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ARTICLE

Chemical shift assignments for *S. cerevisiae* Ubc13

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Abstract The ubiquitination pathway controls several human cellular processes, most notably protein degradation. Ubiquitin, a small signaling protein, is activated by the E1 activating enzyme, transferred to an E2 conjugating enzyme, and then attached to a target substrate through a process that can be facilitated by an E3 ligase enzyme. The enzymatic mechanism of ubiquitin transfer from the E2 conjugating enzyme onto substrate is not clear. The highly conserved HPN motif in E2 catalytic domains is generally thought to help stabilize an oxyanion intermediate formed during ubiquitin transfer. However recent work suggests this motif is instead involved in a structural, non-enzymatic role. As a platform to better understand the E2 catalyzed ubiquitin transfer mechanism, we determined the chemical shift assignments of *S. cerevisiae* E2 enzyme Ubc13.

Keywords Ubc13 · E2 · Ubiquitin transfer · Ubiquitination · NMR chemical shift assignments

Biological context

Eukaryotic cells use ubiquitination for a diverse set of actions including protein degradation, DNA transcription, DNA repair, and the inflammation response(Pickart and Eddins 2004; Schulman and Harper 2009; Berndsen and Wolberger 2014). Given this diverse cadre of roles, it is not surprising that the cellular machinery involved in the ubiquitination process is highly specialized. Ubiquitin must first be activated by an E1 enzyme (Uba1 in yeast) in an ATP-dependent manner (Schulman and Harper 2009). This results in ubiquitin being covalently linked to the E1 enzyme via a thioester bond. The E1 enzyme then transfers ubiquitin to a conserved catalytic cysteine within an E2 ubiquitin conjugating enzyme. E2 enzymes then either transfer ubiquitin to an E3 cysteine or directly ligate ubiquitin to a lysine side chain of the target substrate, with specificity often conferred by an E3 ligase enzyme (Wenzel et al. 2011; Berndsen and Wolberger 2014). The latter, simpler case is the pathway utilized by *S. cerevisiae* Ubc13 and provides a clearer model for posing mechanistic questions.

While the basic ubiquitin transfer pathway is well established, there is considerable uncertainty about both the enzymatic mechanism and the substrate specificity mechanism of ubiquitin transfer by E1, E2 and E3 enzymes. The catalytic domain of E2 enzymes are all very similar, with ubiquitin bound by a thioester linkage to the active site cysteine that is near a highly conserved His-Pro-Asn motif in the active site (Wenzel et al. 2011). Previously proposed mechanisms suggest that the asparagine within the HPN motif stabilizes an oxyanion intermediate during ubiquitin transfer from the E2 enzyme to the substrate lysine (Wu et al. 2003; Eddins et al. 2006). However, examination of multiple E2 enzyme structures reveal that the asparagine sidechain would have to break two hydrogen bond contacts with an active site loop and rotate almost 180 degrees to fulfill this role (Eddins et al. 2006). While such a conformational change is feasible, there is evidence to suggest that this asparagine instead plays a non-enzymatic role in E2 ubiquitin transfer (Berndsen et al. 2013).

Ubc13, an E2 enzyme found in *S. cerevisiae*, is an attractive model system to study this mechanism due to its high enzyme activity, small size, and ability to pass

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ubiquitin onto substrate without the use of E3 ligase (Wu et al. 2003; Eddins et al. 2006). Multiple structural and functional studies have used Ubc13 to assess the role of N79, the putative oxyanion-stabilizing residue, in E2 catalysis and structure (Wu et al. 2003; Eddins et al. 2006; Berndsen et al. 2013). Mutation studies of Ubc13, where more suitable oxyanion hole sidechains are substituted for N79, all result in significantly decreased activity which can be compensated all or in part by addition of the E3 ligase (Berndsen et al. 2013). Additionally, while the active site loop (residues 112 to 123) is well-defined in the wild-type Ubc13 crystal structure, this loop is completely disordered in an N79A mutant structure (Berndsen et al. 2013). Together these studies suggest the HPN motif does not directly contribute to catalysis, but instead acts to stabilize the critically important Ubc13 active site loop.

One way to probe how Ubc13 catalyzes ubiquitin transfer is to monitor the backbone dynamics of both the Ubc13 HPN motif and the active site loop. Movement of the N79 side chain toward the ubiquitin binding site would support a role for this side chain in stabilizing a reaction intermediate. Thus, information on the active site dynamics of Ubc13 will provide deeper insight into the mechanism of ubiquitin transfer. As a first step in coupling protein dynamics information with enzyme structure and mechanism, we present the NMR chemical shift assignments of Ubc13. These data will act as a starting place to examine how Ubc13 active site residues move during catalysis. A full description of such motions can then be used to more accurately model the enzymatic mechanism of ubiquitin transfer of Ubc13 in particular and E2 conjugating enzymes in general.

Methods and experiments

A sequence-verified gene encoding *S. cerevisiae* Ubc13 with a N-terminal TRX-His_6 tag and transformed into BL21(DE3) *Escherichia coli* cells (The TRX-Ubc13 plasmid was a generous gift of Dr. Cynthia Wolberger and Dr. Reuven Wiener). Plasmid-containing cells were grown 5 × 1L in either ^{15}N or ^{15}N, ^{13}C enriched MOPS minimal media (Wright et al. 2005, 2011) ($^{15}NH_4$ and ^{13}C glucose were purchased from Cambridge Isotopes, Andover, MA) to an OD600 of 0.6–0.8 at 37 °C and induced for 4 h at 37 °C with 100 μM IPTG. Cells were pelleted by centrifugation and the cell pellet stored at −20 °C. For purification, the cell pellet was resuspended in 50 mM phosphate and 300 mM NaCl, pH 8.0. After lysis via sonication and clarification by centrifugation, the resulting supernatant was applied over a Ni^{2+} affinity column and Ubc13 was eluted in lysis buffer with an additional 500 mM imidazole. The TRX-His_6 tag was subsequently cleaved by Tobacco Etching Virus (TEV) protease during dialysis into the original lysis buffer overnight at 4 °C. Uncleaved TRX-Ubc13 and TRX-His_6 were removed by passing the protein through a Ni^{2+} affinity column. After dialysis in 20 mM HEPES, 40 mM NaCl, 0.5 mM EDTA and 0.5 mM TCEP at pH 7.2, Ubc13 was further purified over a Q-column (GE Life Sciences). Ubc13 eluted near 600 mM NaCl, and purity was assessed via SDS-PAGE. Protein was dialyzed into 20 mM MES, 50 mM NaCl, and 0.5 mM TCEP at pH 6.50 and stored at −80 °C.

All NMR experiments were collected on a 600 MHz Bruker Avance II spectrometer equipped with a TXI room temperature 5 mm probe with *z*-axis pulse field gradient coils at 298°K. All data was processed using NMRPipe and analyzed using Sparky (Kneller and Kuntz 1993; Delaglio et al. 1995). A 300 μL aliquot of ^{15}N labeled Ubc13 (0.7 mM) was prepared with 10 % D_2O and 0.3 mM NaN_3 to collect a 2D ^{1}H, ^{15}N-HSQC spectrum. 300uL aliquots of ^{15}N, ^{13}C Ubc13 (1 mM) with 10 % D_2O and 0.3 mM NaN_3 were used to collect 3D ^{15}N-TOCSY-HSQC, 3D ^{15}N-NOESY-HSQC, 3D HNHA, 3D H(CCO)NH, 3D CC(CO)NH, 3D CBCA(CO)NH, 3D HNCACB, 3D HNCO, and 3D HN(CA)CO spectra. Backbone resonances were manually assigned using primarily HNCO, HN(CA)CO, HN(CA)CB, CBCA(CO)NH spectra. Side-chain resonance assignments were made using primarily ^{15}N-TOCSY-HSQC, 3D ^{15}N-NOESY-HSQC, 3D CC(CO)NH and H(CCO)NH spectra. Sample reproducibility and protein stability were assessed by through examination of an HSQC before each subsequent 3D experiment. New samples were prepared as necessary, usually after about 4 weeks of data collection. The ^{1}H chemical shifts were referenced to external DSS, the ^{13}C shifts were referenced indirectly to DSS using the frequency ratio $^{13}C/^{1}H = 0.251449527$ and ^{15}N shifts were referenced indirectly to liquid ammonia using $^{15}N/^{1}H = 0.101329118$.

Extent of assignments and data deposition

Ubc13 is 153 amino acids in length and contains 138 non-proline residues. Of these, 120 ^{1}H-^{15}N backbone resonances, all 3 sidechain tryptophans, and 9 of the 12 glutamine and asparagine NH_2 groups are present in the HSQC spectrum (Fig. 1a). All visible resonances on the HSQC were unambiguously assigned. Standard triple resonance assignment strategies provided assignments of 86 % of the ^{1}H and ^{15}N backbone atoms and over 90 % of the C_α, C_β and carbonyl resonances (Fig. 1b). The CC(CO)NH experiment was significantly weaker than other 3D experiments, resulting in assignments of only 58 % of the non-ring γ, δ, and ε sidechain carbons. All C_α and C_β and 64 % of the C_γ and C_δ proline carbons are assigned. 94 % of H_α, 43 % of H_β, and 18 % of H_γ protons

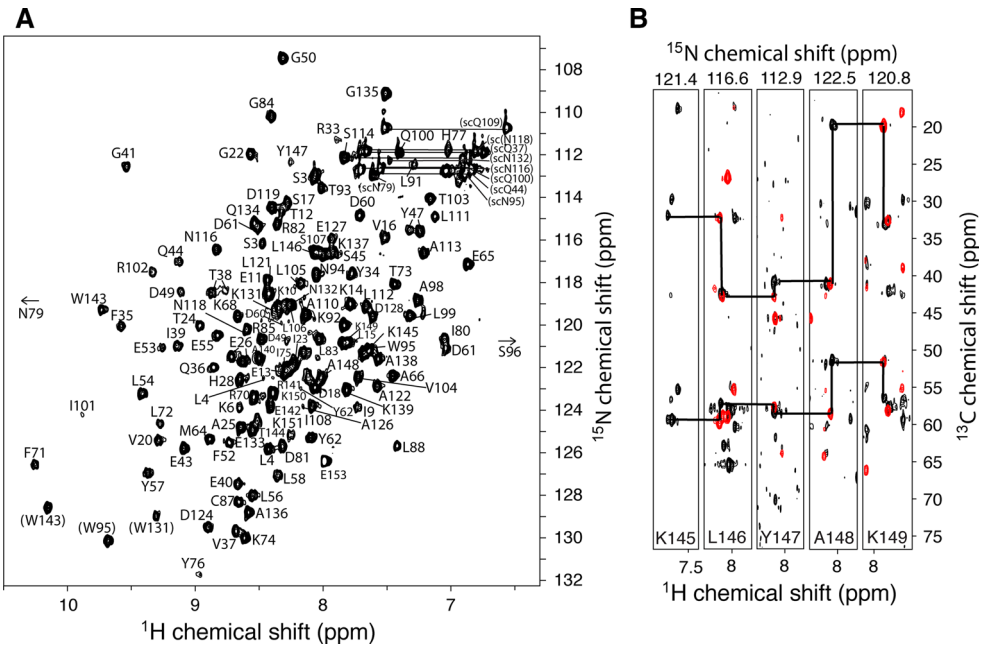

Fig. 1 **a** 2D ^{1}H-^{15}N HSQC collected at 600 MHz of Ubc13 at 298 °K with the backbone assignments included. Backbone ^{1}H-^{15}N correlations are labeled sequence-specifically, and correlations connected by horizontal lines correspond to glutamine and asparagine side-chain NH_2 groups. **b** Strips derived from ^{15}N planes of a 3D ^{15}N-edited CBCA(CO)NH (*in red*) and 3D ^{15}N-edited HNCACB (overlaid in *black*) illustrating through-bond connections of the C_α and C_β carbon atoms from K145 to K149

were assigned. The 18 non-proline residues for which there are no chemical shift assignments (residues 1–2, 7–10, 29, 32, 61–62, 86, 89–90, 123–125, 129–130) are all located either in the E1/E3 interaction site near the N-terminal helix, near a severe alpha helix bend within the C-terminal helix, or in loops or turns (VanDemark et al. 2001) (Fig. 2a). These amino acids are in regions that have elevated B-factors in the Ubc13 crystal structure and may be undergoing some amount of chemical exchange (VanDemark et al. 2001). Importantly, 79 % of the H, C, and N atoms in the catalytic region, the HPN loop, and the active site loop are assigned. Thus, a thorough description

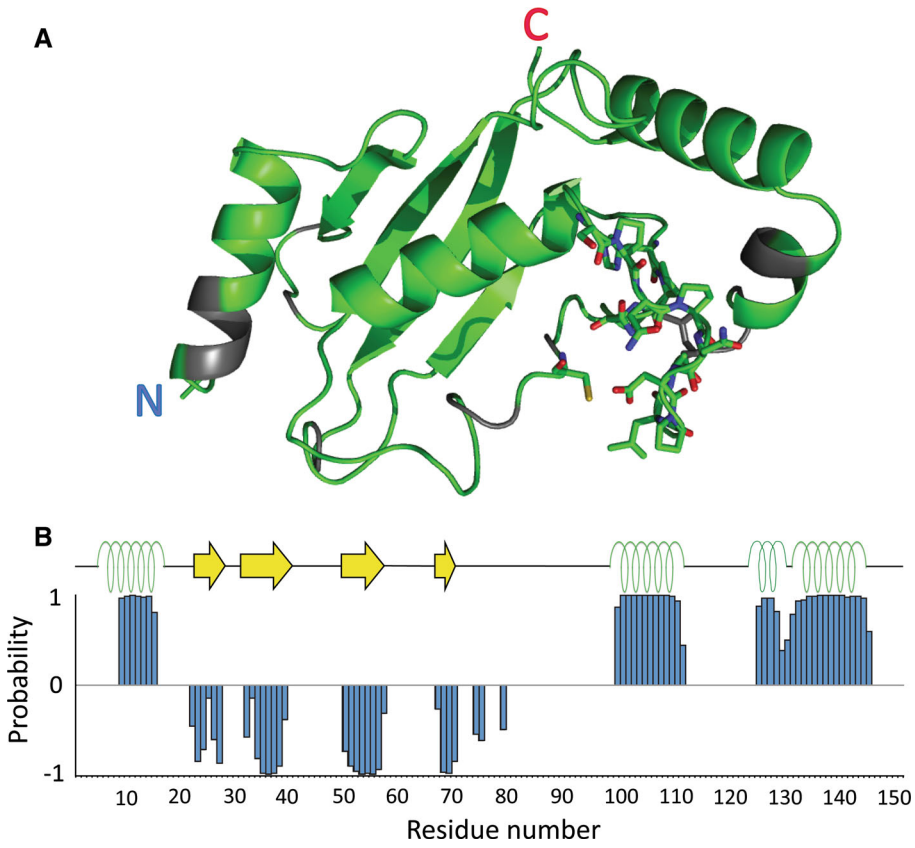

Fig. 2 **a** Extent of assignments mapped onto Ubc13 (pdb 1JBB). The active site cysteine, HPN loops, and active site loop are shown as sticks. *Gray* residues represent those residues not visible in the HSQC. **b** Comparison of secondary structure of the X-ray Ubc13 structure (*top*) with the TALOS+ predicted 2D structure from the assignments (*bottom*). Positive probability indicates the likelihood a residue is in an α-helix and negative probability the likelihood the residue is in a β-strand

of motions within the active site will be possible with these data. As expected, N79 displays the characteristic downfield shift common in E2 ubiquitin ligases (Cook and Shaw 2012). S96 is located proximal to the E1/E3 binding site and the active site. The chemical shift of this residue (near 6 ppm) is conserved in most E2 ubiquitin conjugating enzyme HSQCs (Houben et al. 2004; Ju et al. 2010; Cook and Shaw 2012). The structural reason for this shift in most E2 proteins is still under investigation. Secondary structure of Ubc13, predicted by TALOS+ using our NMR data, agrees with the crystal structure (Shen and Bax 2013) (Fig. 2b). The sequence-specific chemical shift assignments for Ubc13 have been deposited in the BioMagResBank (BMRB) under accession number 19877.

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