Journal of Biomolecular NMR, **18:** 363–364, 2000.
KLUWER/ESCOM

Letter to the Editor: Backbone resonance assignment of human UBC4

Neil A. Farrow[a,†,*], Sharon J. Archer[a,†], Zhongren J. Wu[a], Daniel M. Camac[a], Tom Parsons[b,**], Mark Rolfe[b,**] & Peter J. Domaille[a]
[a]*DuPont Pharmaceuticals Company, Experimental Station, Wilmington, DE 19880-0353, U.S.A.*
[b]*Mitotix, 1 Kendall Square, Building 600, Cambridge, MA 02319, U.S.A.*

Received 28 July 2000; Accepted 7 September 2000

Key words: heteronuclear NMR, resonance assignments, ubiquitination, UBC4

Biological context

Ubiquitin-mediated proteolysis plays an important regulatory role in many cellular processes. The covalent attachment of one or more ubiquitin molecules to lysine residues on a target protein results in the recognition of the ubiquitinated molecule by the 26S proteosome and its subsequent degradation. It is postulated that control of the rate of degradation of key cellular proteins permits the cell to respond to environmental changes in a rapid manner. Ubiquitination plays a role in numerous biochemical pathways, by controlling the degradation of key proteins, such as: the tumor suppressor protein p53; the cell cycle regulation proteins cyclin A, cyclin B and p27; the cystic fibrosis transmembrane conductance regulator; and the transcription factors c-jun, c-fos and IκB/NFκB (Laney and Hochstrasser, 1999).

Ubiquitination of the target protein is the final event in a pathway that involves the sequential transfer of ubiquitin between at least two other enzymes. The initial step of the signaling cascade, common to all target proteins, involves the ubiquitin activating enzyme E1. In an ATP-dependent step, a thioester bond is formed between the active-site cysteine on the surface of E1 and the C-terminal glycine residue of ubiquitin. Subsequent steps in the pathway require target-specific enzymes. In the second step of the ubiquitination cascade, activated ubiquitin is transferred from E1 to a specific cysteine residue on the appropriate ubiquitin conjugating enzyme, referred to as an E2. To date, many such E2s have been identified in humans, the sequences of which are highly conserved around the active-site cysteine, but which diverge in other regions of the protein. The final step in the ubiquitin signaling cascade involves the transfer of the ubiquitin from the E2 to the target protein. This step is accomplished by the action of a ubiquitin ligase, known as an E3. In some cases, the E3-mediated transfer of ubiquitin between the E2 and the target protein involves not only the E3 but also additional proteins that appear to play a role in mediating substrate recognition.

Many pathological conditions involve pathways that are in part regulated by the degradation of key proteins. For this reason, there has been considerable effort toward understanding the mechanisms by which specificity arises within the enzymes involved in the ubiquitination pathway. The multistep nature of the pathway suggests that in order for the appropriate protein to be ubiquitinated there must be a number of protein-protein recognition events – most notably between the E3 and the target protein but also between ubiquitin, the E2 and the E3 proteins.

The human protein UBC4 (hUBC4) was first identified by Rolfe and co-workers (Rolfe et al., 1995) as the E2 involved in the viral E6-dependent ubiquitination of p53. The structure of hUBC4 has not been determined but the primary sequence of the protein is 78% identical to that of *Saccharomyces cerevisiae* UBC4, the structure of which has been determined by X-ray crystallographic methods (Cook et al., 1993). The structure reveals the protein to be a slightly elongated mixed α, β protein with the active-site cysteine located in a shallow groove in the center of the molecule. In addition to *S. cerevisiae* UBC4, the tertiary structures of seven other E2 enzymes have been de-

*To whom correspondence should be addressed. E-mail: Neil.A.Farrow@dupontpharma.com
**Present address: Millennium Pharmaceuticals Inc., 75 Sidney Street, Cambridge, MA 02139, U.S.A.
†Contributed equally to the work reported here.

posited in the Protein Data Bank. Comparison of the structures suggests the class of enzymes is composed of a conserved global fold with specificity encoded in local variations between the structures.

Methods and results

^{13}C, ^{15}N-labeled hUBC4 (accession code: AAC41750) was expressed in *E. coli* BL21 (DE3) cells that had been transformed using a pT7 plasmid encoding for the hUBC4 protein. The cells were grown on a minimal media (MOPS) containing 1 g/l $^{15}NH_4SO_4$ and 2 g/l [^{13}C-6] glucose. The overexpression of hUBC4 was induced by the addition of 0.6 mM IPTG once the cells had grown to an OD_{600} of 0.6. The cells were harvested at an OD_{600} of 1.8 and were lysed using a French Press in a buffer containing: 100 mM sodium phosphate, 1 mM dithiothreitol, 1 mM EDTA, pH 6.0, with trace amounts of protease inhibitors provided by complete protease inhibitor tablets (Boehringer-Mannheim). The soluble portion of the cell lysate was loaded onto an S-Sepharose column and was eluted with a 0–500 mM NaCl gradient. The fraction containing hUBC4 was then further purified using size exclusion chromatography on a Superdex S75 gel filtration equilibrated in a buffer containing: 50 mM sodium phosphate, 100 mM sodium chloride, 5 mM DTT, pH 6.0. A Centriprep 10 concentrator (Amicon) was used to concentrate the fraction containing the purified protein. The final concentration of hUBC4 used for NMR studies was 1 mM.

All data were collected on a Bruker AMX 600 spectrometer equipped with a Nalorac triple resonance z-gradient probe. Data were collected at 303 K. Assignment was accomplished using the following experiments: 2D ^{1}H-^{15}N HSQC, 3D HNCACB, CBCA(CO)NH and CC-TOCSY(CO)NH. Data were processed using NMRPipe software (Delaglio et al., 1995), and the spectra were analysed using a combination of PIPP (Garrett et al., 1991) and NMRView (Johnson and Blevins, 1994). Resonance assignment was performed using standard methods, correlating the inter- and intra-residue $^{13}C\alpha$ and $^{13}C\beta$ shifts and using the data from the TOCSY experiment to confirm the assignment of residue type.

Figure 1 shows the assigned HSQC spectrum. Analysis of the chemical shifts of the Cα and Cβ resonances, using the method described by Wishart and coworkers (1992) suggested that the secondary structure of hUBC4 is similar to the homologous *S. cerevisiae* UBC4 (Cook et al., 1993).

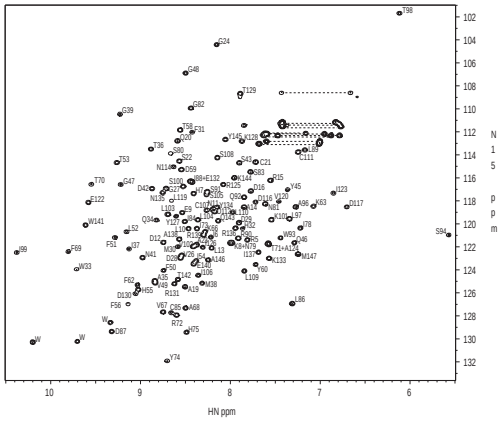

Figure 1. ^{1}H-^{15}N HSQC spectrum of hUBC4 collected at 303 K. The assignments of the resonance peaks are indicated. Tryptophan indole resonances are marked 'W'.

Extent of assignments and data deposition

Unique resonance assignments have been obtained for all non-proline backbone resonances ($^{1}H^{N}$, ^{15}N, $^{13}C\alpha$ and $^{13}C\beta$) with the exception of the four N-terminal residues, which are not detected. Proline $^{13}C\alpha$ and $^{13}C\beta$ resonances were determined from the CBCACONH experiment. Assignments were also possible for the aliphatic side-chain carbons of certain residues using the CC-TOCSY(CO)NH data and characteristic chemical shift databases. The assignments have been deposited in the BioMagResBank databank (http://www.bmrb.wisc.edu) under accession code 4866.

Acknowledgements

We thank Dr. Paul Morin and Carol Budzilowicz for advice regarding the purification of the protein.

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