Backbone ^{1}H, ^{15}N and ^{13}C resonance assignments for an E2 ubiquitin conjugating enzyme-UBE2T

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Abstract

Ubiquitin-conjugating enzyme E2 T (UBE2T) plays important roles in ubiquitination of proteins through participation in transferring ubiquitin to its substrate. Due to its importance in protein modifications, UBE2T associates with diverse diseases and serves as an important target for drug discovery and development. The crystal structure of UBE2T has been determined and the structure reveals the lack of a druggable pocket for binding to small molecules for clinical applications. Despite the challenge, effort has been made to develop UBE2T inhibitors. We obtained UBE2T constructs with and without the C-terminal region which is flexible in solution. Herein, we report the backbone resonance assignments for human UBE2T without the C-terminal region. The backbone dynamics of UBE2T was also explored. The available assignments will be helpful for hit identification, determining ligand binding site and understanding the mechanism of action of UBE2T inhibitors.

Keywords: UBE2T; NMR; resonance assignment; E2 ligase; protein dynamics

Biological Content

Ubiquitination is an important post-translational modification process and is catalysed by several enzymes including ubiquitin activating enzymes (UBE1), ubiquitin binding enzymes (UBE2) and ubiquitin ligating enzymes (UBE3) (Ma et al. 2023, Pickart 2001). Among these enzymes, UBE2 proteins are the central part of the reaction process, interacting with UBE1 and UBE3 enzymes to result in transferring activated ubiquitin to the substrates. The function or the location of proteins can be altered after ubiquitination. Therefore, protein ubiquitination is a tightly regulated process and associates with multiple cellular pathways such as cell cycle and signal transduction. Improper regulation of this process will result in diseases such as cancer (Popovic, Vucic and Dikic 2014).

The UBE2 superfamily contains approximately 40 members, and each member contains a highly conserved domain which is critical for catalysis and named as UBC (Ma et al. 2023). Accumulated studies have shown that UBE2s are linked to various cancers because of their association with DNA repair, apoptosis, cell cycle and oncogenic signalling. UBE2T is a member of the UBE2 superfamily and composed of 197 amino acids. It contains an N-terminal UBC domain critical for its activity and a C-terminal short region formed by approximately 40 residues which are not structured. Its conserved residue K91 near the catalytic site was found to be monoubiquitinated and may play a negative role in regulating UBE2. UBE2T is involved in Fanconi anaemia (FA) through regulating DNA repair (Zhang, Zhou and Huang 2007). In addition, it is identified to link with oncogenesis, metastasis, survival, and prognosis (Ma et al. 2023, Rickman et al. 2015).

UBE2T is shown to be related to diseases and considered as a drug target (Tao et al. 2023). Several inhibitors of UBE2T have been developed through different approaches, but no potent inhibitor for clinical applications is available (Cornwell et al. 2019, Morreale et al. 2017). Structure of the UBC region of UBE2T has been determined by X-ray crystallography and provides insights into the mechanisms of action for the available inhibitors (Morreale et al. 2017). NMR spectroscopy plays important roles in probing target and ligand interactions (Li and Kang 2017). Herein, we report the backbone resonance assignment for UBE2T without the C-terminal region. The available assignment

of the ^{1}H-^{15}N-HSQC spectrum will be useful for confirming the hits identified from other methods, mapping ligand binding sites, and understanding the binding modes of UBE2T inhibitors.

Methods and Experiments

Protein production

The cDNAs of full-length human UBE2T and a short construct containing amino acids 1-154 (UBE2T-UBC) were synthesized (Genscript) and cloned into pET15b, respectively (Kang et al. 2013, Li, Ng and Kang 2015a). To produce isotopically labelled proteins for NMR studies, the plasmids were transformed into *E. coli* BL21(DE3) competent cells and grown in minimal medium containing 1 g/L $^{15}NH_4Cl$ and 2 g/L ^{13}C-glucose (Li et al. 2022). After induction in the presence of 1 mM β-D-1-thiogalactopyranoside (IPTG) at 18 °C overnight, cells were collected by centrifugation at 10 °C and 9,000 ×g for 15 min. Both recombinant proteins were purified using immobilized metal affinity chromatography (IMAC) (Kang et al. 2015). Briefly, the suspended cells from one liter culture in a resuspension buffer containing 20 mM sodium phosphate, pH 7.8, 500 mM NaCl, and 1.0 mM β-mercaptoethanol were broken and the supernatant was mixed with 4 ml of Ni^{2+}-NTA resin. The resin was then washed with the resuspension buffer with 20 mM imidazole and eluted with a buffer that contained 500 mM imidazole, pH 6.5 and 1.0 mM β-mercaptoethanol. The proteins were further purified by size exclusion chromatography using a HiPrep 16/60 Sephacryl S-200 HR column in NMR buffer that contained 20 mM sodium phosphate, pH 6.5, 150 mM NaCl and 1 mM DTT.

NMR experiments for resonance assignment

Backbone resonance assignment was performed using a uniformly $^{15}N/^{13}C$-labeled sample in the NMR buffer (Li et al. 2015a). The experiments were carried out at 298K on a Bruker Avance II spectrometer with a proton frequency of 600MHz and equipped with a cryoprobe. The following spectra including 2D-^{1}H-^{15}N-HSQC, 3D-HNCA, 3D-HNCOCA, 3D-HNCACB, 3D-HNCOCACB, 3D-HBHACONH and 3D-HNCO were collected (Kim et al. 2014, Li et al. 2013). All the pulse sequences are from Bruker pulse library in Topspin (version 2.1). The collected data were processed with NMRPipe (Delaglio et al. 1995) and visualized using NMRView and NMRView java version (Johnson 2004). Secondary structural prediction

(SSP) was obtained using TALOS+ (Shen et al. 2009) using assigned chemical shifts. The experiments to obtain T_1, T_2 and ^{1}H-^{15}N steady-state NOE values (Kay, Torchia and Bax 1989) were collected at 298 K on a Bruker Avance 600 MHz spectrometer (Li et al. 2016, Li et al. 2015c, Li et al. 2015b). Steady-state ^{1}H-^{15}N NOEs (hetNOE) were calculated by analysing two datasets that were collected with and without initial proton saturation for a period of 3 s (Gayen et al. 2011). A pseudo 3D experiment with delays of 0.05, 0.1, 0.2, 0.4, 0.6, 1.0, 1.6, 2.4and 3.0 s were collected for obtaining T_1 values. A pseudo 3D experiment with delays of 0.017, 0.034, 0.051, 0.068, 0.085, 0.102, 0.119, 0.136 and 0.153 s was collected. The pseudo 3D data were spitted into 2D data sets with Topspin (version 2.1) and processed with NMRPipe (Delaglio et al. 1995). The T_1, T_2 and hetNOE values and errors were obtained with NMRView (Johnson 2004).

Extent of assignment and data deposition

To explore UBE2T and ligand interactions, both full-length UBE2T and its UBC region containing the first 154 amino acids were obtained. The ^{1}H-^{15}N-HSQC spectra of both constructs were obtained and the cross-peaks corresponding to the C-terminal region exhibited chemical shifts in the range of 7.5-8.5 ppm in the amide proton dimension (**Fig. 1A**). The narrow dispersion of the chemical shifts and high intensities of those cross-peaks suggest that the residues from the C-terminal region of UBE2T are not structured. The hetNOE experiment of the full-length UBE2T was collected, quite a few residues from the C-terminus exhibited cross-peaks with lower peak intensities or negative cross-peaks in the spectrum after 3s irradiation compared with those in the reference spectrum (**Fig. 1B**). We collected a few 3D heteronuclear NMR spectra for assigning the resonances of the full-length UBE2T, but the weak signals in the spectra made us focus on the shorter construct containing the UBC region.

Backbone resonance assignment of the UBC region of UBE2T was obtained based the 2D and 3D heteronuclear NMR experiments. About 92.7% cross-peaks of non-proline residues in the ^{1}H-^{15}N-HSQC spectrum were assigned except for residues including M1, Q2, G40, E47, K48, G49, V50, R99, T1147 and E148 (**Fig. 2A**). Approximately 84.0% of backbone resonances ($^{13}C\alpha$, and ^{13}CO) were

assigned. About 76.8% of Hα and 89.8% of Cβ chemical shifts were assigned. Due to the low quality of the 3D experiments for assigning resonances of side chains, only a few side chain resonance assignments were obtained.

The secondary structure of UBE2T containing the N-terminal 154 amino acids was obtained based on analysing the assigned backbone resonances using TALOS+ (**Fig. 3**). Similar to the secondary structures determined by X-ray crystallography, UBE2T UBC region in solution contains four α-helices and four β-strands. These helices include α1 formed by residues R3-T16, α2 formed by residues I104-S116, α3 formed by residues A126-Y134 and α4 formed by residues A138-H150 (**Fig. 3**). These β-strands include β1 formed by residues I22-Q26, β2 formed by residues L34-L39, β3 formed by residues V50-I56 and β4 formed by residues Q67-F70. With the assignment of the cross-peaks in the ^{1}H-^{15}N-HSQC spectrum, the ^{15}N relaxation parameters including longitudinal (T_1) and transverse (T_2) relaxation rates and steady-state nuclear Overhauser effect (hetNOE) were obtained (**Fig. 4**). Overall, UBE2T forms a rigid structure in solution because most residues have hetNOE values above 0.6. The last four residues at the C-terminus of the constructs are flexible because of the lower T_1 and hetNOE values and higher T_2 values observed.

We obtained the backbone resonance assignment of UBE2T and explored its dynamics in solution. The availability of these data will be useful for understanding its interaction with inhibitors. The availability of the assignment is also helpful for performing fragment screening and confirming hits identified using other methods. The assignments for the N-terminal 154 amino acid of UBE2T have been deposited in the Biological Magnetic Resonance Data Bank (BMRB) under accession code 52051.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agreed with the content

Availability of data and material

The assignments have been deposited to the BMRB under the accession code: 52051.

Competing interests

The authors declare no conflict of interest.

Authors' contributions

Q.H. and H.Q.N. carried out protein purification. Y.Y.L., Z.K., W.H. L. and C.K. designed the experiments. C.K. analysed data and drafted the manuscript. All authors approved the manuscript.

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Figure Legends

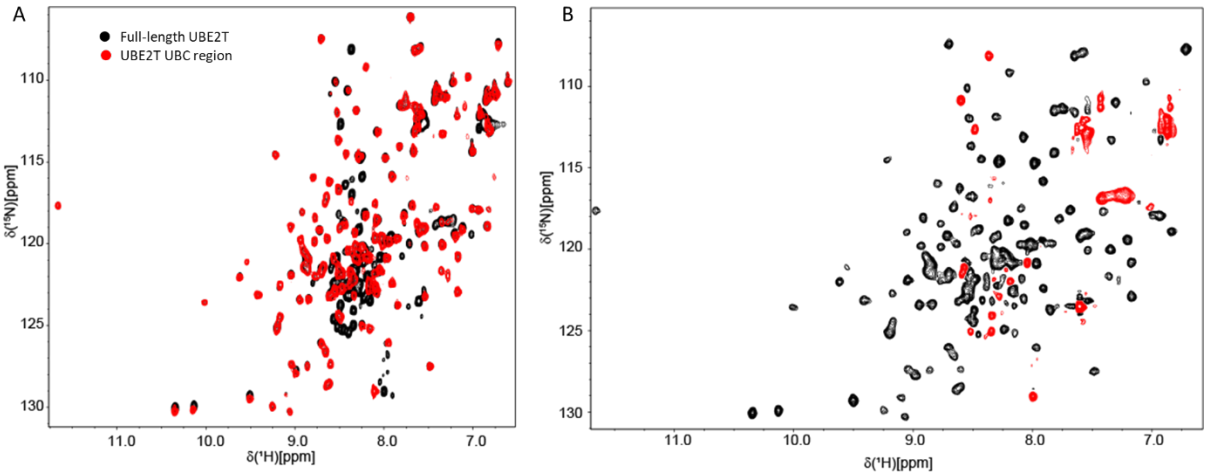

Figure 1. ^{1}H-^{15}N-HSQC spectra of full-length UBE2T and the N-terminal 154 amino acids of UBE2T (UBE2T UBC). A. Overlay of the ^{1}H-^{15}N-HSQC spectra of full-length UBE2T (black) and UBE2T UBC (red). B. The hetNOE spectrum of full-length UBE2T after 3 s proton saturation. The positive and negative cross-peaks are shown in black and red, respectively.

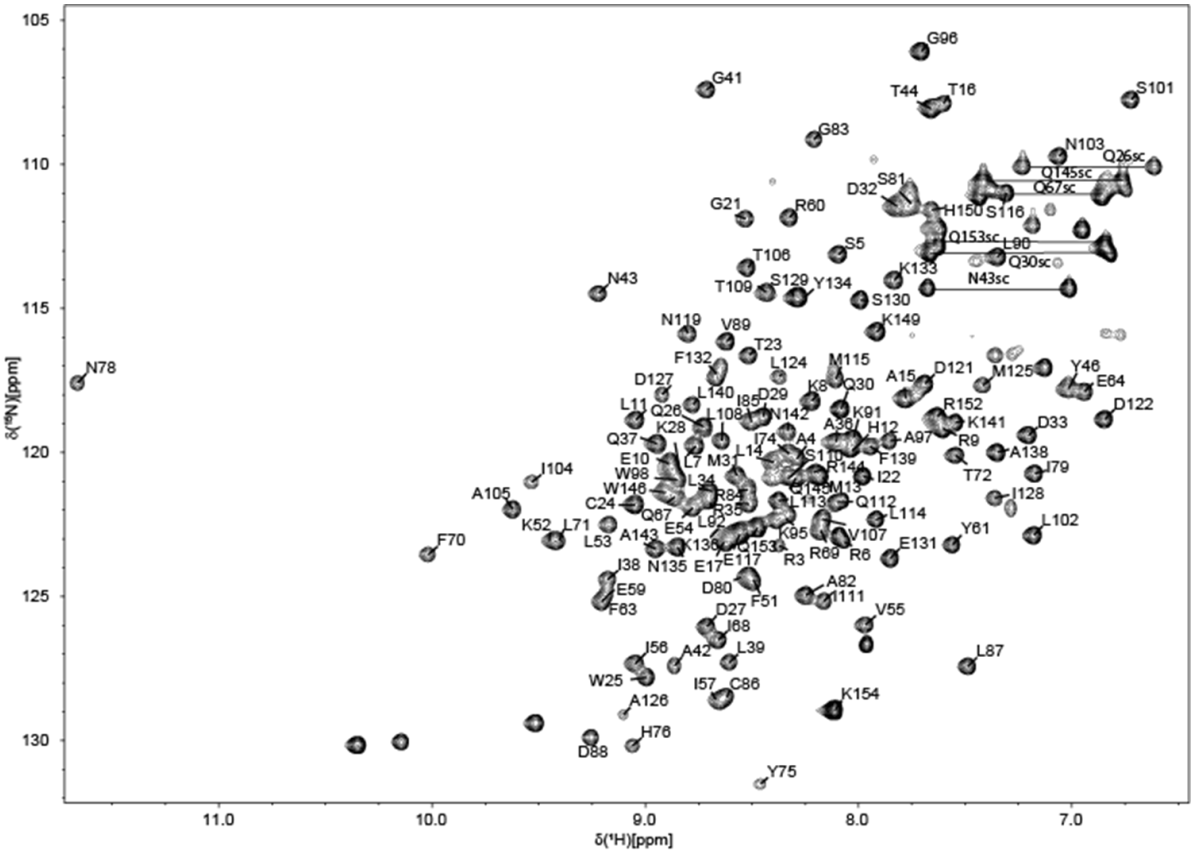

Figure 2. Assignment of UBE2T UBC region. Assignment of the ^{1}H-^{15}N-HSQC spectrum of UBE2T. The cross-peaks in the spectrum are labelled with residue name and sequence number. The spectrum was collected at 600 MHz at 25 °C.

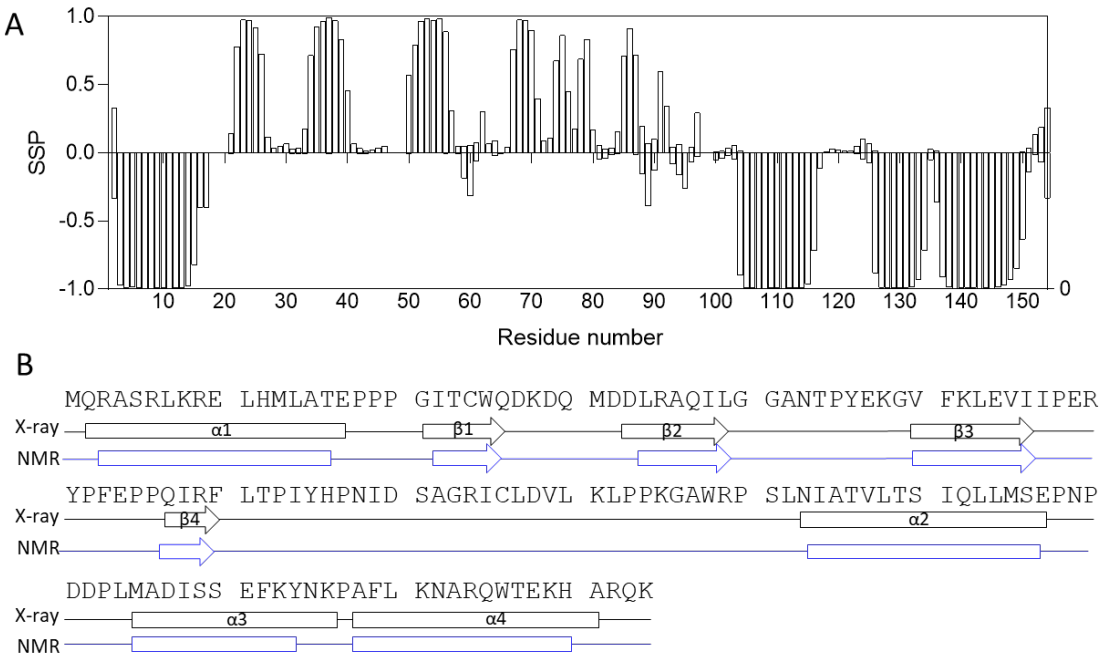

Figure 3. Secondary structure of UBE2T. A. Secondary structure of UBE2T based on NMR assignment. The secondary structures of UBE2T were analysed based on TALOS+ analysis. In the figure, positive SSPs correspond to beta-strands and the negative SSPs correspond to helices, respectively. B. Secondary structures of UBE2T derived from NMR (blue) and X-ray (black) studies. The structural elements of a crystal structure (PDB ID 5NGZ) are shown. The α-helices, β-strands and loops are indicated as boxes, arrows, and straight lines, respectively.

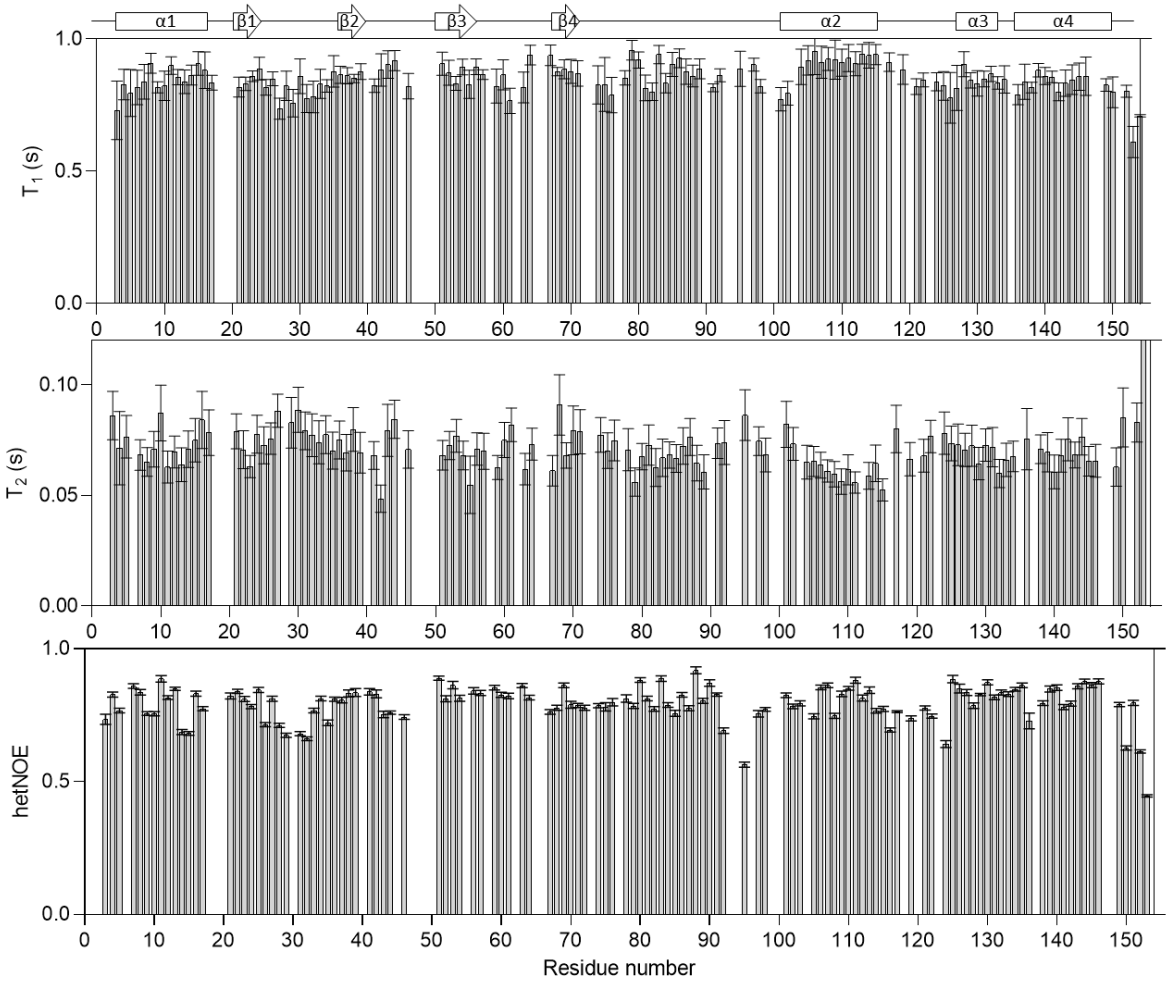

Figure 4. ^{15}N-T_1, T_2 and hetNOE analysis of UBD2T. The data were collected on a Bruker Avance 600 MHz spectrometer equipped with a cryo-probe at 298 K. ^{15}N-T_1, T_2 and hetNOE values of UBE2T are plotted against sequence number. The values of unassigned residues or peaks without a satisfactory curve fit are not shown.

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