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¹H, ¹³C and ¹⁵N resonance assignments of a ribonucleoprotein complex consisting of Prp24-RRM2 bound to a fragment of U6 RNA

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Abstract

Saccharomyces cerevisiae Prp24 is an essential RNA binding protein involved in pre-mRNA splicing. Nearly complete backbone and side chain resonance assignments have been obtained for the second RNA recognition motif (RRM) of Prp24 (RRM2, residues M114-E197) both in isolation and bound to a 6 nucleotide fragment of U6 RNA (AGAGAU). In addition, nearly complete backbone assignments have been made for a Prp24 construct spanning the second and third RRM (RRM23, residues M114-K290), both free and bound to AGAGAU.

Keywords

RRM; protein-RNA complex; splicing; snRNP

Biological Context

Proper eukaryotic gene expression requires successful pre-mRNA splicing, catalyzed by the spliceosome (Will and Luhrmann 2006). The spliceosome is assembled from five RNA-protein complexes (small nuclear ribonucleoproteins, or snRNPs). Each snRNP contains one small nuclear RNA molecule (named U1, U2, U4, U5, or U6) and multiple proteins. The *Saccharomyces cerevisiae* protein Prp24 is required for incorporation of U6 RNA into the spliceosome (Shannon and Guthrie 1991; Raghunathan and Guthrie 1998). Prp24 binds to U6 RNA as a part of the U6 snRNP (Karaduman et al. 2006; Karaduman et al. 2008), and facilitates basepairing between U6 RNA and U4 RNA (Shannon and Guthrie 1991; Ghetti et al. 1995).

Prp24 contains four RNA recognition motif (RRM) domains (Bae et al. 2007). Previous work established that RRM1 and/or RRM2 of Prp24 bind the AGAGAU sequence of U6 RNA (Kwan and Brow 2005; Bae et al. 2007). Interestingly, the canonical RNA binding surface of RRM2 appears to be occluded by inter-domain contacts in a crystal structure (Bae et al. 2007). In order to further investigate RNA binding by RRM2 of Prp24, and the inter-domain orientation between RRM2 and RRM3, we have obtained backbone and side chain assignments for RRM2 free and bound to AGAGAU RNA, and backbone assignments for RRM23 free and bound to AGAGAU.

Ethics and Conflicts: The authors declare that they have no conflict of interest, and that the experiments performed comply with the current laws of the United States of America.

Methods and Experiments

Uniformly unlabeled, ^{15}N , and ^{13}C ^{15}N labeled protein (RRM2-His₆ or RRM23-His₆) was prepared through over-expression from a pET21b plasmid transformed into BL21 *E. coli* (Stratagene). Cells were grown to saturation in 750 mL M9 minimal media supplemented with ^{13}C glucose and/or ^{15}N ammonium chloride as needed. Unlabeled RRM2 was grown in Luria Broth. Protein production was induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 15 hours at room temperature. Cells were pelleted by centrifugation and stored at -80°C . Frozen cell pellets were thawed for 15 hours at 4°C , and then resuspended in 50 mL Wash Buffer (50 mM sodium phosphate pH 7.4, 300 mM sodium chloride, 10 mM imidazole). Lysis was performed on the 50 mL volume using a 30 minute incubation with 1 mg/mL lysozyme at 4°C , followed by 2 minutes sonication on ice (12 cycles of a 10 second pulse followed by a 30 second rest). Lysate was clarified through centrifugation for 30 minutes at $30,000\times G$ and $0.45\text{ }\mu\text{m}$ filtration. Purification was achieved with HisPur cobalt spin columns (Pierce Biotechnology) following the manufacturer's suggested protocol. If necessary based on SDS-polyacrylamide gel electrophoresis and coomassie blue staining, further purification was performed using a Superdex 75 gel filtration column in Gel Filtration Buffer (50 mM Tris-Cl pH 7.5, 150mM sodium chloride, 1 mM dithiothreitol, 5% (v/v) glycerol) on an Akta FPLC. Eluate was dialyzed into 1 liter Protein Storage Buffer (25 mM Tris-Cl pH 7.5, 125 mM sodium chloride, 1.25 mM dithiothreitol, 0.2 mM ethylenediaminetetraacetic acid, 50% (v/v) glycerol) for 2 days at 4°C using a 3,500 MW cutoff dialysis cassette (Pierce Biotechnology), then stored at -20°C . Prior to NMR data collection, the protein was dialyzed into 1 liter NMR Buffer (10 mM Tris-Cl pH 7, 50 mM potassium chloride, 1 mM dithiothreitol) for 2 days at 4°C , and then concentrated using a centrifugal filter device (Millipore Amicon, 5 kDa cutoff).

RNA was purchased from Dharmacon Inc., deprotected following the manufacturer's protocol, desalted over a 15 mL G-25 Sephadex column, and lyophilized to dryness. RNA was then resuspended to 4 mM in water, adjusted to pH 7.0 with sodium hydroxide or hydrochloric acid, and stored at -20°C . Uniformly ^{13}C ^{15}N labeled AGAGAU RNA was prepared through *in vitro* transcription, using purified His₆-tagged T7 RNA polymerase, synthetic DNA oligonucleotides (Integrated DNA Technologies, Inc.) containing either the T7 promoter sequence and seven repeats of AGAGAT or the reverse complement, and isotopically labeled ribonucleotide triphosphates (Silantes GmbH, München, Germany). The transcribed RNA was purified using denaturing 15% polyacrylamide gel electrophoresis, identified through UV shadowing, and excised from the gel. It was then allowed to diffuse out of the gel fragment into 0.3 M sodium acetate (pH 5.0), precipitated with cold ethanol, and desalted on a 15 mL G-25 sephadex column. The purified RNA was then treated with 1 $\mu\text{g/mL}$ RNase A (Fermentas) for 5 minutes at room temperature, cleaving it into seven uniformly ^{13}C ^{15}N labeled AGAGAU 6mers. After the RNase treatment, RNA was lyophilized, purified and desalted as described above (except using 20% PAGE).

All NMR spectra were obtained on 600 MHz, 800 MHz, or 900 MHz Varian spectrometers, or a 750 MHz Bruker spectrometer, at 25°C . Raw data were processed using nmrPipe (Delaglio et al. 1995), and spectra were analyzed using Sparky (University of California-San Francisco). Backbone and side chain assignments for RRM2 with and without RNA were obtained using a sample of 500 μM uniformly labeled ^{13}C ^{15}N RRM2 in 90% NMR Buffer, 10% D₂O. Some samples were prepared in 99% D₂O by diluting a 300 μL sample with 3 mL deuterated NMR buffer (10mM deuterated Tris-Cl pH 7.0, 50 mM potassium chloride, in D₂O) followed by concentration in a Millipore Amicon centrifugal filter device, and repeating three times. A sample in 99% D₂O containing unlabeled RRM2 and unlabeled AGAGAU was also used. For experiments with RNA, a 5 mM stock solution of AGAGAU was added to protein sample to reach the desired final concentration. Standard 2D and 3D NMR experiments (2D ^1H , ^{15}N -

HSQC; 2D ^1H , ^{13}C -HSQC; 3D HBHACONH; 3D CBCACONH; 3D HNCACB; 3D CCONH; 3D HCCONH; 3D HCCH-TOCSY; 3D HNCO; 3D NOESY- ^1H , ^{15}N -HSQC; 3D NOESY- ^1H , ^{13}C -HSQC; and 2D ^1H , ^1H -NOESY) were used to manually assign backbone and side chain resonances.

Backbone assignments for RRM23 with and without RNA were obtained similarly, through manual analysis of 2D ^1H , ^{15}N -HSQC; 3D CBCACONH; 3D HNCA; and 3D HNCACB experiments.

AGAGAU RNA in complex with RRM2 was partially assigned (ribose and aromatic protons, some ribose carbons) to facilitate identification of intermolecular NOEs. Resonances were assigned through analysis of 2D ^1H , ^{13}C -HSQC and ^1H , ^1H -NOESY spectra taken on a sample containing 200 μM ^{13}C ^{15}N labeled AGAGAU and 500 μM unlabeled RRM2 in 90% NMR Buffer/10% D_2O .

^1H peaks were referenced to an internal DSS standard, while ^{13}C and ^{15}N peaks were referenced indirectly using the absolute frequency ratios.

Extent of Assignments and Data Deposition

NH backbone and side chain assignments for RRM2 without and with AGAGAU RNA are shown in Figure 1. Quantifying the changes in peak position (Figure 1C) reveals the RNA binding site on RRM2 (defined as residues with a change of greater than 0.1 ppm). Similar changes in peak position, and therefore a similar binding site, were observed for RRM23 when AGAGAU RNA was added (data not shown).

All statistics exclude the non-native C-terminal His₆ tag. Typically assigned resonances exclude side chain NH groups on arginine and lysine, OH and SH groups, and side chain carbons not bound to a proton (based on BioMagResBank statistics). For RRM2 bound to AGAGAU RNA, >92% of typically assigned proton resonances, >80% of typically assigned carbon resonances, and >95% of typically assigned nitrogen resonances were assigned. Many of the missing carbon resonances are aromatic. All aromatic CH protons, 92% of ribose protons, and 73% of ribose carbons in AGAGAU were assigned (BMRB 16230). In the absence of RNA, >80% of typically assigned proton resonances, >60% of typically assigned carbon resonances, and >87% of typically assigned nitrogen resonances were assigned (BMRB 16246).

For RRM23, 92% of non-proline backbone NH resonance assignments were made in the absence of RNA (BMRB 16243), and 76% in the presence of RNA (BMRB 16244). The majority of missing assignments were located in flexible regions of the protein. The chemical shift deviations from random coil $^{13}\text{C}_\alpha$ values for RRM23 are presented in Fig. 2. This analysis is consistent with the secondary structure observed in the crystal structure (Bae et al. 2007). All chemical shifts fall within the chemical shift distribution histograms published by the Biological Magnetic Resonance Data Bank (<http://www.bmrwisc.edu>).

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Fig. 1.

Amide peak assignments for Prp24-RRM2 with and without bound RNA. **(a)** 2D [^1H , ^{15}N]-HSQC spectrum of ^{15}N -labeled Prp24-RRM2 (protein concentration 0.5 mM, 10 mM Tris-Cl, pH 7.0, 50 mM potassium chloride, 1 mM dithiothreitol. The spectrum was recorded at ^1H frequency of 750 MHz, $T=25^\circ\text{C}$. Side chain amide resonances for asparagines and glutamines are connected by dashed lines. **(b)** Same as in **(a)**, except in the presence of 5 mM AGAGAU RNA. **(c)** Shifts in peak position induced by RNA binding. Values were calculated using:

$$\Delta d = \sqrt{\Delta H^2 + (\Delta N / 9.86)^2}.$$

Residues without data could not be quantified (due to overlap or line broadening).

Fig. 2.

Secondary $C\alpha$ shifts for RRM23. Shifts were calculated as observed minus random coil (Schwarzinger et al. 2001). The secondary structure of RRM23 is shown below based on the previously determined crystal structure (Bae et al. 2007). Arrows indicate beta strands, ovals alpha helices, and hollow rectangles disordered regions.