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^1H , ^{13}C and ^{15}N backbone chemical shift assignments of camelid single-domain antibodies against active state μ -opioid receptor

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Abstract

Nanobodies are single chain antibodies that have become a highly valuable and versatile tool for biomolecular and therapeutic research. One application field is the stabilization of active states of flexible proteins, among which G-protein coupled receptors (GPCRs) represent a very important class of membrane proteins. Here we present the backbone and side-chain assignment of the ^1H , ^{13}C and ^{15}N resonances of Nb33 and Nb39, two nanobodies that recognize and stabilize the μ -opioid receptor (μOR) to opioids in its active agonist-bound conformation. In addition, we present a comparison of their secondary structures as derived from NMR chemical shifts.

Keywords

Camelid antibody; Nanobody; G protein Coupled Receptor

Biological context

Nanobodies are single domain antibodies produced in *Camelidae* (Hamers-Casterman et al. 1993). The single-chain domain architecture of the variable domain of the heavy-chain antibody (VHH) is composed of only three complementary determining regions (CDR) instead of the six CDR of the conventional antibodies. Despite being composed of a single VHH domain of small molecular size (12–15 kDa), nanobodies retain the same full antigen specificity and binding affinity as conventional antibodies with heavy and light chains. Moreover, because of their unique three-dimensional structure, nanobodies have access to cavities or clefts on the surface of proteins usually inaccessible to conventional antibodies (De Genst et al. 2006). Given the versatility, the high expression yield (Salema and Fernandez 2013) and the typically high thermal and chemical stabilities of nanobodies, they have become an attractive tool to identify and trap specific protein conformations, such as those involved in receptor mediated signaling, trafficking, and protein complex assembly. Furthermore, they are more and more developed as therapeutic tools, for example in cancer (Van Audenhove and Gettemans 2016).

Polytopic membrane proteins such as G protein-coupled receptors (GPCRs), transporters and channels are dynamic proteins that exist in an ensemble of functionally distinct conformational states (Deupi and Kobilka 2010). In particular, GPCRs represent the major class of cell surface receptors involved in signal transduction across the cell membrane. GPCRs are structurally dynamic molecules that can be stabilized in functionally distinct states by different ligands. Many GPCRs are known to signal through more than one G protein subtype, as well as through G protein independent pathways such as arrestins. Nanobodies can be evolved to bind to a diverse array of protein structures with high affinity and specificity, and are therefore logical candidates for stabilizing specific GPCR conformations.

Opioid receptors (OR), members of the GPCR superfamily, constitute the major and the most effective target for the treatment of pain (Huang et al. 2015; Melnikova 2010). Both beneficial and side effects are mediated by the activation of μ -opioid receptor (μ OR). To understand the μ OR activation mechanism, nanobodies have been generated to stabilize agonist-bound conformations. A phage display library of nanobodies was prepared from peripheral blood lymphocytes. μ OR-binding nanobodies were identified by selecting phages that bound liposome-reconstituted μ OR in the presence or absence of agonist. One family, which includes Nb33 and Nb39, contains nanobodies that bind to the intracellular surface of μ OR with different affinities and function as G-protein mimetics (Huang et al. 2015). Nb33 and Nb39 differ only by two amino acids, whose side chain does not interact directly with the G-protein interface of μ OR. The Nb39 was used to solve the crystal structure of the agonist-induced active state of μ OR bound to the morphinan agonist BU72, a very potent and highly efficacious agonist of μ OR (Huang et al. 2015; Neilan et al. 2004). Nb33 has a lower affinity towards μ OR but was used to study the conformational dynamics of μ OR in the presence of natural and synthetic agonists by solution-state NMR (Sounier et al. 2015).

In order to obtain further insights into the mechanism by which Nb33 and Nb39 stabilize the μ OR active state, although with different affinities, we have undertaken a detailed elucidation of both μ OR-Nb33 and μ OR-Nb39 structures and dynamics using solution state NMR spectroscopy. Here, we report the ^1H , ^{15}N and ^{13}C assignments of Nb33 and Nb39 backbone and $\text{C}\beta$ atoms with only two differing residues at position 19 and 69 but with significantly distinct HSQC spectra.

Methods and experiments

Cloning, expression and purification of Nb33 and Nb39

The DNA sequences of Nb33/Nb39 were cloned into a modified pMALp2x vector with the inclusion of the cleavage site for 3C protease. Nb33/Nb39 bearing the *E. coli* maltose binding protein (MBP) at the N-terminus and a His6-tag at the C-terminus (MBP-NB_{His6}) was expressed in *E. coli* BL21(DE3) cells using M9 minimal medium supplemented with 1 g.L⁻¹ $^{15}\text{NH}_4\text{Cl}$, 2 g.L⁻¹ ^{13}C -D-glucose at 37°C. Expression was induced by addition of 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) when an OD₆₀₀ 0.6 was reached. Cells were harvested after overnight growth at 20°C by centrifugation at 6,000 g for 30 min. Cells were resuspended in 20 mM HEPES buffer (pH 7.5), 500 mM NaCl, 0.1 mg.mL⁻¹ lysozyme and PMSF was added as a protease inhibitor before lysis by sonication. The cell lysate was

centrifuged at 18,000 rpm for 30 min at 4 °C. The soluble fraction was isolated and was supplemented with imidazole to a final concentration of 20 mM. MBP-Nb_{His6} was purified using a Ni-NTA resin. Subsequently, MBP-Nb_{His6} was washed with 20 mM HEPES buffer (pH 7.5), 100 mM NaCl (HN1 buffer). MBP-Nb_{His6} was eluted with HN1 buffer containing 250 mM imidazole. The eluted protein was cleaved by the addition (1:50) of 3C protease with an overnight incubation period at 4 °C. The digested sample was dialyzed for 2 h against 1 L of HN1 buffer. Cleaved MBP was separated from Nb33/Nb39 by additional amylose purification and size exclusion chromatography with Superdex-200 (GE healthcare). Selected fractions of high purity and protein content were then concentrated in a 3.5-kDa centrifugal filter unit. The sample protein concentration was approximately 1.2 mM (Nb33) and 1.3 mM (Nb39), in 20 mM HEPES, 40 mM NaCl, pH 6.8, 10% D₂O, 0.01% maltose-neopentyl glycol (MNG) and 0.001% cholesteryl hemisuccinate (CHS). The latter additives were added to enable future monitoring of the interaction with the μ -opioid receptor.

NMR spectroscopy

All NMR experiments were performed at 298 K on a Bruker Avance III spectrometer (Bruker, Rheinstetten, Germany) operating at ¹H frequency of 699.97 MHz, using a 5 mm cryogenic H/C/N/D probe with Z-axis gradient. ¹H chemical shifts were referenced with respect to DSS while ¹⁵N and ¹³C chemical shifts were calibrated indirectly from the absolute frequency ratios of ¹⁵N/¹H = 0.101329118 and ¹³C/¹H = 0.251449530 (Wishart et al. 1995) following the IUPAC conventions (Markley et al. 1998). Gifa (Pons et al. 1996) and NMRPipe (Delaglio et al. 1995) were used for NMR data processing and CCPNMR for subsequent analysis (Vranken et al. 2005). For sequential backbone assignment of Nb33 and Nb39, we used the following 3D experiments HNCO, HNCA, HN(CO)CA, CBCA(CO)NH. The assignment of the remaining aliphatic and aromatic resonances was accomplished using combinations of 3D HBHA(CO)NH, 3D ¹H-¹⁵N TOCSY-HSQC, 3D ¹H-¹⁵N-NOESY-HSQC (100 ms mixing time), 3D (H)CCH-TOCSY (10.9 mixing time) and ¹³C-¹H-NOESY-HSQC (100 ms mixing time) as well as ¹H-¹³C HSQC.

Extent of assignments and data deposition

The Nb33 and Nb39 proteins are both 132 amino acid long (N-terminal extra-residues included), with only two differing residues, namely R19^{Nb33} and L19^{Nb39} on one side, and I69^{Nb33} and T69^{Nb39} on the other side. However, their ¹H-¹⁵N HSQC fingerprint spectra are significantly different (Fig. 1), and resonance assignment required the recording of a full set of 3D experiment as well as their thorough step-by-step analysis for both proteins. We have assigned 98% of the expected backbone ¹H-¹⁵N correlations (119 out of 122 non-proline residues) for both nanobodies. In addition, 98% (94%) of H^α, 98% (99%) of C', 98% (97%) of C^α and 98% (91%) of C^β resonances have been assigned for Nb33 (Nb39). Additionally for both nanobodies, all the asparagine and the glutamine NH₂ side-chain resonances and the tryptophan side-chain ¹H-¹⁵N correlations have been assigned.

The missing residues are G₄₁, S₁₀₅ and S₁₂₃ amide pairs for both proteins. S₁₀₅ and S₁₂₃ are included in serine stretches, ¹⁰³QSSSP and ¹²¹VSSL respectively, making their assignment

more difficult than resonances belonging to other residues. G41 is part of a solvent exposed loop region based on the structure of Nb39 in complex with μ -opioid receptor (PDB 5C1M). Three resonances displayed chemical shifts shifted toward the high fields and differing more than 1.5 times the standard deviation, namely W35-H^{el}, W52-H^{el} and W113-H^{el}. By looking at the crystal structure of Nb39, we observed that the proximity of the aromatic ring of Y31 could explain the pronounced upfield shift of the side-chain indole of W52 at 8.64 ppm. Furthermore, W113-H^{al} is accessible to the solvent and in close proximity to F36 and Y110 aromatic rings that would explain a less pronounced upfield shift at 9.18 ppm. Moreover, the W35 side-chain HN is packed in the hydrophobic core of the protein that would explain prominent upfield shift at 7.73 ppm.

Both domains appear to have the same overall fold (Fig. 2), with however a significantly different ¹H-¹⁵N HSQC spectra. As can be seen from Fig. 2, major chemical shift changes are observed at the mutation sites (residues 19 and 69) as expected, but are also propagated to more distant regions, with major impacts centered around the 10th and 80th residues. These segments correspond to beta strands paired with the beta strands containing the mutations.

The assignments of Nb33 and Nb39 have been deposited in the BMRB under the accession number 26936 and 26937, respectively.

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Fig. 1. Amide resonance assignments of Nb39 and Nb33

2D [^{15}N - ^1H]-HSQC spectra of U -[^{15}N , ^{13}C]-Nb39 (a) and U -[^{15}N , ^{13}C]-Nb33 (b) recorded on a Bruker Avance III 700 MHz spectrometer with cryogenic probe at 25 °C. Sequence specific resonance assignments are indicated by number for the backbone amide atoms and the one-letter amino acid code. Pairs of side-chain NH_2 resonances are connected by *horizontal lines* and aliased side-chain resonances are *boxed*. The three tryptophan side chain indole peaks (We_1) are also indicated.

Fig. 2. The Secondary structure propensities and the backbone ^1H , ^{15}N chemical shift difference between Nb39 and Nb33

The chemical shifts of H^α , C^α , C^β and C' of Nb39 (a) and Nb33 (b) were used to calculate the secondary structure propensity using the program TALOS-N (Shen and Bax 2013). Positive and negative values of RCI indicate higher β -sheet and α -helical propensities, respectively. (c) Bar plots showing the combined chemical shift difference calculated as

$$\Delta\delta_{\text{NH}}^{\text{Nb33-Nb39}} = \sqrt{\frac{1}{2} \left(\delta_{\text{H}}^{\text{Nb33}} - \delta_{\text{H}}^{\text{Nb39}} \right)^2 + 0.15 \times \left(\delta_{\text{N}}^{\text{Nb33}} - \delta_{\text{N}}^{\text{Nb39}} \right)^2}$$
 and plotted as a function of the protein sequence. The two differing residues at position 19 and 69 are indicated by vertical dash lines. On top of each panel, the secondary structure of Nb39 in complex with μ -opioid receptor is shown. Residues that are located in the β -strands and α -helix are shown with white arrows and black rectangles. The disulfide bond between C23 and C95 is indicated by a black line at the top of the figure. The locations of the complementarity determining regions (CDR1, CDR2 and CDR3) are indicated by black zigzag rounded lines. Proline residues are indicated by asterisk.