amino acid residues that could not be detected are color coded in red in **Figure 2c**. Gα can be divided into two sub-domains, the Ras-like domain and the α-helical domain. In the GTP-bound state, the two domains are firmly docked against each other, wrapping around the nucleotide. In the GDP-bound state, the arrangement is less well defined, leading to chemical exchange processes that eventually cause line broadening. Consequently, we see that resonances that are located around the nucleotide binding site in the Ras-like domain are severely broadened (residues 36-41, 46-52, 197-202, 218-223. 244-250). Furthermore, signals

corresponding to residues located in the α-helical domain that are in direct transient contact with the Ras domain are broadened (residues 73-89, 127-131, 136-140, 154-157). These observed effects are consistent with a model where the two subdomains show high intrinsic flexibility as well as no fixed orientation relative to each other.

Side chain methyl ILVA resonance assignment of Gαi1Δ31 in complex with GDP

Next, we aimed at methyl group resonance assignments of $G\alpha_{i,1}\Delta31$:GDP with selectively methyl-labeled Ala-β, Ile-δ1, Leu-δ2 and Val-γ2 protein in a highly deuterated and ^{12}C background. We previously assigned the corresponding methyl resonances of $G\alpha_{i,1}\Delta31$ in complex with GTP-analogues (Goricanec et al. 2016). Due to minor chemical shift differences between the GTP and the GDP-bound form (**Fig. 3a**), we were able to transfer the assignments. For Ala-β resonances, we confirmed the ^{13}C chemical shift assignments in a 2D-[^{13}C,^{1}H]-HMQC spectrum by comparison with the ^{13}C chemical shift assignments obtained from a 3D-HNCACB experiment. Compared to GTP-bound $G\alpha_{i,1}\Delta31$ a subset of resonances is severely broadened in the GDP complex (labeled resonances in **Fig. 3a**), further highlighting the notion that Gα proteins are highly dynamic if not bound to GTP. Nonetheless, we were able to assign most resonances in the 2D-[^{13}C,^{1}H]-HMQC spectrum as shown in Figure 3b-c for the isoleucine δ1, alanine β and valine-γ/leucine-δ region of the spectrum. Using methyl resonances as probes for studying the interaction and dynamics of G-protein alone and in complex with a GPCR will be crucial to obtain data of sufficient quality, as demonstrated previously on this system (Goricanec et al. 2016).

The obtained backbone and methyl resonance assignments have been deposited at the BMRB data bank under accession code 27672.

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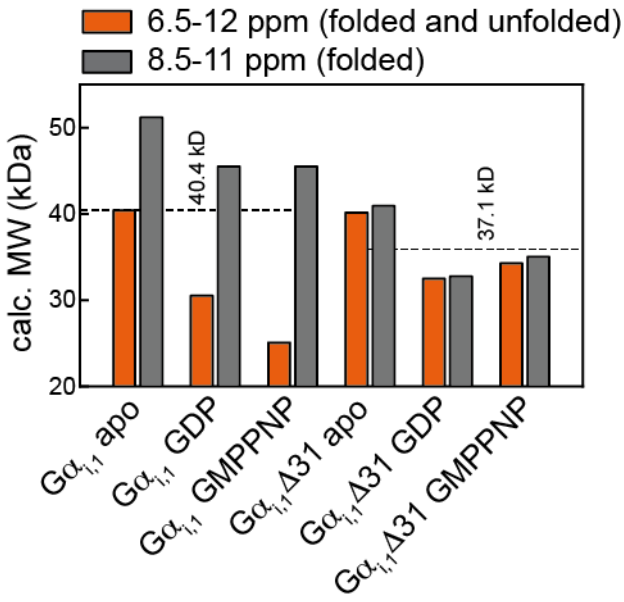
Figure legends

Fig. 1 Optimization of protein construct of $G\alpha_{i,1}$ by TRACT experiments (Lee et al. 2006). Calculated molecular weight using TRACT relaxation rates of $G\alpha_{i,1}$ or $G\alpha_{i,1}\Delta31$ in the apo form or in complex with GDP or GMPPNP, respectively. Proton amide signals have been either integrated in the full amide proton spectral range (6.5-12.5 ppm) or only in the non-random coil region (8.5-11 ppm). Only if both regions give rise to the same calculated molecular weight, no significant portion of unfolded random coil regions are present in the protein. The theoretical molecular weight of both constructs in indicated by broken lines. Figure adapted from Ref. (Goricanec et al. 2016)

Fig. 2 Backbone resonance assignment of $G\alpha_{i,1}\Delta31$ in the GDP-bound form. (a) 2D-[^{15}N,^{1}H]-TROSY spectrum of ^{2}H,^{13}C,^{15}N-labeled $G\alpha_{i,1}\Delta31$ with assigned resonances labeled with residue number. 73 % of all non-proline backbone amide amino acid resonances could be assigned. (b) Secondary chemical shift ($\Delta\delta^{C\alpha}$-$\Delta\delta^{C\beta}$) plotted against the $G\alpha_{i,1}\Delta31$ amino acid sequence. The secondary structure in the crystal structure of $G\alpha_{i,1}$ in complex with GDP (PDB ID: 1BOF) is indicated by a dotted line (negative: β-sheet; positive: α-helix) (c) Residues that are not visible in the NMR spectra are color-coded in red in the structure of $G\alpha_{i,1}\Delta31$:GDP.

Fig. 3 Methyl assignment of $G\alpha_{i,1}\Delta31$ in complex with GDP and comparison with the GTPγS-bound state. (a) 2D-[^{13}C,^{1}H]-HMQC spectra of $G\alpha_{i,1}\Delta31$ in complex with GDP (left) or GTPγS (right). Resonances that are not visible in the GDP-bound state are labeled. (b-d) Spectral regions in a 2D-[^{13}C,^{1}H]-HMQC spectrum for Ile-δ1, Ala-β and Val-γ2/Leu-δ2 methyl resonances with labeled assignments.

Figure 1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8.5-11 ppm (folded)
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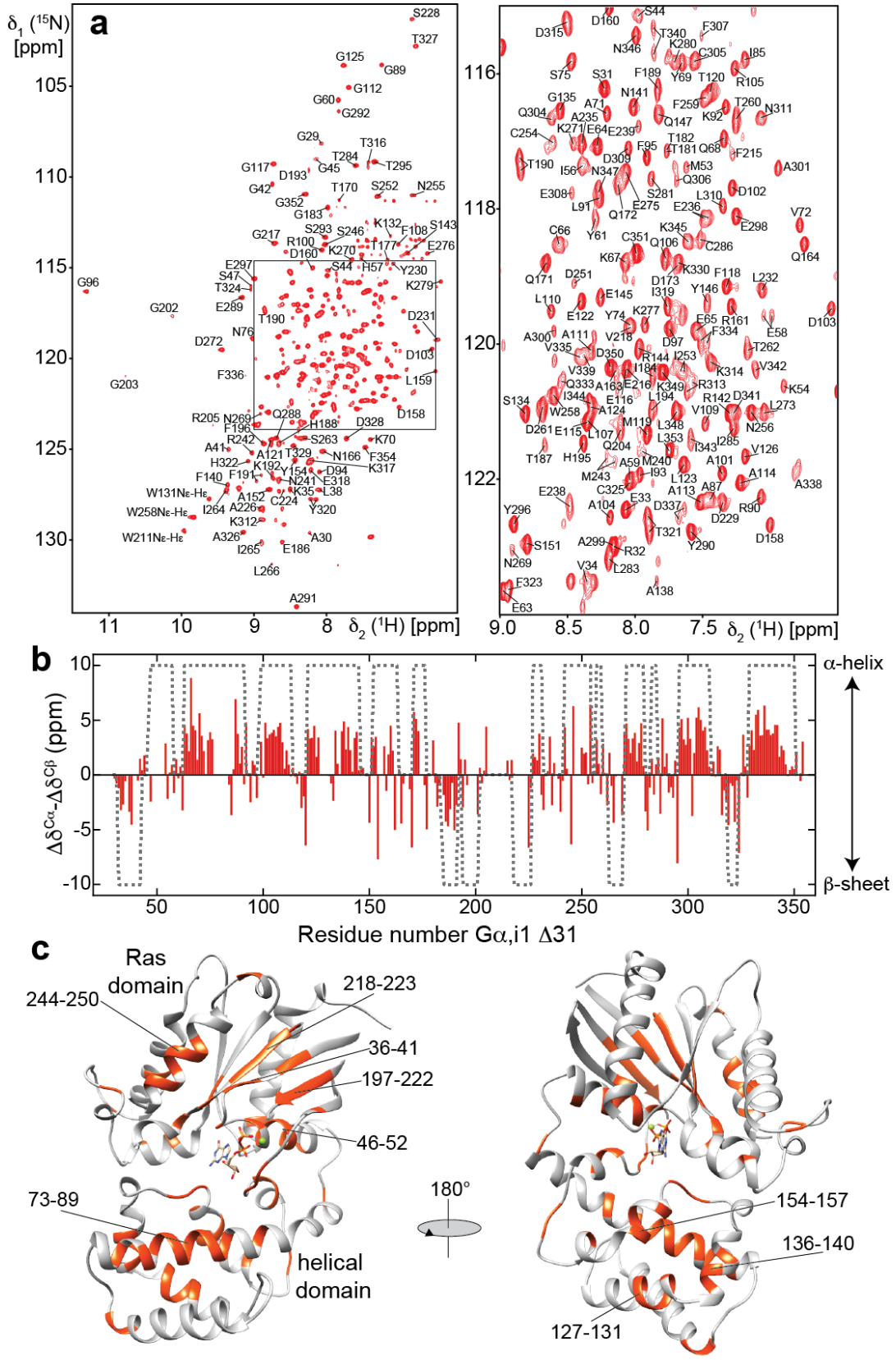
40
30
20
40.4 kD
37.1 kD
Gα i,1 apo
Gα i,1 GDP
Gα i,1 GMPPNP
Gα i,1 Δ31 apo
Gα i,1 Δ31 GDP
Gα i,1 Δ31 GMPPNP

Figure 2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δ1 (15N) [ppm]
δ2 (1H) [ppm]
b
ΔδCα-ΔδCβ (ppm)
α-helix
β-sheet
Residue number Gα,i1 Δ31
c
Ras domain
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197-222
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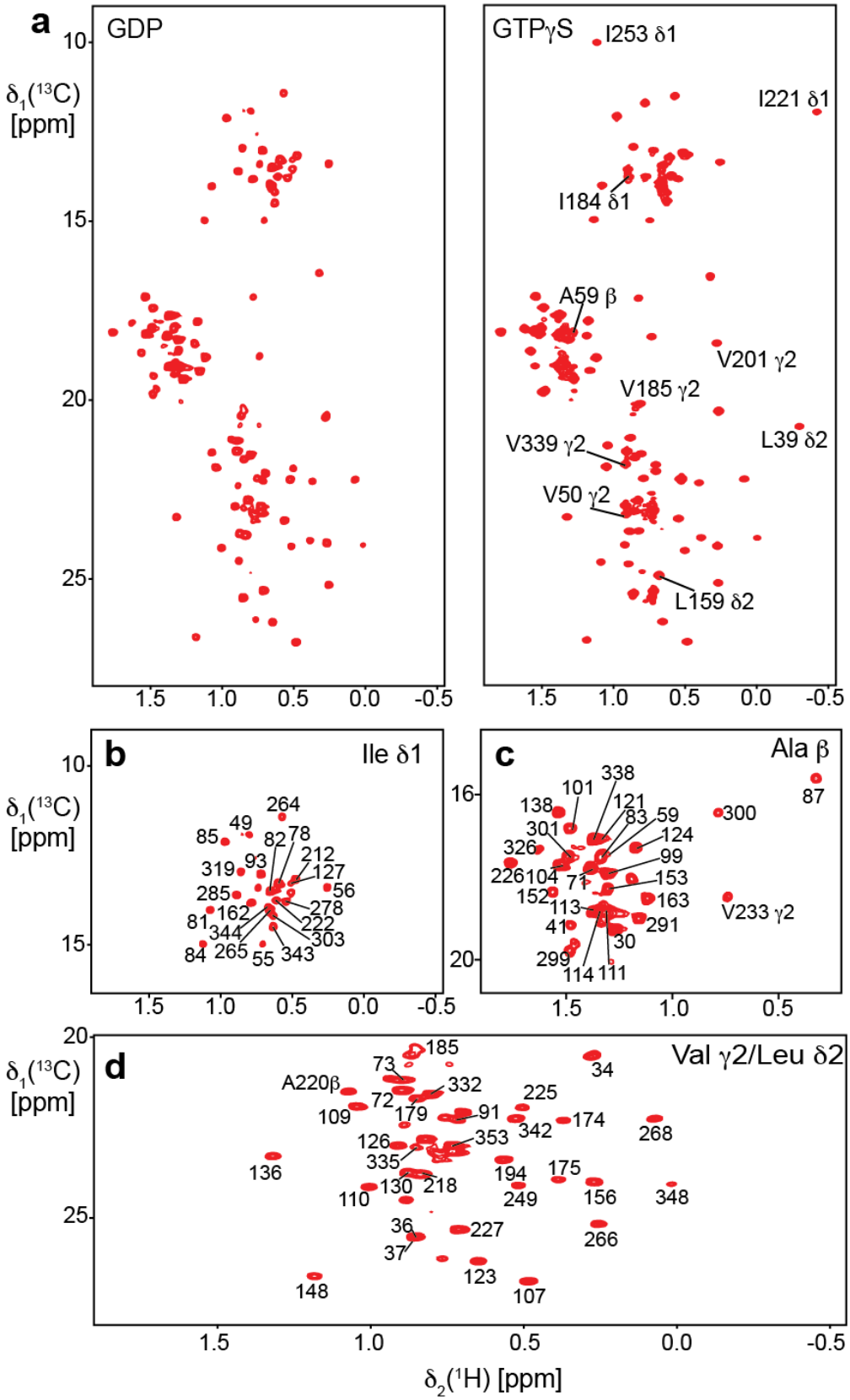
73-89
helical domain
180°
154-157
136-140
127-131

Figure 3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