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¹H, ¹³C and ¹⁵N NMR assignments of cyclophilin LRT2 (OsCYP2) from rice

Lucila Andrea Acevedo¹ and Linda K. Nicholson^{1, iD}

¹Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853, USA

Abstract

Cyclophilins are enzymes that catalyze the isomerization of a prolyl–peptide bond and are found in both prokaryotes and eukaryotes. LRT2 (also known as OsCYP2) is a cyclophilin in rice (*Oryza sativa*), that has importance in lateral root development and stress tolerance. LRT2 is 172 amino acids long and has a molecular weight of 18.3 kDa. Here, we report the backbone and sidechain resonance assignments of ¹H, ¹³C, ¹⁵N in the LRT2 protein using several 2D and 3D heteronuclear NMR experiments at pH 6.7 and 298 K. Our chemical shift data analysis predicts a secondary structure like the cytosolic wheat cyclophilin TaCypA-1 with 87.7% sequence identity. These assignments will be useful for further analysis in the NMR studies for function and structure of this enzyme.

Keywords

NMR resonance assignments; TALOS-N prediction; Rice cyclophilin; Lateral root development; Protein chemical shift assignment

Biological context

Cyclophilins are a conserved class of peptidyl prolyl isomerases found in mammals, plants, insects, fungi and bacteria (Wang and Heitman 2005). The primary function of Cyclophilins is to catalyze the isomerization of prolyl–peptide bonds, making this class of enzymes important in fundamental processes such as protein folding, gene transcription, protein degradation, and cell signaling. The cis/trans isomerization of the X-Pro peptide bond has been studied as a molecular switch and timing device in diverse biological processes (Lu et al. 2007), and the enzymes that regulate this switch can represent the rate-limiting step in these processes. Mammalian cyclophilins have been extensively studied, in particular Human Cyclophilin A which has been studied by NMR in order to determine the residues involved in its interaction with substrate and to investigate its mechanism of action (Eisenmesser et al. 2002).

In rice (*Oryza sativa*), there have been reported 27 Cyclophilin genes which encode 46 protein products (Kumari et al. 2013). One of these protein products is the LRT2 protein.

Correspondence to: Linda K. Nicholson.

Linda K. Nicholson <http://orcid.org/0000-0002-2701-1530>

Expression of LRT2 has been induced by multiple abiotic stresses, and overexpression of it has been shown to enhance multiple stress tolerance in *E. coli* and *S. cerevisiae*, and salinity stress tolerance in rice, making it a target protein for study of stress resistance mechanisms in plants (Kumari et al. 2009; Ruan et al. 2011). Also, LRT2 has recently been associated to the C2HC-type Zinc finger protein (OsZFP, Os01g0252900) by yeast two hybrid cDNA library screening, a protein that has an important regulatory role in lateral root development (Cui et al. 2017). Importantly, LRT2 has been reported as the cyclophilin that affects lateral root development in rice, where LRT2 knock out or mutation shows defects on lateral root development (Kang et al. 2013). In the same context, knock out of the auxin response (Aux/IAA) gene that codes for the transcriptional repressor protein OsIAA11 affects lateral root development (Zhu et al. 2012). Auxin is a class of phytohormones that regulate essentially all developmental processes in plants through their action in feedback inhibition circuits that control the expression of auxin-responsive genes. Notably, auxin is required for the E3 ubiquitin ligase SCF^{TIR1/AFB} complex to bind specifically with the *cis* isomer of a highly conserved Trp-Pro degron motif in Aux/IAA proteins such as OsIAA11, thereby targeting these transcription repressor proteins for proteasomal degradation. In vitro interaction between LRT2 and OsIAA11 and LRT2 catalysis of the Trp-Pro peptide bond in the Aux/IAA degron motif of OsIAA11 has been reported (Jing et al. 2015). This interaction is of importance in the field of plant biology, since LRT2 catalysis of *cis*/trans isomerization of OsIAA11 could accelerate ubiquitin-mediated proteasomal degradation of OsIAA11 via the *cis* conformation of the conserved Aux/IAA degron motif (Jing et al. 2015). This degradation is crucial for transcription regulation since OsIAA11 is a repressor protein that regulates lateral root formation (Dinesh et al. 2016). Therefore, if LRT2 is disrupted, OsIAA11 would not be degraded at the appropriate rate and could still repress gene expression in the absence of Auxin.

We report here the backbone and sidechain resonance assignments of LRT2 at room temperature in a 6.7 pH phosphate buffer. LRT2 represents a defined *cis*/trans switch and molecular timer that has been linked to a clear phenotype (Kang et al. 2013), thus offering an opportunity to study the relationships between a specific isomerization rate and a developmental outcome in lateral roots.

Methods

Protein expression and purification

The cDNA for the LRT2 gene (Os02g0121300) was custom synthesized by Cyagen Biosciences (Santa Clara, CA). This gene was amplified by PCR using primers that introduced restriction enzyme digestion sites for NdeI at the 5' terminus and HindIII at the 3' terminus to allow insertion of this gene into a PET28 vector with Kanamycin resistance, an N-terminal His₆-tag, and a TEV cleavage site. Expression of protein was carried out in M9 minimum media enriched with ¹⁵NH₄Cl and ¹³C-Glucose. Protein expression was induced by addition of 0.88 mM IPTG when the culture reached 0.8 O.D.₆₀₀. Expression proceeded over night at 17 °C. Cells were spun down and recovered in 20 ml lysis buffer [wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM Imidazole, and 0.1 mM TCEP pH 8.0], plus 100 µl P.I. cocktail (Thermo scientific), 1 mM TCEP, 2.5 mM MgCl₂, 0.5 mM

CaCl₂), and frozen. Lysis of cells was carried out by addition of 0.02 g lysozyme, 100 μ l Dnase, and 100 μ l Rnase. The solution was kept on ice for 30 min; further lysis was carried by 15 cycles of sonication with pulse on for 10 s and off for 15 s. Separation of soluble proteins was achieved by spinning down the solution at 15,000 rpm for 45 min at 4 °C. Filtered supernatant was added to 2 ml nickel beads pre-equilibrated in wash buffer. The binding was carried out for 45 min, the column was washed with 50 ml of wash buffer and protein was eluted with ~ 10 ml wash buffer + 300 mM Imidazole. A desalting column was used to exchange the eluent into TEV buffer (50 mM NaPO₄, 100 mM NaCl, 1 mM TCEP, pH 8.0), followed by TEV cutting of His₆-Tag for 48 h. 10 mM imidazole and 50 mM KCl were added to the solution, which was run again through the nickel column. The flow through of this process was run through a desalting column to exchange into “NMR” buffer (50 mM KCl, 20 mM KPO₄, 0.1 mM TCEP, pH 6.67). This sample of ¹³C/¹⁵N-labeled LRT2 was concentrated to 1.16 mM in NMR buffer (plus 5 mM NaN₃, 0.1% P.I. cocktail, 1 mM TCEP, and 8% D₂O).

NMR spectroscopy

For backbone and side-chain assignments, a suite of 2D and 3D NMR experiments were acquired. The ¹³C/¹⁵N-LRT2 sample was used to acquire 2D ¹⁵N-¹H-HSQC and ¹³C-¹H-HSQC spectra, and to acquire 3D HNCACB, HN(CO)CA, HNCA, (H)C(CO)NH, H(CCO)NH, HCCH-TOCSY, and HNCO spectra. All spectra were collected at 25 °C using a Varian Inova 600 MHz spectrometer with a (H, C, N) Z-axis gradient probe. Spectra were processed with nmrPipe (Delaglio et al. 1995) and analyzed with Sparky (<http://www.cgl.ucsf.edu/home/sparky/>).

Results and discussion

For LRT2, chemical shift assignment of backbone and side-chain resonances were made for 163 of the 164 non-proline residues in the ¹⁵N-¹H HSQC spectra (Fig. 1). The only residue for which the main chain amide peak was not assigned was E88. Assignments were determined for 99% of C ^{α} , C ^{β} and C', for 93% of non-aromatic and non-carbonyl side-chain carbons, for 91% of non-aromatic side-chain hydrogens, and for 98% of H ^{α} . The backbone and side chain resonance assignments were automatically assigned using PINE Server (<http://pine.nmrfarm.wisc.edu/>) followed by manual validation and correction. The chemical shift assignments of LRT2 have been deposited in the BioMagResBank (<http://www.bmrb.wisc.edu>) under accession number 27159.

Our assigned LRT2 chemical shifts were used to predict the corresponding secondary structure using the TALOS-N Server (Shen and Bax 2013). LRT2 has 87.7% sequence identity to the cytosolic wheat cyclophilin TaCypA-1, for which its crystal structure has been solved [PDB entry 4HY7 (Sekhon et al. 2013)]. As expected, LRT2 chemical shift assignments predict a secondary structure similar to TaCypA-1 (Fig. 2). In future work we will use the chemical shift assignments to study the complete thermodynamic cycle of the LRT2 enzyme acting on the OsIAA11 degron motif as well as on other Aux/IAA proteins of rice.

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Fig. 1. ^{15}N - ^1H HSQC spectrum of 1.16 mM ^{13}C , ^{15}N labeled LRT2 recorded at 298 K at 600 MHz in 93% H_2O /7% $^2\text{H}_2\text{O}$, 20 mM sodium phosphate buffer, pH 6.7, 50 mM KCl, 5 mM NaN_3 , 1 mM TCEP. The cross peaks are labeled with single letter amino acid residue and number according to the native sequence. Correlations arising from NH_2 spins of Asn and Gln residues are shown with dashed green lines

Fig. 2. Probabilities for the secondary structure of LRT2 (yellow, α -helices; grey, β -strands) predicted from chemical shift values using TALOS-N are plotted as a function of residue number. Probability values below 0.5 are not displayed. Position of secondary structure elements in the crystal structure of TaCypA-1 (PDB entry 4HY7 and 87.7% sequence identity to LRT2) are indicated on the top