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^{1}H, ^{13}C and ^{15}N backbone and side-chain resonance assignments of *Drosophila melanogaster* Ssu72

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Abstract

Ssu72 helps regulate transcription and co-transcriptional mRNA processing by dephosphorylating serine residues at the 5th position in the heptad repeats of the C-terminal domain of RNA polymerase II. Here we use multidimensional, multinuclear NMR experiments to assign the backbone and side-chain resonances of the 23 kDa Ssu72 from *Drosophila melanogaster* in the phosphate-bound state, and use NMR titrations to examine the phosphate-binding properties of three active site mutants.

Keywords

Ssu72; RNA Polymerase II; CTD phosphatase

Biological context

The C-terminal domain (CTD) of RNA polymerase II consists of multiple copies of a heptad repeat with the consensus sequence Y1-S2-P3-T4-S5-P6-S7 and acts as an unstructured platform for association with proteins that regulate transcription and co-transcriptional processes. Phosphorylation of the CTD ties the recruitment of specific factors to the correct stage of transcription, with phosphorylation at the S5 position (pS5) predominating during initiation and S2 phosphorylation (pS2) increasing during elongation. Ssu72 is a CTD phosphatase with activity directed specifically at the pS5 position. Ssu72 activity is essential in yeast and affects the initiation, elongation and termination stages of transcription as well as co-transcriptional 3′-end processing of nascent mRNA. Recently, we and others reported crystal structures of Ssu72 in complex with pS5 CTD (Werner-Allen et al. 2011; Xiang et al. 2010), which show that Ssu72 contains the scaffold of the low molecular weight protein tyrosine phosphatases (LMW PTPs) with unique additions, giving structural support for a common catalytic mechanism of dephosphorylation. The complexes also reveal a remarkable substrate conformation with the pS5-P6 motif of the CTD in the *cis* isomer, which exists at only ~10% in solution, providing the first example of a *cis* proline-specific enzymatic activity and a surprising explanation for the in vivo connection between the activities of Ssu72 and the proline isomerase Ess1 (Pin1 in humans).

Despite these recent findings, many aspects of Ssu72 function have yet to be elucidated at the molecular level. For example, Ssu72 binds to symplekin (Pta1 in yeast), a scaffolding

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component of the large complex that catalyzes 3′-end cleavage and polyadenylation of mRNA transcripts, and this interaction significantly stimulates in vitro pS5 CTD dephosphorylation by Ssu72 (Xiang et al. 2010). However, a Ssu72-symplekin complex structure shows that the symplekin binding site is far from the active site of Ssu72 (~25Å) and that complex formation does not cause any significant changes in the Ssu72 structure (Xiang et al. 2010), suggesting that dynamics may play a dominant role in the allosteric mechanism. Ssu72 has also been reported to physically interact with several other proteins, including the general transcription factor TFIIB. The association of Ssu72 with TFIIB may contribute to the interaction of initiation factors with the 3′-end processing machinery during gene looping, a proposed mechanism for transcription reinitiation that tethers the promoter and terminator regions of a gene. NMR should provide a crucial tool for mapping the protein–protein interaction sites of Ssu72 and for understanding how these binding events affect the structure and dynamics of the enzyme in order to modulate its role in transcription.

Methods and experiments

Drosophila melanogaster

Ssu72 protein was expressed and purified as previously described (Werner-Allen et al. 2011). Isotopically enriched samples were prepared from cells grown in M9 minimal media with ^{15}N-NH_4Cl and ^{13}C-glucose as the sole nitrogen and carbon sources. Perdeuterated Ssu72 was expressed in D_2O M9 minimal media with ^{15}N-NH_4Cl and ^{2}H/^{13}C-glucose, with the addition of 85 mg [3-^{2}H] ^{13}C-α-ketoisovalerate and 50 mg [3,3-$^{2}H_2$] ^{13}C-α-ketobutyrate ~1 h prior to induction for selective protonation of ILV methyl groups (Goto et al. 1999). A sample prepared from 10% ^{13}C-glucose M9 minimal media was used to stereospecifically assign valine and leucine methyl groups (Neri et al. 1989). All isotopes were purchased from Cambridge Isotope Laboratories, Inc. The Ssu72 construct contains an N-terminal His tag followed by a thrombin cleavage site. Proteolytic removal of the His tag leaves three extra residues N-terminal to the natural protein sequence, which are disordered and do not contribute peaks to the ^{1}H/^{15}N-HSQC-TROSY spectrum. In the figures and discussion below, residue numbering corresponds to the natural protein sequence. NMR samples for resonance assignment were prepared by extensive buffer exchange into 25 mM sodium phosphate pH 8.0, 25 mM KCl, 2 mM dithiothreitol, with either 5 or 100% D_2O, and brought to a final protein concentration of ~1 mM. NMR samples for titrations with inorganic phosphate were prepared in 25 mM Tris–HCl, 25 mM KCl, 2 mM dithiothreitol and 5% D_2O.

NMR data were collected on 600 and 800 MHz Varian Inova spectrometers equipped with triple-resonance, cryogenically-cooled probes at 30°C. FIDs were processed with NMRPIPE (Delaglio et al. 1995) and datasets were analyzed with CARA (Keller 2004).

Assignments and data deposition

Backbone amide resonances of phosphate-bound *D. melanogaster* Ssu72 were assigned with a ^{2}H/^{13}C/^{15}N-labeled protein sample using a suite of TROSY-based, 3-D triple-resonance experiments—HNCO, the ‘just-in-time’ HN(CA)CO (Werner-Allen et al. 2006), HNCA, HN(CO)CA, HN(CA)CB, and HN(COCA)CB—and the PACES algorithm to identify resonance connectivity (Coggins and Zhou 2003). Amide assignments were confirmed through analysis of a 4-D NH–NH diagonal-suppressed TROSY-NOESY-TROSY experiment (Werner-Allen et al. 2010), and through resonance connectivity in TROSY-based HN(CA)HA and HN(CO-CA)HA experiments collected with a ^{13}C/^{15}N-labeled sample. Overall, every identifiable backbone amide peak in the ^{1}H/^{15}N-HSQC-TROSY spectrum was assigned (Fig. 1), representing 93% (177 of 190) of the non-proline residues

in Ssu72. Residues without amide signals are found in flexible regions, including the N-terminus (residues 1–3) and the active site loop (residues 14–15 and 18–19). Secondary structure predictions based on ^{1}H and ^{13}C backbone chemical shifts agree well with the secondary structures observed in the crystal structure (Supplemental Figure 1). The perdeuterated NMR sample also contained protonated valine γ methyls and leucine and isoleucine δ methyls, which were assigned with a 4-D HC(CCO)NH experiment.

Side-chain resonances were assigned using a 3-D HCCH TOCSY experiment, which worked well for shorter side-chains with efficient magnetization transfer. Longer side-chain assignments were confirmed and extended using intra-residue NOEs in a $^{1}H/^{13}C$-NOESY dataset. Aromatic ring ^{1}H and ^{13}C resonances were assigned using a structure-guided analysis of NOEs in both aromatic and aliphatic 3-D $^{1}H/^{13}C$-NOESY datasets; however, several of these ring resonances were broadened or missing. Methyl groups of valine and leucine residues were stereospecifically assigned using fractional ^{13}C-labeling (Neri et al. 1989). As a result of backbone and side-chain resonance assignment, ~82% of the protons in Ssu72 were assigned, including 95% of the backbone protons and 77% of side-chain protons, along with 80% of the ^{13}C and ^{15}N atoms. These chemical shifts have been deposited in the BioMagResBank database (http://www.bmrb.wisc.edu) under BMRB accession number 17510.

We also used NMR titrations to explore the phosphate-binding properties of wild-type Ssu72 and mutant enzymes with alanine, serine, and aspartate replacements of the catalytic cysteine residue (C13). The catalytic cysteine is part of a conserved CX_5R motif also found in protein tyrosine phosphatases. The C13 side-chain is held as a negatively charged thiolate group and initiates dephosphorylation through nucleophilic attack of the substrate phosphorous atom, while the five following residues form the phosphate-binding active site loop. Together with the side-chain of the conserved arginine (R19), the backbone amides of the active site loop hold the substrate phosphate group in place with strong electrostatic interactions. Catalysis by Ssu72 also requires an aspartate residue (D144) on a flexible loop positioned near the active site (‘the aspartate loop’), which protonates the leaving phosphate to regenerate the enzyme. Surprisingly, we observed a large number of significant perturbations upon titration of wild-type Ssu72 with inorganic phosphate (Supplemental Figure 2A). Given the small size of the phosphate group and the large number of residues affected, it is likely that these perturbations are caused by conformational changes associated with phosphate binding, rather than direct interaction with phosphate. Furthermore, the set of perturbed residues is very similar to the set of residues perturbed by titration of a catalytically-dead C13D/D144N Ssu72 mutant with synthetic pCTD peptide (Werner-Allen et al. 2011), suggesting that phosphate binding alone drives an analogous conformational change.

Next, we tested C13A, C13S and C13D Ssu72 mutants for phosphate binding. While C13D Ssu72 exhibited phosphate-induced chemical shift perturbations similar to the WT enzyme, neither C13A nor C13S showed any perturbations (Fig. 2 and Supplemental Figure 2B). Additionally, no perturbations were observed in a titration of C13S dSsu72 with a synthetic pS5 CTD peptide (data not shown). Notably, a catalytic cysteine to serine mutation in protein tyrosine phosphatase 1B (C215S PTP1B) has been reported to dramatically distort the apo conformation of the active site loop, causing it to flip open into the substrate-binding space (Scapin et al. 2001). It is possible that the loss of a negatively charged catalytic side-chain in the C13A and C13S mutants severely alters the conformation and dynamics of the active site loop of Ssu72, rendering it unable to bind substrate effectively. Interestingly, a recent structure of the catalytic cysteine to serine mutant of human Ssu72 in a ternary complex with pCTD substrate and the scaffolding protein symplekin (Xiang et al. 2010) shows that the active site loop properly recognizes the substrate phosphate group. Therefore,

the requirement of a negatively charged catalytic side-chain may be a species-specific characteristic of *D. melanogaster* Ssu72. Alternatively, the active site loop of the human mutant may be stabilized by the allosteric association with its symplekin binding partner in the ternary crystal complex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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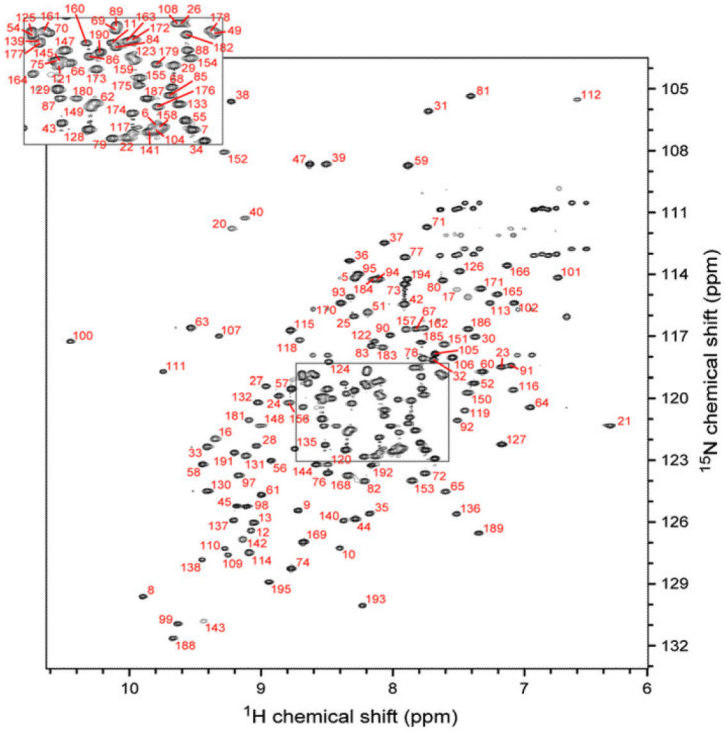

Fig. 1.

Assigned ^{1}H/^{15}N-HSQC-TROSY spectrum of phosphate-bound *D. melanogaster* Ssu72 collected at 800 MHz and 30°C

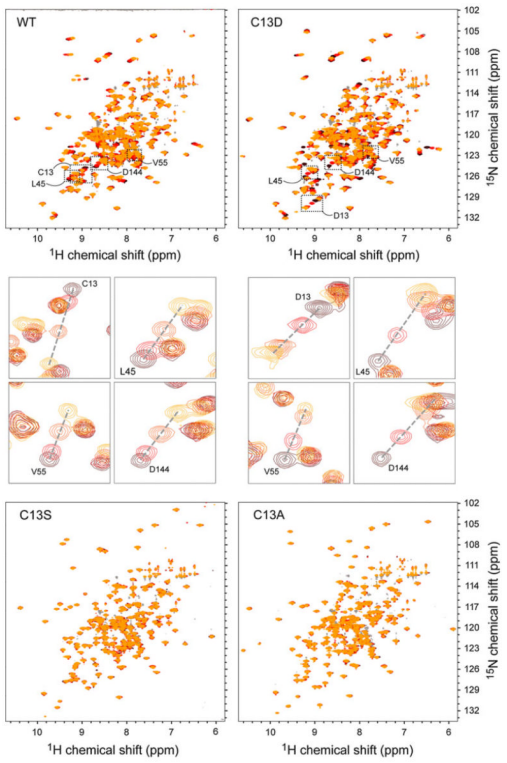

Fig. 2.

The effect of active site mutations on phosphate binding. The phosphate titration of WT dSsu72 is shown alongside identical titrations of C13D, C13S and C13A mutants. Brown to yellow coloring denotes ^{1}H/^{15}N-HSQC-TROSY spectra for apo enzyme to a 25:1 molar ratio of phosphate to enzyme, respectively. Close-up views of peak perturbations for four residues in the WT and C13D titrations are shown below the full spectra