Observation of conformational changes that underlie the catalytic cycle of Xrn2

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Nuclear magnetic resonance (NMR) methods that quantitatively probe motions on molecular and atomic levels have propelled the understanding of biomolecular processes for which static structures cannot provide a satisfactory description. In this work, we studied the structure and dynamics of the essential 100-kDa eukaryotic 5′→3′ exoribonuclease Xrn2. A combination of complementary fluorine and methyl-TROSY NMR spectroscopy reveals that the apo enzyme is highly dynamic around the catalytic center. These observed dynamics are in agreement with a transition of the enzyme from the ground state into a catalytically competent state. We show that the conformational equilibrium in Xrn2 shifts substantially toward the active state in the presence of substrate and magnesium. Finally, our data reveal that the dynamics in Xrn2 correlate with the RNA degradation rate, as a mutation that attenuates motions also affects catalytic activity. In that light, our results stress the importance of studies that go beyond static structural information.

5′→3′ exoribonucleases are essential enzymes that progressively remove nucleotides from the 5′ end of RNA. In the nucleus, this process is carried out by the exoribonuclease Xrn2 (Rat1), which is important for the removal of aberrant pre-mRNAs, rRNA maturation and decay, the processing of small nuclear RNA (snRNA), transcription termination, telomere length regulation and the degradation of microRNAs and hypomodified transfer RNAs (tRNAs). This multitude of cellular functions renders Xrn2 essential in Saccharomyces cerevisiae and Schizosaccharomyces pombe and during development of Caenorhabditis elegans.

The molecular basis of RNA degradation by Xrn2 and its cytoplasmic parologue Xrn1 has been established by several studies. Both enzymes are highly selective for substrates with exposed 5′-monophosphorylated ends and require divalent cations to catalyze the hydrolysis of phosphodiester bonds. Xrn enzymes are highly processive and remove tens of nucleotides per second from the substrate.

The combination of processivity and speed is important, as this enables Xrn1 to closely follow ribosomes in the substrate translocation step, with a particularly pronounced rearward movement taking place at the N-terminal α-helix of Xrn1 (ref. 30). This helix is functionally important, and it has been postulated to constitute a steric hindrance for double-stranded RNA. The functional relevance of the observed helix dynamics is emphasized by a mutant enzyme in which motions and catalytic activity are reduced. Although no structural data have been reported for Xrn2 in a substrate-bound state, the conservation of CR1 and CR2 between Xrn1 and Xrn2 indicate that both enzymes share a common molecular mechanism, in which the N-terminal α-helix can adopt two functionally important conformations.

In this work, we studied the interplay of structure and dynamics in the 100-kDa Xrn2 enzyme. To that end, we combined X-ray crystallography with NMR relaxation dispersion measurements on 13CH3-labeled and 19F-labeled Xrn2 samples. We found that the N-terminal region of the apo enzyme transiently populates an excited state that resembles the pre-hydrolysis substrate-bound state. The functional relevance of the observed helix dynamics is emphasized by a mutant enzyme in which motions and catalytic activity of Xrn2 are reduced.

Results

Chaetomium thermophilum Xrn2 is a canonical 5′→3′ exoribonuclease. High-resolution NMR studies that address protein movements between the apo and substrate-bound states.

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structure is similar to the core structures of the Xrn1 paralogues from *D. melanogaster*4, *Kluyveromyces lactis*30 and *S. cerevisiae*25 (Supplementary Fig. 3).

The active site of the enzyme is positioned in a pocket in front of the ‘tower’ domain helix (helix 4) (Fig. 1c). Seven acidic residues provide a coordination platform for the two catalytically important Mg²⁺ ions (Fig. 1d). Furthermore, we found that His61 and Trp706 are correctly positioned above the active site to form a continuous π–π stack with the RNA substrate, as has been observed for Xrn1 in complex with a pseudo-substrate DNA27 and an mRNA substrate in the context of a stalled ribosome28 (Supplementary Fig. 4).

dynamics require isotope-labeled samples that are stable over multiple days at protein concentration in the high μM range. Obtaining such samples is often a bottleneck, especially for large eukaryotic enzymes. Here, we found that the Xrn2 core from the thermophilic yeast *Chaetomium thermophilum* (CtXrn2, residues 1–875, molecular weight 100 kDa; Fig. 1b and Supplementary Table 1) fulfills those conditions. To lay the structural foundation for our NMR experiments, we first determined the crystal structure of CtXrn2 (Fig. 1c and Supplementary Table 7). In agreement with the high sequence conservation, we found that the CtXrn2 structure closely resembles those of *S. pombe*25 and *C. elegans*26. In addition, the CtXrn2 structure is similar to the core structures of the Xrn1 paralogues from *D. melanogaster*4, *Kluyveromyces lactis*30 and *S. cerevisiae*25 (Supplementary Fig. 3).
C. thermophilum Xrn2 contains a functional zinc finger. In our structure of the Xrn2 enzyme, electron density is lacking for three regions: (1) the top of the tower domain (residues 135–158) that is also less well defined in other Xrn2 structures; (2) the linker region between CR1 and CR2 (residues 423–568) that is highly flexible in solution (Extended Data Fig. 1); and (3) a short loop between residues 266 and 304 that harbors a putative CCHC zinc finger (ZnF) motif. Such a CXCX₅CXXC₆C ZnF motif (where C, H and X correspond to a cysteine, a histidine and any amino acid, respectively) is present in Xrn2 homologues from other Ascomycota (including Neurospora crassa and Aspergillus fumigatus). However, the ZnF is missing in Xrn2 homologues from other Ascomycota (including S. pombe and S. cerevisiae) and is also generally absent in Xrn1. To obtain insights into the functional importance of the Xrn2 ZnF, we solved the NMR structure of this region (Fig. 1f). The structural ensemble (Supplementary Fig. 5 and Supplementary Table 8) displays a well-defined core (residues 269–284) that is similar to ZnFs that interact with (deoxy-)ribonucleotides (Supplementary Fig. 6). In the context of the full-length protein, the ZnF is localized on the side of the enzyme that also contains the active site (Fig. 1c and Extended Data Fig. 2), which prompted us to determine if the Xrn2 ZnF is important for substrate recruitment and/or turnover. To test this, we first measured the binding affinity of 5-mer and 10-mer RNAs to Xrn2. These experiments were recorded in the absence of Mg²⁺ to prevent the rapid degradation of the RNA substrate, and we found that the substrates bind with low nanomolar affinities (Fig. 1g). We next ‘inactivated’ the ZnF by using an Xrn2 ΔZnF construct. This increased the dissociation constant (reduced the affinity) by a factor of 1.5 (5-mer) to 3 (10-mer), corresponding to a ΔG < 2.7 kilojoules per mole (kJ/mol) at room temperature (Fig. 1g). Second, we investigated if the ZnF plays a role in Xrn2 activity (Supplementary Fig. 7). To that end, we compared the degradation rates of the Xrn2 wild-type (WT) protein with a version of the enzyme that lacks the ZnF: As the importance of the ZnF might depend on the extent of secondary structure in the substrate, we performed degradation assays using five RNA substrates with different stem–loop stabilities (Fig. 1b). We found that RNAs with a stem–loop of low stability were degraded with similar rates in the presence or absence of the ZnF. For RNAs that contain increasing numbers of ZnFs that interact with (deoxy-)ribonucleotides (Supplementary Table 6). The finding that the motions in Xrn2 are most sensitive to the exchange parameters (Supplementary Fig. 12) and temperature-dependent parameters for excited state populations (Supplementary Table 6 and Extended Data Fig. 4). The precision of the extracted population is limited, as can be judged from the shallow minimum in the reduced χ² surface (Extended Data Fig. 4). Taken together, our data establish that the α₁–ratchet-helix of Xrn2 samples multiple states in solution in the absence of a substrate, whereas other parts of the enzyme are largely devoid of motions on the millisecond time scale (Supplementary Fig. 10).

19F NMR provides an independent measure of α₁-helix dynamics. The methyl CPMG RD experiments provided accurate information with a high spatial resolution. However, one drawback of those experiments is the extended measurement time that is required (>2 days per dataset). This prevents the execution of NMR measurements in the presence of substrates, as these would be turned over before the acquisition of the data is finished. Recently, we showed that ¹⁹F RD data can be recorded in substantially less time and that this approach can provide accurate insights into bio-molecular exchange processes. Here, we introduced a cysteine in Xrn2 at position 12 that is located at the end of the α₁-helix and exposed on the surface (Fig. 3a,b), and we labeled this residue with bromotrifluoroacetone (BTFA) (Supplementary Fig. 11).

Based on time-efficient one-dimensional (1D) ¹⁹F NMR spectra (Fig. 3c), we recorded CPMG and on-resonance R₁ relaxation dispersion data at ¹H Larmor frequencies of 500 MHz and 600 MHz and at five temperatures between 303 K and 313 K (Fig. 3d,e and Extended Data Fig. 5). For all temperatures, we observed strongly enhanced effective transverse relaxation rates at CPMG/spin-lock frequencies below 500 Hz. We fit all data simultaneously with a single global chemical shift difference parameter Δω and temperature-dependent parameters for excited state populations pₑₛ and exchange rates kₑₛ (Supplementary Figs. 12 and 13). We obtained |Δω| = 0.15±0.01 p.p.m. and found that kₑₛ increased from 445±67 s⁻¹ to 913±108 s⁻¹ between 303 K and 313 K (Fig. 3e and Extended Data Fig. 6). The population of the excited state is high (>~20%) for all temperatures, although it is not possible to determine pₑₛ with high accuracy, as was the case for the ¹³C data. We note that the CPMG data contain less information than the on-resonance R₁ data, as for the latter, it is possible to record more data points at low (spin-lock) frequencies that are most sensitive to the exchange parameters (Supplementary Fig. 12 and Supplementary Table 6). The finding that the motions in Xrn2 are localized to a region around the active site is corroborated by measurements on samples where the ¹⁹F label has been positioned at other sites in Xrn2 (Supplementary Fig. 14). In addition, we found...
that $^{19}$F RD profiles that we recorded for a specific position are the same for enzymes that have been labeled using BTFA or trifluorothioanethiol (TET) (Fig. 3f). Together, these observations establish that our $^{19}$F RD profiles are suitable for detecting dynamic processes of the region around the active site of Xrn2. The exchange rates at 313 K ($k_{ex}$) that we extract from the $^{19}$F data and from the methyl-TROSY data at 313 K are compatible ($k_{ex}^{19F} = 710 \pm 63$ s$^{-1}$ and $k_{ex}^{19F} = 913 \pm 108$ s$^{-1}$), whereas $p_{ES}$ is, in both cases, relatively high. The consistency of the $^{13}$C and $^{19}$F measurements is further confirmed by a global fit of the $^{13}$C and $^{19}$F RD data (Extended Data Fig. 7), from which we obtained an exchange rate of $864 \pm 112$ s$^{-1}$ (Supplementary Table 6).
Substrate binding changes the structure and dynamics of Xrn2. The fast degradation of RNA by Xrn2 prevents the NMR characterization of a complex between Xrn2 and an RNA substrate. To investigate the structural changes that occur in Xrn2 upon substrate interaction, we used three substrates with strongly attenuated degradation rates: 3′,5′-bisphosphoadenosine (pAp), a 5-mer DNA and an exoribonuclease-resistant RNA (xrRNA). It is important to note that we use the catalytically active WT Xrn2, as versions of the enzyme that lack the catalytically important magnesium ions likely display altered interactions and/or motions.

First, we used pAp, which is hydrolyzed to adenosine 5′-monophosphate (AMP) at a rate of 0.05 min⁻¹ (Fig. 4a). This allowed us the measurement of HMQC spectra of Ile6-[13CH₃]-labeled Xrn2 in the presence of an excess of pAp (Fig. 4b). The binding of pAp to Xrn2 resulted in substantial chemical shift perturbations (CSPs) of the methyl group resonances that are in agreement with a conformational change around the active site of the enzyme (Fig. 4c). Interestingly, the carbon chemical shifts of the excited apo state that we extract from the CPMG experiments (Fig. 2c) correlate linearly with the carbon chemical shifts that we measure in the presence of pAp (Fig. 4d). Likewise, the 19F chemical shift difference upon the addition of pAp to Xrn2-deltaZnF-N12C(11) at 0.10 p.p.m. is in agreement with pAp•Δω = 0.07–0.12 p.p.m. for 0.5 < pAp < 0.8 and |Δω| = 0.19 p.p.m. that we determined from the RD experiments in the absence of the substrate (Fig. 4e). These findings support the notion that the apo enzyme transiently populates a state that is structurally similar to the pAp-bound conformation. Interestingly, 19F line widths and RD experiments reveal that the dynamics in Xrn2 are substantially reduced upon substrate binding (Fig. 4f). Taken together, our data reveal that Xrn2 locks into a state that is structurally similar to the pAp-bound conformation.

![Diagram of Xrn2 with labeled residues](image1)

![Diagram of Xrn2 with labeled residues](image2)

**Fig. 3** | 19F NMR supports the sampling of an excited conformational state by the α1-helix. 
- **a**: Close-up view of the α1-helix, where the mutation N12C was introduced to allow for BTFA labeling. Hydrophobic residues are shown in blue; charged and polar solvent-exposed residues are shown in yellow. 
- **b**: Helical wheel projection for residues 3–12 in the α1-helix. Coloring as in **a**. 
- **c**: 19F NMR spectrum of Xrn2, Helical a1-helix. Coloring as in **wheel projection for residues 3–12 in the a1-helix.** 
- **d**: CPMG and on-resonance R₁ dispersion profiles for Xrn2 at 313 K. The data were fit with the Δ dispersion profiles of Xrn2, CPMG s.d., derived from 500 Monte Carlo simulations (Extended Data Fig. 5, Supplementary Figs. 12 and 13 and Supplementary Table 6).

![Graph showing dispersion profiles](image3)

![Graph showing dispersion profiles](image4)
Fig. 4 | Substrate binding induces a conformational change to a more rigid Xrn2 state. a, Left: 1H NMR spectrum showing the H1' resonance of pAp (orange) and AMP (purple). Right: linear fit of the integrated peak intensities reveals a turnover rate of 0.05 min$^{-1}$. Data points are shown as integrated peak intensities; error bars represent spectral noise. b, Overlay of methyl-TROSY spectra in the absence (black) and presence (dark blue) of pAp. Upon interaction with the substrates, the 19F line width is reduced from 55 Hz to 36 Hz (pAp) and 32 Hz (pdA5), respectively. Data points are shown as mean ± s.d., derived from 500 Monte Carlo simulations (Extended Data Fig. 7 and Supplementary Table 6). c, 19F NMR spectra of Xrn2 ZnF N12CBTFA without ligand (black) and bound to pAp (light blue) or pdA5 (dark blue). Upon interaction with the substrates, the 19F line width is reduced from 55 Hz to 36 Hz (pAp) and 32 Hz (pdA5), respectively. f, CPMG RD profiles of Xrn2 ΔZnF N12CBTFA in the absence (gray) and presence (light blue) of pAp. Data points are shown as mean ± s.d., derived from three duplicate NMR measurements. g, Overlay of methyl-TROSY spectra in the absence (black) and presence (dark blue) of pdA5 (that is degraded to pdA2). CSPs are observed in the Ile region; M704 experiences a characteristic shift in the 1H dimension. h, CPMG RD profiles of Xrn2 ΔZnF N12CBTFA in the absence (gray) and presence (dark blue) of pdA5/pdA2. Data points are shown as mean ± s.d., derived from three duplicate NMR measurements. i, Overlay of methyl-TROSY spectra in the absence (black) and presence (yellow) of an xrRNA. The shift of M704 as well as Ile-δI CSPs close to the active site and the RNA entry site show that the complex is locked in the pre-translocation conformation. a.u., arbitrary unit.
into a stable active conformation (the post-translocation state) upon substrate recruitment. As a consequence of the more rigid nature of the Xrn2:substrate complex, we were able to assign nine additional Xrn2 methyl resonances (Fig. 4c) that were broadened beyond detection in the apo state due to conformational exchange. To obtain additional information on the structural and conformational changes in Xrn2 upon substrate binding, we next made use of a 5′-phosphorylated DNA pentanucleotide (pA5) that is readily degraded into a 2-mer DNA (pA2) and free nucleotides (Supplementary Fig. 15). pA2 is one nucleotide longer than the pAp that we used above (Fig. 4b) yet induced similar chemical shift perturbations in the HMQC spectrum (Fig. 4g and Supplementary Fig. 16). In analogy to pAp binding, the binding of pA2 to Xrn2 also abolished the extensive motions in the enzyme (Fig. 4h). pAp and pA2, thus, both induce the post-translocation conformation in Xrn2. Interestingly, in the presence of pA2, we noticed a CSP of Met704 that was absent in the pAp-bound states (Fig. 4g). As pA2 is one base longer than pAp, we concluded that the CSP of Met704 reports on the location of the second base of the Xrn2 substrate. In the structure of Xrn2, Met704 is positioned in direct proximity to the conserved Trp706 residue, which stacks with the third nucleotide base in the substrate-binding pocket of Xrn1 (ref. 39). The addition of Xrn2 to this xrRNA results in the rapid hydrolysis of the unstructured 5′ region and the subsequent formation of a stable complex between Xrn2 and the xrRNA pseudoknot that is sterically hindered to translocate into the active state. (Supplementary Fig. 17). The HMQC spectrum of this complex shows overall reduced peak intensities that are particularly pronounced for resonances of residues between the α-helix and the active site (Fig. 4i and Supplementary Fig. 18). The reduced resonance intensities can be attributed to the increase in molecular weight and the large number of protons from the xrRNA that come close to Xrn2 methyl groups and, thereby, enhance relaxation rates. Nevertheless, we can conclude that the active post-translocation state of the enzyme is not stably formed, as the signature chemical shifts of the active state (Fig. 4b) are not fully observed. Met704, on the other hand, shows a clear CSP, which confirms that the 5′ end of the trimmed xrRNA interacts in the substrate-binding pocket. Taken together, these data show that the Xrn2 enzyme is locked in a pre-translocation state in the presence of the xrRNA product, as was observed in the cryo-EM structure of Xrn1 in the presence of a stalled ribosome30.

In summary, our data reveal that the apo Xrn2 enzyme is in a dynamic equilibrium between the pre-translocation and post-translocation states. Upon recruitment of a substrate and in the presence of Mg2+, Xrn2 mainly adopts the post-translocation state. The A5F mutation alters Xrn2 dynamics and activity. To investigate how the dynamics in Xrn2 are correlated with RNA substrate degradation, we introduced single point mutations in or close to the α1-helix with the aim of changing the dynamics around the active site (Supplementary Table 5). We found that the A5F mutation led to substantially reduced relaxation dispersions (Fig. 5a), indicative of substantial changes in the energy landscape of the enzyme. It is important to note that this mutation does not interfere with the overall structure of the enzyme or with the interaction between the substrate and the enzyme (Extended Data Fig. 9). These data can be explained by a higher energy barrier between the pre-translocation and post-translocation states. Interestingly, we found that the A5F mutation decreases the activity of Xrn2 by 30~50%, depending on the substrate (Fig. 5b). Based on these findings, we conclude that the A5F mutation results in changes in the dynamics of Xrn2 and, at the same time, in a decrease in the enzymatic turnover rates.
Regulation of Xrn2 activity. In previous studies, it was shown that the activity of Xrn2 can be enhanced by the interaction with binding partners. On the one hand, Xrn2 in higher eukaryotes can recruit XRN2-binding domain (XTBD) containing binding partners that have been proposed to stabilize the Xrn2 fold in the absence of a substrate. However, in our crystal structure of apo CtxRN2, this XTBD segment is already fully folded (Supplementary Fig. 19), and the chemical shifts of Ile687 and Ile688 δ 1-methyl groups clearly deviate from the shift of Ile61-methyl groups in unstructured regions (Fig. 2a). As XTBD-containing proteins have only been identified in metazoa, our findings suggest that the mechanisms that regulate the catalytic activity of Xrn2 differ between yeast and metazoa. On the other hand, yeast Xrn2 directly interacts with the pyrophosphohydrolase Rai1 in S. pombe; this interaction enhances the activity of the enzyme due to the Rai1-induced stabilization of the Xrn2 fold. Xrn2 and Rai1 also form a stable complex in C. thermophilum (Supplementary Fig. 20); however, we observe that the recruitment of CtxRai1 to CtxRN2 does not result in an enhancement of Xrn2 activity (Supplementary Fig. 21), and neither are the structure or motions in the active site of Xrn2 influenced by Rai1 binding (Extended Data Fig. 10). In S. pombe, Rai1 enhances Xrn2 activity especially for RNA substrates that contain stable secondary structure elements, indicating that Rai1 possesses RNA unwinding activity. In C. thermophilum, this unwinding activity appears to have moved from the Xrn2-interacting protein Rai1 to the ZnF domain in the enzyme.

Discussion
Static macromolecular structures provide important information about biochemical mechanisms. It is, however, important to realize that these static structures (1) do not necessarily represent the ground state conformation of the macromolecule in solution and (2) fail to reveal the dynamical processes that underlie allostery or catalysis. In that regard, it is, thus, of central importance to complement our understanding of molecular function with information regarding dynamic processes, although this can be technically challenging.

A catalytic cycle of a chemical reaction comprises multiple steps, where the overall rate of the reaction is limited by the slowest of these steps. Enzymes can increase the speed of these reactions substantially, by reducing the rate-limiting step that typically involves the breaking or formation of chemical bonds. In an enzyme-catalyzed chemical reaction, the slowest step can then change from the chemical step to a conformational change in the enzyme. This has been shown to be the case for, for example, dihydrofolate reductase (DHFR), which needs to rearrange from a closed conformation where catalysis can take place to an occluded conformation that is required for product release. Likewise, the opening of a lid domain was found to constitute the rate-limiting step in the reaction cycle of hyperthermophilic and mesophilic homologs of the enzyme adenylate kinase.

In this study, we exploited methyl-TROSY (Figs. 2 and 4) and 19F (Figs. 3, 4 and 5) NMR techniques, and we show that the N-terminal α1-helix in the 5′→3′ exoribonuclease Xrn2 is highly dynamic. In the apo state, the enzyme adopts the pre-translocation (ground) state (Fig. 1a and Supplementary Fig. 2) between 50% and 80% and the post-translocation (active/excited) state between 20% and 50% (k<sub>ES</sub> = 864 s<sup>−1</sup>; 173 s<sup>−1</sup> < k<sub>ES</sub> < 432 s<sup>−1</sup> and 432 < k<sub>GS</sub> < 691 s<sup>−1</sup> ).

Substrate binding shifts the conformational equilibrium to the post-translocation state, likely by reducing the free energy of this conformation. As a result, the backward rate (k<sub>GS</sub>) will be substantially reduced (to less than 10 s<sup>−1</sup>) while the forward rate (k<sub>ES</sub>) remains unchanged. This scenario is in full agreement with the changes in the 19F RD profile that we observe upon substrate binding (Fig. 4f,h). Interestingly, for RNA substrates without strong secondary structure elements (AU hairpin RNA), the turnover rate (127–163 s<sup>−1</sup>) is close to the rate with which the enzyme transitions from the pre-translocation state to the post-translocation state (k<sub>ES</sub>). This suggests that this transition can constitute the rate-limiting step in the catalytic cycle. This notion is further corroborated by the observation that k<sub>ES</sub> and k<sub>GS</sub> both linearly change with temperature (Extended Data Fig. 6). Finally, the central importance of the dynamics of the N-terminal helix is revealed by the ASF mutant enzyme that results in changes in the energy landscape of Xrn2 (Fig. 5) that coincide with a reduction in the turnover rates.

In summary, we made extensive use of NMR methods to quantify and localize structural changes in Xrn2. It should be noted that Xrn2 (875 residues, 100 kDa) is among the largest single-chain enzymes that have so far been studied using solution-state NMR methods. We exploited methyl labeling strategies, which provide a higher spatial resolution, and 19F labeling, which is able to provide information regarding protein motions in short times. It is important to note that positioning of the fluorine label was directly guided by data obtained from the methyl-labeled samples. We found that this approach that combines methyl group and fluoride labeling is highly efficient. However, we would also like to stress the importance of cross-checking that the methyl and fluoride labels report on the same molecular process. Here, we ensured this by comparing the exchange parameters that were extracted from 19F-based and 19F-based RD measurements and by labeling the enzyme with two independent fluorine-containing compounds (BTFA and TET). Importantly, the rapid (1-hour) 19F experiments allowed us to obtain information on catalytically active Xrn2 in the presence of substrates, before those were fully processed. We anticipate that the combination of complementary NMR labeling techniques together with information on static structures will be an important approach to obtain fundamental insights into biologically important dynamics in large enzymes.

Online content
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Methods

Molecular biology. The protein-coding sequence of Xrn2 from C. thermophilum (National Center of Biotechnology Information (NCBI) reference sequence XP_0086691140.1) was synthesized by GenScript with codon optimization for expression in E. coli and provided in a pUC19 vector. A construct encompassing amino acids 1–875 (Supplementary Table 1) was cloned into a pET28b+ vector (Novagen) using NdeI and XhoI restriction sites (hereafter referred to as CxXrn2). The vector carries no additional amino acids at the N-terminus of the construct and a C-terminal His6-tag constitutive by the sequence LEHHHHHHH, where the amino acids LE are a remnant from the restriction site.

A construct lacking the residues 263–300, corresponding to the Znf loop (hereafter referred to as CxXrn2 ΔZnf) was prepared using Gibson assembly cloning. All point mutations were introduced using site-directed mutagenesis (hereafter referred to as CtXrn2).

The 13C/15N-labeled ZnF was expressed in M9 medium containing 0.5 g L−1 of 2H12C-labeled glucose) culture to an OD of 0.1–0.15. For labeling with 3-bromo-μM T7 RNA polymerase (prepared with Coot53 and Phenix54, respectively. Structure factors and coordinates have been deposited in the PDB under accession code 7OPK.

Cysteine fluorine labeling. Proteins were fluorine labeled after gel filtration at 100 μM. For labeling with 1,1,1-trifluoroacetone (BTA), the sample was cooled on ice, and BTA was added to a final concentration of 10 mM. The reaction was incubated for 30 minutes on ice and quenched with 20 mM DTT, and the sample was purified over a PD10 or PD Minipart G-25 desalting column pre-equilibrated with GF buffer. NMR samples were then treated with 0.03% NaN3, and 5% D.O. For labeling with (CuPh), the sample was cooled to 4 °C; dichloro-(1,10-phenanthroline)-copper (II) (CuPh) was added to a final concentration of 20 μM; and TET was added to a final concentration of 500 μM. The reaction was followed in 1H spectra until free TET was depleted.

RNA preparation. 5′-monophosphorylated RNAs were prepared by in vitro transcription (IVT) in the presence of an excess of GMP over GTP. In brief, a template DNA oligonucleotide with the desired RNA sequence in reverse complement followed by a reverse complementary 17 promoter sequence at the 3′ end (5′-TATAGTGACGCTACATAGG-3′; Supplementary Table 4) was used in equimolar amounts with an antisense T7 promoter oligonucleotide (5′-CGTAAACGCAGCCTATAGG-3′) in the reaction at a concentration of 1 μM. The reaction included 40 mM Tris (pH 8.0), 5 mM DTT, 1 mM spermidine, 0.01% Triton X-100, 10–40 mM MgCl2, and 0.2 μM T7 RNA polymerase (prepared in-house). GMP was included at a concentration of 10 mM, and the required nucleoside triphosphates were included typically at 4 mM concentrations, with the exception of GTP, which was used at 1 mM. The reaction mixtures were brought to 37 °C for 4 hours to overnight, after which 50 mM EDTA was added to dissolve the magnesium-pyrophosphate precipitate. Reaction products were precipitated with 0.7 reaction volumes of isopropanol and 0.1 reaction volumes of 2H12C-labeled glucose 0.1 M NaOAc (pH 5.3) with subsequent cooling at −20 °C for at least 30 minutes. The precipitate was pelleted by centrifugation, washed with cold 70% ethanol, air dried and resuspended in 5 M urea and 20 mM Tris (pH 8.0). The solution was applied to a preparative anion exchange DNAac column (Dionex) and separated by size with a NaCl gradient. Fractions containing RNA were analyzed with urea polyacrylamide gel electrophoresis, and the fractions of interest were pooled, precipitated with isopropanol, washed with 70% ethanol and resuspended in 10 mM H2O. The samples were supplemented with 0.03% NaN3 and 5% D2O.

For fluorescence anisotropy measurements, RNAs (5-mer 5′-GGAGU-3′ and 10-mer 5′-GGAGAGAGAGUG-3′) were fluorescently labeled with 6-iodoacetamido-fluorescein. To this end, IVTs were carried out in the presence of 200 μM 4-thiouridine, and the RNA was precipitated, washed with 70% ethanol, resuspended in 0.1 M sodium phosphate (pH 8.0) and incubated with 10 mM iodoacetamido-fluorescein at room temperature overnight. The RNA was twice precipitated and resuspended in H2O and purified with 0.1% bromophenol blue and 0.1% xylene cyanole and analyzed by urea polyacrylamide gel electrophoresis.

Activity assays. RNase assays were performed in GF buffer (25 mM HEPES (pH 7.3), 125 mM NaCl and 1 mM DTT) with 10% protein concentrations of 5–125 μM. Protein samples used for activity assays were prepared freshly and dialyzed against 50 mM NaH2PO4 (pH 7.4), 150 mM NaCl and 1 mM DTT with simultaneous TEV protease cleavage after the Na-afinity chromatography step. The cleaved His6-G81 tag was removed by applying the solution to a second Ni-afinity chromatography step, where the resin was equilibrated in buffer B (50 mM Na phosphate (pH 7.4), 150 mM NaCl and 300 mM imidazole), until a Bradford test did not show staining anymore, and 2 μM EDTA was added to the eluent. The elution was concentrated to a volume of 1–1.5 mL and purified by size-exclusion chromatography using a 16/600 Superdex 200 column in GF buffer (25 mM HEPES (pH 7.3), 125 mM NaCl and 1 mM DTT). The target fractions were combined and concentrated. For deuterated samples, the buffer was exchanged in this concentration step by multiple rounds of addition and precipitation with a 250 mM NaCl and 1 mM DTT in D2O and subsequent concentration. NMR samples were supplemented with 0.03% NaN3, and 5% D2O (for samples not in 100% D2O).

The 1C/15N-labeled ZnF was expressed in M9 medium containing 0.5 g L−1 of NH4Cl and 2 g L−1 of H3C-labeled glucose. The purification followed the procedure described above but included a dialysis step against 50 mM Na phosphate (pH 7.4), 150 mM NaCl and 1 mM DTT with simultaneous TEV protease cleavage after the Ni-afinity chromatography step, where the resin was equilibrated in buffer A. The flow-through of the column was collected, concentrated and purified using a 16/600 Superdex 75 column equilibrated in GF buffer. The target fractions were pooled and concentrated again, and the sample was supplemented with 0.03% NaN3 and 5% D2O.

Crystal structure determination. Crystallization of C. thermophilum Xrn2 1-875 was carried out with vapor diffusion in sitting drops, derived from 0.3 μL of Xrn2 (36 mg mL−1) in 25 mM HEPES (pH 7.3), 125 mM NaCl, 0.5 mM DTT and 0.1 μL of the precipitant solution (20% w/v precipitant in 0.1 M NaCl, 0.1 M NaH2PO4, 25% 2H12C-labeled glucose and 10% 2H12O). Crystals grew as thin needles within 2–4 weeks. Crystals were briefly incubated in the reservoir solution with 30% glycerol added for cryoprotection before harvesting. Diffraction data were collected at 100 K on the PXII beamline at the Swiss Light Source with a wavelength of 1 Å and processed with XDS5. S. pombe Xrn2 (Rat1, Protein Data Bank (PDB) entry 3PQ3) was used as a search model for molecular replacement, which was performed with the CCP4 utility Phaser5. The final structure was obtained after performing several cycles of iterative model building and refinement with Coot6 and Phenix7, respectively. Structure factors and coordinates have been deposited in the PDB under accession code 7OPK.
Articles

166.7 Hz, 250 Hz, 333.3 Hz, 416.7 Hz, 500 Hz, 666.7 Hz, 833.3 Hz, 1,000 Hz, 1,166.7 Hz, 1,333.3 Hz, 1,666.7 Hz and 2,000 Hz. All datasets were recorded either two or three times to obtain error estimates for all data points. 1H-19F TROSY spectra of Xnr2 and Xnr2 linker (residues 265–293) were recorded with a Bruker Avance III 600 MHz spectrometer equipped with a TXI room temperature probehead.

19F spectra were recorded with Bruker Avance III 500-MHz and 600-MHz spectrometers equipped with nitrogen-cooled TCI probeheads. 1D spectra were recorded with the aring pulse sequence from Bruker. 1H-19F CPMG and 1H-19F R1 experiments were recorded in a pseudo-2D and pseudo-3D fashion, respectively, using previously published pulse sequences that include an aring sequence. Data were recorded with a recycle delay of 1.0 second. All 19F-CPMG experiments were recorded with a constant time T1CPSM of 16 ms and CPMG frequencies of 62.6 Hz, 125 Hz, 187.5 Hz, 250 Hz, 312.5 Hz, 375 Hz, 437.5 Hz, 500 Hz, 562.5 Hz, 1,000 Hz, 1,500 Hz, 2,000 Hz, and 5,000 Hz (experiments on the 500-MHz spectrometer) and CPMG frequencies of 62.5 Hz, 125 Hz, 187.5 Hz, 250 Hz, 312.5 Hz, 375 Hz, 437.5 Hz, 500 Hz, 625 Hz, 1,000 Hz, 1,500 Hz, 2,000 Hz, 2,500 Hz, 3,000 Hz, 3,500 Hz, 4,000 Hz, 4,500 Hz, and 5,000 Hz (experiments on the 600-MHz spectrometer), respectively. 19F-R1 experiments were recorded with five different spin-lock times TSL (0, 4, 8, 16, and 32 ms) and 18 different spin-lock fields on the 600-MHz spectrometer (50 Hz, 75 Hz, 100 Hz, 150 Hz, 200 Hz, 250 Hz, 300 Hz, 400 Hz, 500 Hz, 600 Hz, 700 Hz, 800 Hz, 1,000 Hz, 2,500 Hz, 3,000 Hz, 5,000 Hz and 8,000 Hz) as well as 18 different spin-lock fields on the 600-MHz spectrometer (50 Hz, 75 Hz, 100 Hz, 150 Hz, 200 Hz, 250 Hz, 300 Hz, 400 Hz, 500 Hz, 600 Hz, 700 Hz, 800 Hz, 1,000 Hz, 2,000 Hz, 3,000 Hz, 5,000 Hz and 8,000 Hz). The 1F frequency carrier was centered on the respective peak maximum in the 1D 19F spectrum. All 19F-R1 relaxation dispersion datasets were recorded in triplicate to obtain experimental errors for all data points, with the exception of V416C (Supplementary Fig. 14), where the errors in the intensities were determined based on the noise level of the spectra. NMR spectra were processed with the NMRPipe/NMRDraw software.

NMR structure calculations of the ZnF. All spectra used for the structure calculation were acquired at 288 K on Bruker Avance III 500-MHz and 600-MHz spectrometers equipped with nitrogen-cooled TCI probeheads. Assignment of the ZnF (residues 265–293) backbone and sidechain resonances were recorded with in-house-written MATLAB scripts as described previously. Minimum standard deviations were set to 2 Hz to avoid excessive weighting of data points with accidentally low errors.

To extract errors in the parameters extracted from the NMR relaxation data, 500 cycles of Monte Carlo simulations were carried out by random variation of R2 rates according to their standard deviation and subsequent execution of the nonlinear fitting routine on the resulting pseudo-3D datasets. The errors in the intensities were determined based on the noise level of the spectra. NMR spectra were processed with the NMRPipe/NMRDraw software.

Data processing, analysis and fitting. Methyl-TROSY CPMG pseudo-3D data were processed with NMRPipe and analyzed with in-house-written MATLAB scripts. In brief, peak intensities I corresponding to CPMG frequencies νCPMG were extracted from 2D planes with NMRPipe and normalized to a reference intensity I0 obtained by omitting the CPMG element. The normalized intensity I/I0 was then converted into effective transverse relaxation rates R2,eff by assuming an exponential decay. The data were fitted numerically to a global two-state model to extract the exchange parameters pR, pM, ΔωM, and ΔωR, R2,M and R2,R where the index i refers to the residue number. A minimum standard deviation of 0.2 Hz was assumed for the fit.

1F CPMG pseudo-2D datasets were processed with NMRPipe and analyzed with in-house-written MATLAB scripts as described previously. Minimum standard deviations were set to 2 Hz to avoid excessive weighting of data points with accidentally low errors.

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Author contributions
J.H.O and R.S. designed experiments. J.H.O. performed experiments and analyzed and interpreted data. D.S. measured turnover rates. A.-L.F. helped with solving the crystal structure of Xrn2. J.P.W. helped with recording and analyzing NMR data of the ZnF and with the NMR structure calculations. J.H.O. and R.S. wrote the manuscript.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Remco Sprangers.
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Extended Data Fig. 1 | $^1$H-$^{15}$N TROSY spectra of Xrn2 (residues 1-875) and the Xrn2 linker (residues 265-293) that connects CR1 and CR2. The $^1$H-$^{15}$N TROSY NMR spectrum of Xrn2 (black; residues 1-875) displays only $^1$H-$^{15}$N correlations from highly flexible parts of the protein; the $^1$H-$^{15}$N resonances in the protein core are broadened beyond detection due to the high molecular weight of the enzyme. The $^1$H-$^{15}$N spectrum from the isolated Xrn2 linker region (yellow; residues 265-293) largely overlaps with the spectrum from Xrn2 (1-875), proving that the CR1-CR2 linker region is flexible and disordered in the context of the full length protein.
Extended Data Fig. 2 | The ZnF transiently interacts with the Xrn2 core. Overlay of methyl TROSY NMR spectra of the Xrn2 enzyme with (black, WT) and without (purple, Delta ZnF) the ZnF. Removal of the ZnF does not perturb the structure of the Xrn2 enzyme, as can be concluded from the limited number of residues that experience CSPs. The observed CSPs upon deletion of the ZnF can be explained by the proximity of these residues to the (deleted) ZnF (for example I235) and by a transient interactions between the ZnF and a region of the enzyme that is located around the active site (I89, I59, I166, I314 and I850). In summary, these data thus show that the ZnF is loosely associated with Xrn2 and transiently interacts with the region of the enzyme that also contains the active site.
Extended Data Fig. 3 | $^{1}H$-$^{13}C$ methyl TROSY spectrum of Xrn2 at 293 K and exemplary CPMG RD curves. Methyl TROSY NMR spectrum of Xrn2 recorded at 293 K. Resonances that could only be assigned at 293 K are indicated in magenta. At 313 K these resonances could not be assigned due to strong exchange broadening. The residues that appear at 293 K display elevated relaxation rates at lower CPMG frequencies in both $^{13}C$ SQ and $^{1}H$-$^{13}C$ MQ CPMG RD experiments. Due to overall enhanced relaxation and increased peak overlap at 293 K, a faithful fit of the CPMG RD data could not be reliably performed at the lower temperature. Data points are shown as mean ± s.d., as derived from 2 duplicate NMR measurements.
Extended Data Fig. 4 | Reduced $\chi^2$ surfaces obtained from constrained parameter optimization and distribution of fit parameters obtained from Monte Carlo trials: $^{13}$C data. (A) One dimensional reduced $\chi^2$ surfaces for $^{13}$C (SQ and MQ) CPMG RD data, where the fit is restricted by the exchange rate $k_{ex}$ (left) or the ground state population $p_{GS}$ (right). The reduced $\chi^2$ as a function of $k_{ex}$ exhibits a well defined minimum around 700-800 s$^{-1}$, indicating that the exchange rate is well defined by the experimental data. The plot of reduced $\chi^2$ as a function of $p_{GS}$ on the other hand reveals a shallow minimum around $p_{GS} = 82\%$. In the current exchange regime the precision with which $p_{GS}$ can be determined strongly depends on the lowest CPMG frequency that can be recorded, which is in turn limited by the fast relaxation rates of the very large enzyme. Note that $p_{GS}$ is well defined towards higher ground state populations (that is $\chi^2$ strongly increases for larger $p_{GS}$), but loosely defined towards lower ground state populations (that is $\chi^2$ only moderately increases for lower $p_{GS}$). (B) Subplots show the distribution of the exchange constant $k_{ex}$ versus the ground state population $p_{GS}$, as well as versus the carbon chemical shift differences $|\Delta\omega_C|$ for the isoleucine residues that were used in the fitting. The distributions are based on a global fit of two RD experiments (SQ and MQ) for the 5 indicated residues at 3 magnetic field strengths (500, 600 and 800 MHz proton frequency). Note the typical correlation between $\Delta\omega$ and $p_{GS}$ in the residues specific plots. The extracted fitting parameters, including errors are summarized in Supplementary Table 6.
Extended Data Fig. 5 | Temperature dependent $^{19}$F relaxation dispersion. $^{19}$F CPMG (left) and $R_1^\rho$ (right) relaxation dispersion of Ctxrn2 1-875 ΔZnF N12C87K at temperatures between 303 K and 313 K and $^1$H Larmor frequencies of 500 MHz and 600 MHz. Fit parameters of the global fit and separate fits of CPMG and $R_1^\rho$ datasets are included in Table S6. Note that the $R_1^\rho$ data contains more datapoints at low frequencies. In the CPMG experiments the number of possible frequencies is determined by the relaxation delay and can only be a multiple of $1 / T_{relax} = 62.5 \text{Hz}$ (for the $T_{relax}$ of 16 ms that was used). The $R_1^\rho$ experiment, on the other hand, can use arbitrary spin-lock frequencies and is thus able to sample the RD curve at low frequencies better. Note that very low spinlock frequencies cannot be used in $R_1^\rho$ experiment, as then the signal decay is no longer mono-exponential. The extracted fitting parameters, including errors are summarized in Supplementary Table 6. Data points are shown as mean ± s.d., as derived from 3 duplicate NMR measurements.
Extended Data Fig. 6 | The exchange rate $k_{\text{ex}}$ is linearly correlated with the activity in the Xrn2. The activity (turnover rate) and exchange rates ($k_{\text{ex}}$) were measured with an Xrn2 ΔZnF N12C-BTFA sample. Activities are derived from HPLC assays, exchange rates are derived from the global fit of 19F relaxation dispersion data (Fig. 3E). The linear correlation between $k_{\text{ex}}$ ($k_{\text{ex}} = k_{\text{GS-eS}} + k_{\text{S-GS}}$) and turnover rates suggests that the exchange and degradation rates are functionally linked. In the apo-state of the enzyme, the population of the excited (active) state is between 20 and 50%. The forward rate ($k_{\text{GS-eS}}$) is thus between 0.2*$k_{\text{ex}}$ and 0.5*$k_{\text{ex}}$. For a 20% excited state the $k_{\text{GS-eS}}$ rates (as measured) in the apo-enzyme correlate directly with the turnover rate (activity) of the AU10 RNA, indicating that the translocation of the substrate from the pre-translocation state II to the active state is rate limiting during the catalytic cycle. The degradation rates of RNAs with a stable GC stemloop structure (GC12 RNA) is considerably slower, as the rate limiting step in the catalytic cycle moves from the dynamics in the N-terminal helix to the time it takes to melt the secondary structure in the RNA substrate. Data points are shown as mean ± s.d. The errors in the activities are derived from 2 to 4 independent experiments; the errors in the rates are derived from 500 MC simulations (Extended Data Fig. 5, Supplementary Figs. 12 and 13 and Table S6).
Extended Data Fig. 7 | Reduced χ² surfaces obtained from constrained parameter optimization and distribution of fit parameters obtained from Monte Carlo trials: simulations for ¹⁹F and ¹³C RD data. (A) One dimensional reduced χ² surfaces for the simultaneous analysis of ¹³C CPMG, ¹⁹F CPMG and ¹⁹F R₁ relaxation data. Reduced χ² values were obtained as a function of the exchange rate and the ground state population after optimization of all remaining free parameters. (B) Distribution of fit parameters obtained from MC simulations, where ¹³C CPMG, ¹⁹F CPMG and ¹⁹F R₁ relaxation data were fitted simultaneously. The extracted fitting parameters, including errors are summarized in Supplementary Table 6.
Extended Data Fig. 8 | In the absence of Mg\textsuperscript{2+} ions pdA5 interacts with the RNA binding pocket, but does not induce the active conformation in Xrn2.

Overlay of the \textsuperscript{1}H-\textsuperscript{13}C HMQC methyl-TROSY spectra of the apo state (black) and after addition of pdA5 (green), recorded at 18.8 T and 313 K. CSPs of M704 (right panel) clearly report on the interaction of pdA5 with the RNA-binding pocket. Spectra are recorded in the absence of Mg\textsuperscript{2+}. The absence of CSPs around the active site (as observed in the presence of Mg\textsuperscript{2+}; Fig. 4G) indicates that the stable active conformation is not formed when Mg\textsuperscript{2+} is not present. Binding of pdA5 in the absence of Mg\textsuperscript{2+} does not change the dynamics of the \alpha\textsubscript{1}-helix (opposed to what was observed in the presence of Mg\textsuperscript{2+}; Fig. 4H), as shown by the overlay of \textsuperscript{19}F CPMG data in the absence (black) and presence (green) of pdA5. Data points are shown as mean ± s.d., as derived from 3 duplicate NMR measurements.
Extended Data Fig. 9 | The ASF mutation does not interfere with the Xrn2 structure or with the binding of the substrate to Xrn2. (A) Structure of Xrn2 with the mutation site A5 highlighted in orange and the Cβ atom depicted as a sphere. (B) Xrn2 ASF interacts with a 10mer RNA with very similar affinities as the WT enzyme (compare: Fig. 1G of the main text). Data points are shown as mean ± s.d., as derived from 3 independent experiments. (C) HMQC spectra of the Xrn2 WT (black) and Xrn2 ASF enzyme (orange). (D) HMQC spectra of the Xrn2 WT:pAp complex (black) and Xrn2 ASF:pAp complex (orange). Minor CSPs are visible around the N-terminal α1-helix, that are due to the ASF point mutation. The overall structural of the active state is, however, maintained in the presence of the ASF mutation (the black and orange spectra are highly similar in the absence and presence of substrate).
Extended Data Fig. 10 | Changes in the Xrn2 enzyme spread from the Rai1 interface along the central β-sheet. (A) $^1$H CPMG RD profile of Xrn2 ΔZnF N12C$^{[15}$Ni$]$-Rai1 sample at 313 K. The fit yields an exchange rate of $k_{ex} = 984 \pm 94 \text{ s}^{-1}$, which matches the exchange rate of Xrn2 ΔZnF N12C$^{[15}$Ni$]$ in the absence of Rai1 ($k_{ex} = 913 \pm 108 \text{ s}^{-1}$). Binding of Rai1 thus does not influence the motions in Xrn2. Data points are shown as mean ± s.d., as derived from 3 duplicate NMR measurements. (B) HMQC spectrum of Ile$^{96}$-$[13$CH$_3]$ and Mete-$[13$CH$_3]$ methyl labeled Xrn2 in the absence (black) and presence (orange) of unlabeled, protonated Rai1. The resonances in the active site of Xrn2 are not influenced, indicating that Rai1 does not affect the active site of Xrn2. (C) Structure of Xrn2. Ile$^{96}$, methyl groups that show pronounced changes in the HMQC spectrum upon interaction with Rai1 are highlighted in orange. The putative Rai1 interaction surface, inferred from the structure of the Xrn2:Rai1 complex in S. pombe (PDB 3FQD), is highlighted in blue. CSPs upon Rai1 interaction reach from the Rai1 binding site towards a region that is remote from the active site. Pronounced changes in the HMQC spectrum upon interaction with Rai1 are highlighted in orange. The putative Rai1 interaction surface, inferred from the structure of the Xrn2:Rai1 complex in S. pombe (PDB 3FQD), is highlighted in blue. CSPs upon Rai1 interaction reach from the Rai1 binding site towards a region that is remote from the active site.
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