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ARTICLE

^{1}H, ^{13}C, ^{15}N backbone and side-chain resonance assignment of the native form of UbcH7 (UBE2L3) through solution NMR spectroscopy

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Abstract
Ubiquitination is a post-translational modification that regulates a plethora of processes in cells. Ubiquitination requires three type of enzyme: E1 ubiquitin (Ub) activating enzymes, E2 Ub conjugating enzymes and E3 ubiquitin ligases. The E2 enzymes perform a variety of functions, as Ub chain initiation, elongation and regulation of the topology and the process of chain formation. The E2 enzymes family is mainly characterized by a highly conserved ubiquitin conjugating domain (UBC), which comprises the binding region for the activated Ub, E1 and E3 enzymes. The E2 enzyme UbcH7 (UBE2L3) is a known interacting partner for different types of E3 Ub ligases such as HECT, RING and RBR. A structural analysis of the apo form of the native UbcH7 will provide the structural information to understand how this E2 enzyme is implicated in a wide range of diseases and how it interacts with its partners. In the present study we present the high yield expression of the native UbcH7 E2 enzyme and its preliminary analysis via solution NMR spectroscopy. The E2 enzyme is folded in solution and nearly a complete backbone assignment was achieved. Additionally, TALOS+ analysis was performed and the results indicated that UbcH7 adopts a $\alpha\beta\beta\beta\beta\alpha\alpha\alpha$ topology which is similar to that of the majority of E2 enzymes.

Keywords UbcH7 · Solution NMR spectroscopy · E2 enzyme · UBC · Secondary structure · UBE2L3

Biological context

The findings of the regulation of protein degradation by Hershko and co-workers in 1983, highlighted the importance of three enzymatic reactions, undergone in an ATP-dependent manner, required for the addition of a small protein, ubiquitin, to the target protein substrate (Hershko et al. 1983). This mechanism is known as protein ubiquitination and is a post-translational modification that regulates a plethora of homeostatic processes (Karve and Cheema 2011) and disease pathways (Lecker et al. 2006) in cells. The ubiquitination pathway cascade is a versatile machinery that involves three enzymes: an Ub-activating enzyme E1, an Ub-conjugating enzyme E2 and an E3 Ub ligase (Hershko et al. 1983).

E2s function at the heart of Ub transfer choreography being responsible for the diversity of Ub cellular signalling performing multiple functions, such as Ub chain initiation and elongation (Christensen et al. 2007; Rodrigo-Brenni and Morgan 2007). Moreover, E2s have the ability to regulate the topology and the process of chain formation, a highly dynamic reaction (Page et al. 2012; Pruneda et al. 2011), thereby determining the outcome of the ubiquitination of the targeted proteins. E2 enzymes are responsible for providing the appropriate scaffold to select the correct E1, in order to conjugate Ub and form a complex with E3s fetching the target substrate for ubiquitination. Thus the ultimate result of the Ub pathway strongly depends on the E2 enzymes, which are controlling mandatory processes (Karve and Cheema 2011; Mukhopadhyay and Riezman 2007) for the biology of the eukaryotic cells. The E2 enzymes family is mainly characterized by a highly conserved 14–18 kDa UBC, which provides the binding platform for the activated Ub, E1s and E3s (Burroughs et al. 2008) (Fig. 1). UBCs have a rigid structure, comprising a functionally essential conserved 3_{10} helix near the active site cysteine, where the Ub moiety is conjugated, a four stranded anti-parallel β-sheet and three or

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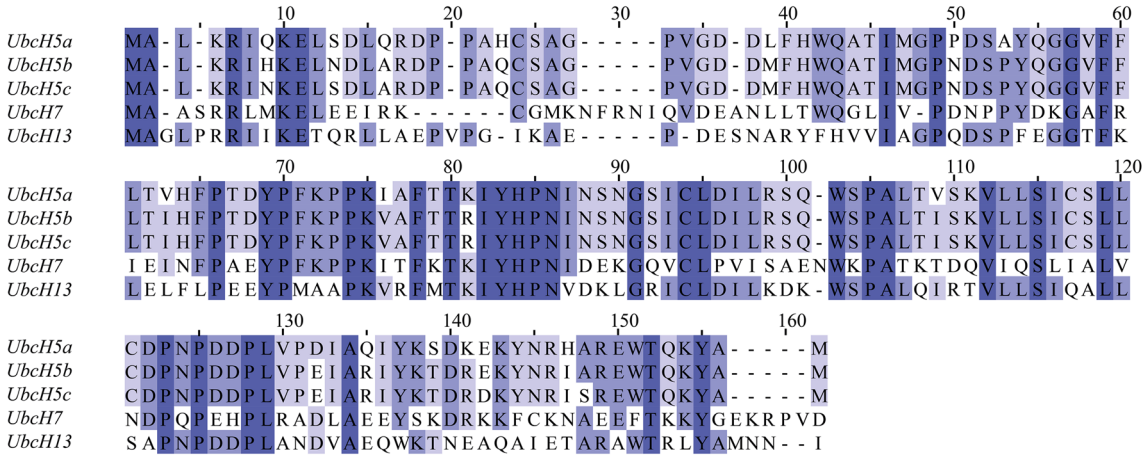

Fig. 1 Sequence alignment of UBC domains UbcH5a, UbcH5b, UbcH5c, UbcH7 and UbcH13. All the aligned sequences are colored according to their identity percentages (> 80% mid blue, > 60% light blue, > 40% light grey, ≤ 40% white)

four α-helixes (Stewart et al. 2016). Historically, the E2 family has been divided into four classes according to the presence of additional extensions at the N- and/or C-termini of the main UBC domain (van Wijk and Timmers 2010). Class I has only the main UBC domain, class II has an extension at the C-terminus of the UBC, class III has an extension at the N-terminus of the UBC and class IV has extensions at both C- and N-termini of the UBC domain. These extensions give to E2s the alteration and differentiation in their function at the subcellular level.

A representative of the class I of the E2 enzymes is the UbcH7 (UBE2L3) which is a known interacting partner for different types of E3 Ub ligases such as HECT (homologous to the E6AP carboxyl terminus), RING (Really Interesting New Gene) and RBR (RING-between-RING). A well-studied example of a HECT E3 ligase that interacts with UbcH7 is E6AP, a HECT ligase involved in human papilloma virus-induced degradation of p53 and mutated in Angelman syndrome (Huang et al. 1999). Furthermore there are some RING ligases that seem to interact with UbcH7 such as the E3 casitas B-lineage lymphoma (CBL) a negative regulator of receptor Tyr kinase signalling pathway (Zheng et al. 2000). A few years ago UbcH7 has been discovered as the critical player for DNA double-strand break (DSB) repair by regulating the steady state and proteasome-dependent degradation of the tumour suppressor p53-binding protein 1 (53BP1) (Han et al. 2014). Moreover, UbcH7 is the E2 enzyme of choice for many RBR E3 ligases. RBR E3s harbour three tandem zinc-binding domains termed RING1, in between RING and RING2. RBR E3 ligases such as HHARI (human homolog of Ariadne) function through a RING-HECT hybrid mechanism (Dunkerley and Shaw 2017; Yuan et al. 2017). Recently genome-wide association studies identified single-nucleotide polymorphisms in the genetic locus of UbcH7 associated with many autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, psoriasis etc. (Stahl et al. 2010; Tsoi et al. 2012; Wang et al. 2012). Interestingly the implication of UbcH7 with the stability of 53BP1 provides a new strategy to amplify the anticancer effect of chemotherapy or radiotherapy (Han et al. 2014). Thus a thorough structural analysis of the non-complexed form of the native UbcH7 will provide the structural information to understand the mechanism that gives this E2 enzyme such a broad involvement in diseases and interacting partners.

Here we report an extensive ^{1}H, ^{13}C and ^{15}N backbone, side-chain and aromatic assignment of the native form of the 154 amino acids polypeptide UbcH7. The assignment of this E2 enzyme will allow the NMR-driven studies towards the understanding of the structural machinery that provides the selectivity of this enzymes providing thus new, valuable insights into the ubiquitin proteasome pathway (UPP).

Methods and experiments

Protein expression and purification

The UBE2L3 gene for the full length UbcH7, amino acids 1–154, was synthesized, codon optimized (GenScript) and subcloned using *BamHI* and *XhoI* restriction enzymes into pGEX-4T-1, which included a thrombin protease recognition site (LVPRGS) between the glutathione *S*-transferase (GST) tag and UbcH7 sequence. *E. coli* (*E. cloni®)* EXPRESS BL21(DE3) cells (Lucigen), transformed with the pGEX-UbcH7 vector, were used for protein expression. Cells were grown in minimal medium containing $^{15}NH_4Cl$ (1 g/L) and D-[$^{13}C_6$] glucose (4 g/L), NaCl (0.5 g/L), 1 M $MgSO_4$ (1 mM/L), Solution Q (2 mL/L) [40 mM HCl, $FeCl_2$ $4H_2O$ (50 mg/L), $CaCl_2$ $2H_2O$ (184 mg/L), H_3BO_3 (64 mg/L), $CoCl_2$

$6H_2O$ (18 mg/L), $CuCl_2$ $2H_2O$ (4 mg/L), $ZnCl_2$ (340 mg/L), Na_2MoO_4 $2H_2O$ (605 mg/L), $MnCl_2$ $4H_2O$ (40 mg/L)] and 1 mL ampicillin (100 mg/mL). Bacterial culture was grown at 37 °C and shaken at 200 rpm and protein expression was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (final concentration). Cells were harvested four hours later by centrifugation at 6000 rpm for 10 min and pellets stored at −20 °C until use. Cell pellets were suspended in phosphate buffered saline (PBS) pH 7.4 containing 1 mM TCEP (100 mM stock) and protease inhibitors cocktail (Sigma Aldrich®) and lysed by sonication (Sonicator Ultrasonic Processor-QSONICA, LLC) for 2:30 min (10 sec pulse on, 2:30 min pulse off). Cellular debris was separated by centrifugation at 14,000 rpm x 20 min at 4 °C. Clarified lysate was applied to Glutathione Sepharose Fast Flow (FF) resin (GE Healthcare) and washed with PBS. Protease thrombin (100NIH Units, Merck) was applied to the column and incubated at 25 °C for 16 h to remove the GST-tag. Cleaved UbcH7 was washed from the column with PBS pH 7.4 and concentrated using Amicon® Ultra 15 mL centrifugal filter with a molecular weight cut off of 10 kDa (Merck). UbcH7 was further purified by size exclusion chromatography using FPLC AKTA Purifier System (GE Healthcare) with a Superose 12 10/300 column (GE Healthcare). The column was equilibrated with 50 mM K_2HPO_4, 50 mM KH_2PO_4 pH 7 and the protein was eluted according to its molecular weight.

Data acquisition, processing and assignment

For the NMR experiments ^{15}N and ^{13}C/^{15}N labelled samples were prepared with a concentration of 0.9 mM. All samples were in a mixed solvent of 90% H_2O and 10% D_2O

(50 mM K_2HPO_4, 50 mM KH_2PO_4) at pH 7 and a bacterial protease inhibitor cocktail (Sigma Aldrich®) used. 0.25 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was used as internal standard for the reference of all nuclei according to ^{1}H chemical shift of DSS signal at 0.0 ppm. All NMR experiments were recorded on a Bruker Avance III HD 700 MHz NMR spectrometer equipped with a four-channel 5 mm cryogenically cooled TCI gradient probe at 298 K.

The sequence specific assignment of UbcH7 domain obtained using conventional backbone and side chain assignment methods (Ferentz and Wagner 2000; Wüthrich 1986) using the following experiments: 2D [^{1}H–^{15}N]-HSQC and TROSY, 2D [^{1}H–^{13}C]-HSQC, 3D HNCA, 3D HN(CO)CA, 3D CBCA(CO)NH, 3D CBCANH, 3D HNCO, 3D HN(CA)CO, 3D HBHA(CO)NH, 3D HNHA, 3D (H)CCH-TOCSY, 3D ^{15}N-edited NOESY and modified versions of the 3D CBCA(CO)NH experiment for the effective correlation of [NH](i) and [CBCA](i-1) when the (i-1) residue lacks an aliphatic Cγ atom (Ala, Asn, Asp, Cys, Gly, Ser and aromatic residues) or a γCO (Ala, Cys, Ser and aromatic residues). All experiments are included in the Bruker pulse program library. For the assignment of the aromatic nucleus were used the 2D (HB)CB(CGCD)HD and 2D (HB)CB(CGCDCE)HE, also included in the Bruker pulse program library.

All NMR data were processed with Topspin 3.5 pl5 software and analysed with CARA (Keller 2004) and XEASY (Bartels et al. 1995).

Results

Assignment and data deposition

The ^{1}H-^{15}N HSQC spectra of UbcH7 (Fig. 2a) showed an extensive chemical shift dispersion and narrow line widths, indicative of a well-folded monomeric polypeptide. The analysis of the heteronuclear NMR experiments of the double isotopically labelled sample with the conventional backbone methodology, resulted in the sequence specific assignment of almost all the backbone ^{1}H-^{15}N resonances, with the exception of Met 1. The assignments of the ^{1}H and ^{13}C aliphatic chemical shifts resulted in the identification of 92.3% and 97.9% of the anticipated nucleus resonances, respectively. Furthermore 96.1% of all the backbone carbonyl ^{13}C chemical shifts were assigned from the analysis of the 3D HNCO and 3D HN(CA)CO experiments and 56.3% and 30.9% of aromatic ^{1}H and ^{13}C resonances were identified, respectively. These resulted in an overall backbone assignment of 97.1% as well as a 79.9% side-chain assignment, with an overall chemical shift assignment reaching 86.1%.

The analysis of the 3D CBCA(CO)NH, 3D CBCANH, 3D HBHA(CO)NH and 3D (H)CCH-TOCSY spectra of the proline residues resulted in the identification of the *cis* or *trans* conformation. The comparison of the prolines $^{13}C_\beta$ and $^{13}C_\gamma$ chemical shifts to average reference values in the literature (*trans*-Pro: $^{13}C_\beta$ 31.75 ± 0.98, $^{13}C_\gamma$ 27.26 ± 1.05; *cis*-Pro: $^{13}C_\beta$ 34.16 ± 1.15, $^{13}C_\gamma$ 24.52 ± 1.09) (Schubert et al. 2002) clearly indicated that 9 of them adopt *trans* conformation (Pro 44 $^{13}C_\beta$ 30.7, $^{13}C_\gamma$ 27.4; Pro 58 $^{13}C_\beta$ 31.2, $^{13}C_\gamma$ 27.5; Pro 65 $^{13}C_\beta$ 31.3, $^{13}C_\gamma$ 26.0; Pro 77 $^{13}C_\beta$ 32.6, $^{13}C_\gamma$ 26.6; Pro 88 $^{13}C_\beta$ 31.4, $^{13}C_\gamma$ 26.9; Pro 97 $^{13}C_\beta$ 32.1, $^{13}C_\gamma$ 27.3; Pro 115 $^{13}C_\beta$ 31.7, $^{13}C_\gamma$ 26.0; Pro 117 $^{13}C_\beta$ 32.3, $^{13}C_\gamma$ 27.2; Pro 152 $^{13}C_\beta$ 31.6, $^{13}C_\gamma$ 27.4) and 4 *cis* conformation (Pro 45 $^{13}C_\beta$ 33.4, $^{13}C_\gamma$ 25.6; Pro 62 $^{13}C_\beta$ 33.2, $^{13}C_\gamma$ 24.8; Pro 120 $^{13}C_\beta$ 33.2, $^{13}C_\gamma$ 25.6). Pro 41 ($^{13}C_\beta$ 33.2, $^{13}C_\gamma$ 28.7) and Pro 66 ($^{13}C_\beta$ 32.2, $^{13}C_\gamma$ 25.3) chemical shifts comparison with the average values cannot indicate unambiguously the conformational state of these prolines (Fig. 3). Moreover, the oxidation state of all cysteines was identified based of the analysis of the 3D CBCA(CO)NH and 3D CBCANH

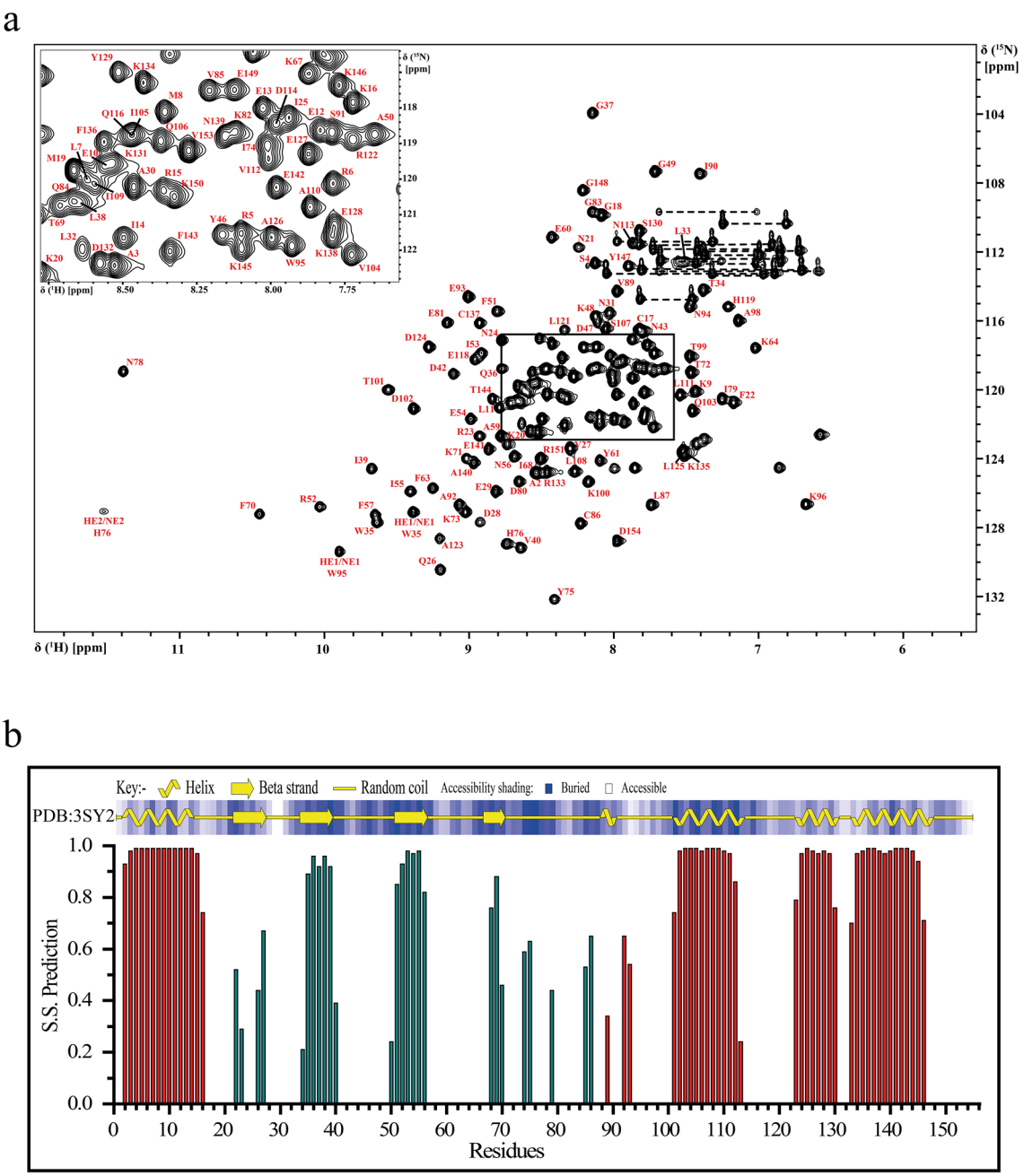

Fig. 2 **a** 2D [^{1}H-^{15}N] HSQC spectrum at 298 K of UbcH7 (UBE2L3). *Left top* magnification of the central region of the 2D [^{1}H-^{15}N] HSQC spectrum. **b** Secondary structure prediction of UbcH7 (UBE2L3) analyzed with TALOS+ using the chemical shift assignment resonances (*red* and *green* bars indicate α-helix and β-strands, respectively) in comparison with the secondary structure of UbcH7 in complex with *Salmonella* E3 ubiquitin ligase SopA (PDB id: 3SY2) according to PROCHECK analysis.

experiments and the comparison with the $^{13}C_{\alpha}$ and $^{13}C_{\beta}$ average values in the literature (Kornhaber et al. 2006). All three cysteine residues of UbcH7 are in their reduced state. All ^{1}H, ^{15}N and ^{13}C chemical shifts of UbcH7 protein have been deposited in BioMagResBank (http://www.bmrb.wisc.edu) under the accession number 28028

UbcH7 chemical shifts were analysed with TALOS+ (Shen et al. 2009) in order to identify the secondary structure elements of the polypeptide. The analysis indicated that UbcH7, in its apo form, consists of 4 α-helix, 4 main β-strands and a small 1 turn α-helix, where in many E2 enzymes this appears as a 3_{10} helix. E2 enzymes have a highly conserved UBC domain leading to a $\alpha\beta\beta\beta\beta\alpha\alpha\alpha$ overall topology of the protein fold (Fig. 1), typical for the majority of E2 enzymes and almost identical with the one predicted by the assigned chemical shifts. Moreover the

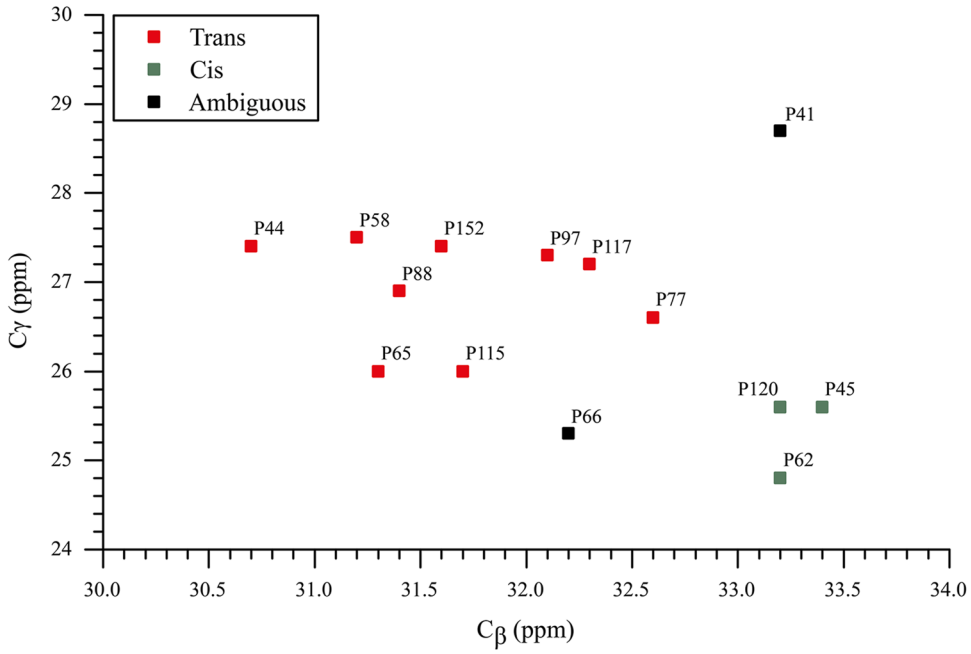

Fig. 3 Bivariant Plot of the 14 Pro Cγ/Cβ chemical shift pairs indicating the Pro cis/trans conformation. Pro 41 and Pro 66 chemical shifts cannot indicate unambiguously the conformational state of these prolines.

comparison of TALOS+ result with the secondary structure elements of the crystal structure of the native UbcH7 in complex with *Salmonella* E3 ubiquitin ligase SopA (PDB id:3SY2), point out the extensive agreement of the predicted secondary structure elements (Fig. 2b).

To summarize, we present in this work a biological method to express and purify in high yield the native recombinant UbcH7. NMR analysis indicated that the polypeptide is well folded and in monomeric state (according to chromatograms and relaxation data; not shown). The almost complete sequence-specific assignment was obtained from the 3D NMR spectra and was deposited under the Accession Number 28028. TALOS+ analysis indicated that UbcH7 adopts a similar fold to that of the majority of E2 enzymes. These results may contribute to the assessment of the NMR solution structure of the non-complexed form of UbcH7, as well as to the biochemical and functional characterization of the E2 family that play a key role in the UPP machinery.

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