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Backbone ^1H , ^{13}C , and ^{15}N resonance assignments of deubiquitinase A in non-phosphorylated and phosphorylated forms

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Abstract

Ubiquitination is one of the most prevalent forms of post-translational modifications that are important for regulating many cellular processes in eukaryotes. Deubiquitinases are proteases that hydrolyze the isopeptide or peptide bonds formed between ubiquitin and the target proteins or within a polyubiquitin chain. Deubiquitinase A (DUBA) is a deubiquitinase known to be a negative regulator of innate immune responses in humans by suppressing production of type I interferons (INF-I). Excess INF-I production has been associated with autoimmune diseases. Phosphorylation of a single serine residue at position 177 is essential for the protease activity of DUBA. The structural and mechanistic basis of DUBA activation by phosphorylation and substrate specificity is not well understood. Here, we report the backbone resonance assignments of the isoform 2 of DUBA in both non-phosphorylated and phosphorylated forms. The reported assignments form the basis for future NMR studies on the structural and dynamical properties of both active and inactive forms of DUBA.

Keywords

deubiquitinase; phosphorylation; enzyme activation; substrate specificity

Biological context

Conjugation of ubiquitin, a 76-amino acid protein, and ubiquitin-like molecules to target proteins, namely ubiquitination, is an important form of post-translational modifications (PTMs) that regulates numerous cellular processes in eukaryotes, such as protein degradation, cell signaling, DNA damage response, intracellular trafficking and immune responses (Komander and Rape 2012; Streich and Lima 2014). A cascade of enzymes, ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3), are responsible for attachment of monoubiquitin or polyubiquitin chains to the lysines of the target protein, whereas deubiquitinases (DUBs) execute the opposing function of removing ubiquitin modifications. Malfunction and deregulation of DUBs are implicated in a wide range of human diseases, including cancer (Pinto-Fernandez and Kessler 2016),

neurodegenerative disorders (Todi and Paulson 2011) and inflammatory diseases (Lopez-Castejon and Edelmann 2016).

DUBs are proteases that hydrolyze the isopeptide bonds formed between ubiquitin C-terminus and the lysines of target proteins or between single ubiquitins within a polyubiquitin chain. Deubiquitinase A (DUBA), also named OTUD5 (EC 3.4.19.12), is a cysteine protease that belongs to the ovarian tumor (OTU) subfamily of DUBs (Clague et al. 2013). The protease activity of DUBA requires phosphorylation of at least one serine residue at position 177 (Huang et al. 2012). DUBA and several other DUBs, including A20 and CYLD, participate in immune responses (Sun 2008). DUBA was initially identified as a negative regulator of type I interferon (INF-I) production by cleaving the K63-linked polyubiquitin chains attached to tumor necrosis factor receptor-associated factor 3 (TRAF3), which is an E3 ubiquitin ligase critical for INF-I production (Kayagaki et al. 2007). A new role of DUBA as a negative regulator of interleukin-17 production in T cells has recently been reported (Rutz et al. 2015).

In a polyubiquitin chain, individual ubiquitins are linked through isopeptide bonds formed between the C-terminal carboxylate of ubiquitin and the ϵ -amino group of one of the 7 lysines (K6, K11, K27, K29, K33, K48, K63) or the α -amino group of the N-terminal methionine. *In vitro* enzyme activity assays indicate that DUBA shows robust activity towards polyubiquitin chains of K48 and K63 linkages (Mevisen et al. 2013), which are the two most well characterized linkage types in terms of their physiological functions. K48-linked polyubiquitin chains serve as signals for protein degradation (Hershko and Ciechanover 1998), whereas K63-linked chains are involved in non-degradative functions, such as activation of protein kinase cascades (Chen and Sun 2009). So far, no crystal structures of diubiquitin bound to DUBA are available and therefore the structural basis for ubiquitin chain linkage specificity is not known.

Protease activities of DUBs are spatially and temporally regulated (Sahtoe and Sixma 2015) because uncontrolled activation can lead to severe consequences, given the broad roles that DUBs play in key cellular processes. The catalytic activities of many DUBs are regulated by PTMs (Kessler and Edelmann 2011), which enables the cross talk between the ubiquitin system and other PTMs. For majority of the DUBs, the structural mechanisms by which the protease activities are modulated by PTMs, including phosphorylation, are not clear (Mevisen and Komander 2017). Previous NMR studies on the isoform 1 of DUBA showed that chemical shift perturbations induced by phosphorylation are confined to the residues near the phosphorylation site, indicating small differences in the ensemble-averaged structures of DUBA in the active (phosphorylated) and the inactive (non-phosphorylated) forms. It has been hypothesized that the catalytic turnover requires the opening and closing of DUBA structure (Mevisen and Komander 2017). Solution NMR techniques are powerful tools for characterizing functional dynamics of proteins on a wide range of time scales (Mittermaier and Kay 2006). To enable NMR characterization of the dynamical properties of DUBA, we assigned backbone resonances of isoform 2 of DUBA in both phosphorylated and non-phosphorylated forms. Previous work (Huang et al. 2012) reported only the $^1\text{H}^{\text{N}}$, ^{15}N , $^{13}\text{C}^{\alpha}$ resonances on isoform 1 of DUBA. Despite the small difference in amino acid sequence, the assignments of isoform 1 cannot be directly transferred to the isoform 2. In

this work, we prepared samples of deuterated DUBA, which display higher sensitivity and facilitate the detection of $^{13}\text{C}\beta$ resonances from residues undergoing chemical exchange.

Methods and experiments

Protein expression and purification

The construct contains the catalytic domain (residue 172–344) of DUBA with residues 304 – 308 missing in isoform 2 compared to isoform 1, an N-terminal His₆-tag and a TEV protease cleavage site. The plasmid, OTUD5, was a gift from Cheryl Arrowsmith (Addgene plasmid # 28270). *Escherichia coli* BL21 (DE3) cells transformed with the plasmid were grown in 5 mL LB medium at 37 °C to an OD₆₀₀ of 0.7. To produce [^2H , ^{13}C , ^{15}N]-labeled protein, the cells were transferred to 20 mL minimal M9/H₂O medium containing 1 g/L $^{15}\text{NH}_4\text{Cl}$ (Cambridge Isotope Laboratories; CIL) and 3 g/L $^{13}\text{C}_6$ -glucose (CIL) and grown at 37 °C to an OD₆₀₀ of 0.6. The cells were pelleted by centrifugation at 4000 rpm for 25 min and transferred to 100 mL M9/D₂O medium, which was prepared with 99 % D₂O (CIL) and contain 1 g/L of $^{15}\text{NH}_4\text{Cl}$ and 3 g/L $^{13}\text{C}_6$ glucose. At OD₆₀₀ of 0.6, the culture was diluted to 400 mL with M9/D₂O, grown to an OD₆₀₀ of 0.6, and then further diluted to a final volume of 1 L with M9/D₂O medium (Tugarinov et al. 2002). When OD₆₀₀ reached 0.7, the culture was cooled to 18 °C prior to induction. Protein expression was initiated with addition of 0.6 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) at OD₆₀₀ of 0.9 and lasted for 18 – 24 h at 18 °C. Cells were harvested by centrifugation at 6000 rpm for 30 min at 4 °C. [^2H , ^{15}N]-labeled protein was produced using the same set of protocols except that the medium was prepared with natural abundance glucose. ^{15}N -labeled protein was produced by preparing the medium with H₂O.

In order to purify DUBA for NMR studies, previously reported protocols (Huang et al. 2012) were slightly modified to ensure purity of resulting protein samples. Cells were re-suspended in the lysis buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 10 mM imidazole) supplemented with 1 mM 1,4-dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) (MilliporeSigma) and 125 U DNase I (Thermo Scientific). Cells were lysed by sonication on ice for 8 min. The cell debris were removed by centrifugation at 12000 rpm for 25 min at 4 °C. The supernatant was filtered with a 0.45- μm syringe filter and applied to a 5-ml HisTrap column (GE Healthcare) equilibrated with the wash buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM imidazole and 1 mM DTT). The column was washed with 100 ml wash buffer and the protein was eluted with the elution buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 300 mM imidazole and 1 mM DTT). His₆-tag was cleaved from the purified protein with 10 U of ProTEV plus (Promega) per mg DUBA, while being dialyzed against the buffer containing 50 mM Tris-HCl pH 8.0, 50 mM NaCl and 1 mM DTT at 4 °C for overnight. The cleaved DUBA was separated from the TEV protease, His₆-tag, and uncleaved DUBA with a 5-ml HisTrap column equilibrated with the wash buffer. The purified protein was dialyzed against the buffer containing 50 mM Tris-HCl, pH 8.5, 50 mM NaCl and 1 mM DTT. The dialyzed protein was further purified by the HiTrap Q HP column (GE Healthcare) pre-equilibrated with 50 mM Tris-HCl pH 8.5, 50 mM NaCl and 1 mM DTT. The protein was eluted by a 50 - 500 mM NaCl gradient. The purity of the protein was confirmed by SDS-PAGE and the position of the protein band is consistent with the

molecular weight of approximately 22 kDa, which was calculated from protein sequence with the substitution of the naturally most abundant isotopes with 100% ^2H , ^{13}C and ^{15}N . The protein concentration was determined by absorbance at 280 nm with the extinction coefficient $23,380 \text{ M}^{-1} \text{ cm}^{-1}$.

Preparation of phosphorylated DUBA

Phosphorylation reaction was carried out in the buffer containing 2 mM ATP in 20 mM Tris-HCl pH 7.5, 50 mM KCl, 0.1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) and 10 mM MgCl_2 , at room temperature for 24 h on a nutator. 350 U of casein kinase II (CKII) (New England Biolabs) was used for each milligram of DUBA. EGTA was included in the reaction mixture to chelate trace amount of Ca^{2+} , which may cause protein to precipitate (Peck 2006). At the end of the reaction, the reaction mixture was dialyzed against the buffer containing 50 mM Tris-HCl, pH 8.5, 50 mM NaCl and 1 mM DTT. Phosphorylated DUBA was separated from other components, including non-phosphorylated DUBA, on a HiTrap Q HP column with a 50 – 500 mM NaCl gradient. The masses of ^{15}N -labeled DUBA in both phosphorylated and non-phosphorylated have been confirmed by ESI mass spectrometry analysis (Fig. S1) and are 20047.6 Da and 19968.8 Da, respectively, which are consistent with phosphorylation at a single site. The expected molecular weight of non-phosphorylated DUBA is 19966.1 Da, which is in good agreement with experimentally measured mass. The purity of phosphorylated DUBA was confirmed by the ^1H - ^{15}N TROSY spectrum. Fig. S2 shows that cross-peak of unphosphorylated S177 is absent from the TROSY spectrum on the phosphorylated DUBA, indicating that the sample does not contain non-phosphorylated DUBA detectable by NMR. For all other detectable serines, the chemical shifts did not change upon phosphorylation, consistent with the expectation that S177 is the only site that has been phosphorylated.

NMR Spectroscopy

All NMR experiments for sequential assignment were performed on [^2H , ^{13}C , ^{15}N]-labeled DUBA samples (400 μM protein concentration). The sample buffer contains 50 mM sodium phosphate, pH 7.0, 100 mM NaCl, 1 mM Tris(2-carboxylethyl)phosphine (TCEP), 0.03% (w/v) NaN_3 , 90% H_2O /10% D_2O (v/v). In addition, ^1H - ^{15}N HSQC was acquired on a ^{15}N -labeled sample to ensure that missing cross-peaks are not due to incomplete back exchange in the deuterated samples.

All NMR data were acquired on a 700-MHz Varian spectrometer, equipped with a triple-resonance z-gradient cryoprobe. The sample temperature was calibrated using methanol-d4 (CIL) (Findeisen et al. 2007). TROSY-detected 3D HNCA, HNCO, HN(CO)CA, HNCACB, HN(CA)CO, HN(COCA)CB (Zhu et al. 2004) experiments were performed at 296.3 K on the non-phosphorylated DUBA sample. TROSY-detected 3D HNCA, HNCACB, HNCO, HN(CA)CO, together with 3D ^{15}N -edited NOESY-TROSY with 100 ms mixing time, were performed on the phosphorylated DUBA sample at 296.3 K. A subset of 3D experiments, including HNCACB and HNCO, were performed at 299.6 K to detect resonances broadened by chemical exchange. ^1H chemical shifts were referenced to 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) signal; ^{15}N and ^{13}C chemical shifts were indirectly referenced using the

gyromagnetic ratios. Data were processed using NMRPipe (Delaglio et al. 1995) and analyzed with SPARKY (Lee et al. 2015).

Results

DUBA contains a catalytic domain, the OTU domain, and a ubiquitin interaction motif (UIM) domain separated by a long and disordered linker. The phosphorylated OTU domain displays catalytic activity similar to the full length DUBA (Huang et al. 2012). In order to define the effects of phosphorylation on the structural and dynamical properties of DUBA and to understand the molecular mechanism of DUBA activation, we performed backbone chemical shift assignment on the OTU domain of the isoform 2 of DUBA. The OTU domain contains 163 non-proline residues. Fig. 1a shows the ^1H - ^{15}N TROSY spectrum of phosphorylated DUBA labeled with assigned residues. For both phosphorylated and non-phosphorylated DUBA samples, approximately 120 cross-peaks can be detected and the rest of the expected cross-peaks are missing due to line broadening induced by chemical exchange. Fig. S3 shows the overlay of the ^1H - ^{15}N HSQC spectrum of the ^{15}N -labeled DUBA and ^1H - ^{15}N TROSY spectrum of $[^2\text{H}, ^{15}\text{N}]$ -labeled DUBA. The similarity between the two spectra indicates that the missing resonances are not due to incomplete back exchange of deuterium to proton at the amide sites. 116 amide resonances can be site-specifically assigned, which account for 97% of all detectable amide resonances. For the phosphorylated form, 128 $^{13}\text{C}^\alpha$ and 112 $^{13}\text{C}^\beta$ resonances have been assigned; for the non-phosphorylated form, 126 $^{13}\text{C}^\alpha$ and 114 $^{13}\text{C}^\beta$ resonances have been assigned. The backbone ^1H , ^{13}C , ^{15}N chemical shifts of assigned residues in both non-phosphorylated and phosphorylated DUBA have been deposited in the Biological Magnetic Resonance Bank (BMRB) (<http://www.bmrb.wisc.edu>) under accession codes 27494 and 27495, respectively. The residues with undetectable amide resonances are mainly located in the helix α_5 and helix α_6 . In addition, some residues in helix α_3 , helix α_4 , helix α_7 , the loop between β_4 and β_5 , strand β_5 (Fig. 1b) cannot be detected. The crystal structures of both free DUBA and DUBA covalently linked to ubiquitin aldehyde through the cysteine at the active site have previously been reported (Huang et al. 2012). The primary difference between the two crystal structures (PDB ID: 3PFY and 3TMP) is that helices α_1 and α_6 are only visible in the bound form, though α_1 can be detected in the TROSY spectra of free DUBA. Helix α_1 contains the single phosphorylation site, S177, and helix α_6 contains two positively charged residues (R272 and K273), which interact with the phosphate group on the phosphorylated S177.

Fig. 1c shows the overlaid ^1H - ^{15}N TROSY spectra of phosphorylated and non-phosphorylated DUBA, which display only minor differences. Consistent with previous NMR studies on the isoform 1 of DUBA (Huang et al. 2012), the chemical shifts of only a few residues near S177, including G174, Y175 and E178, are perturbed in the phosphorylated DUBA. The highly similar TROSY spectra suggest that DUBA is activated in the absence of significant structural changes. Besides the local chemical shift perturbations, at least eight residues located before and in the helix α_1 , and the loop (residues E189-D192) between helices α_1 and α_2 display two conformers that are both highly populated in the phosphorylated form (Fig. 1a inset and Fig. S4), whereas only one major conformer is observed for these residues in the non-phosphorylated form. Fig. 2

shows the predicted secondary structure of non-phosphorylated DUBA by TALOS-N (Shen and Bax 2013). The phosphorylated DUBA displays essentially the same predicted secondary structure, which is in good agreement with the crystal structures of both non-phosphorylated DUBA in free form (PDB ID: 3PFY) and phosphorylated DUBA covalently linked to ubiquitin aldehyde (PDB ID: 3TMP). The only observable difference in the secondary structures from NMR data and the crystal structure is that in the free form, helix α_1 starts from residue E180 rather than from E178 in the bound form, which is consistent with the expectation that phosphorylation sites are located in disordered regions (Iakoucheva et al. 2004). Further NMR studies on DUBA, including quantification of conformational dynamics, hold the promise of revealing the molecular mechanisms of DUBA activation and can shed light on the structural basis of substrate specificity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

a ^1H - ^{15}N TROSY spectrum of [^2H , ^{13}C , ^{15}N]-labeled and phosphorylated DUBA recorded at 296.3 K, with assigned amide resonances labeled. For residues showing two conformers, “u” and “d” denote the cross-peaks upfield and downfield shifted in the ^1H dimension, respectively. The resonances corresponding to arginine side chains (sc) are labeled with R-sc. The inset contains expanded views of a few residues showing two conformers. The TROSY spectra recorded at 296.3 K and 299.6 K were combined to yield optimal resolution for these cross-peaks. **b** Crystal structure of DUBA covalently linked to ubiquitin aldehyde (PDB ID: 3TMP). The residues with missing or unassigned amide resonances are highlighted in red. The catalytic triad and the phosphorylated S177 (pS177) are shown in stick representation. For clarity, the ubiquitin aldehyde is not shown. **c** Comparison of ^1H - ^{15}N TROSY spectra of phosphorylated DUBA (red) and non-phosphorylated DUBA (blue) recorded at 299.6 K. All spectra were recorded at 700 MHz ^1H frequency.

Fig. 2.

The secondary structure prediction of non-phosphorylated DUBA from $^1\text{H}^{\text{N}}$, ^{15}N , $^{13}\text{C}^{\alpha}$, $^{13}\text{C}^{\beta}$ chemical shifts, using TALOS-N (Shen and Bax 2013). The red bars represent probability for the α -helix, and the green bars represent probability for the β -strand. The open bars represent residues with unassigned or missing resonances, whose secondary structure was predicted from amino acid sequence by the default method implemented in TALOS-N. The secondary structure from the crystal structure (PDB ID: 3TMP) of phosphorylated DUBA covalently attached to ubiquitin aldehyde was annotated using POLYVIEW-2D web server (<http://polyview.cchmc.org>) (Porollo et al. 2004) and displayed above the plot for comparison. The construct that yielded the crystal structure contains residues 172 – 351 from the isoform 1 of DUBA (Huang et al. 2012).