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# Carbon-Detected $^{15}$N NMR Spin Relaxation of an Intrinsically Disordered Protein: FCP1 Dynamics Unbound and in Complex with RAP74

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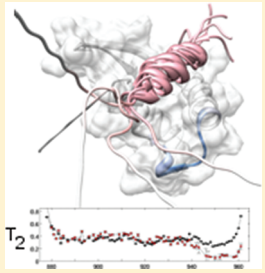
Supporting Information

**ABSTRACT:** Intrinsically disordered proteins (IDPs) lack unique 3D structures under native conditions and as such exist as highly dynamic ensembles in solution. We present two $^{13}$C-direct detection experiments for the measurement of $^{15}$N NMR spin relaxation called the CON(T1)-IPAP and CON(T2)-IPAP that quantify backbone dynamics on a per-residue basis for IDPs in solution. These experiments have been applied to the intrinsically disordered C-terminal of FCP1, both free in solution and while bound to the RAP74 winged-helix domain. The results provide evidence that most of FCP1 remains highly dynamic in both states, while the 20 residues forming direct contact with RAP74 become more ordered in the complex. Parallel analysis of RAP74 backbone $^{15}$N NMR spin relaxation reveals only very limited ordering of RAP74 upon FCP1 binding. Taken together, these data show that folding-upon-binding is highly local in this system, with disorder prevailing even in the complex.




**SECTION:** Biophysical Chemistry and Biomolecules

Intrinsically disordered proteins (IDPs) do not adopt a unique 3D conformation under native conditions, necessitating an ensemble description of their solution state.[1,2] Rather than being rare exceptions in the proteome, IDPs function routinely in transcription, translation, and cellular signal transduction.[3] The ensembles that IDPs adopt, as well as the disorder-to-order transitions that often accompany their interaction with other macromolecules, must be understood if molecular insight into their function is to be gained.[4,5] Conformational dynamics are an integral component of the ensemble description of IDPs, making solution NMR spectroscopy a uniquely attractive biophysical technique for their characterization. In practice, most IDPs suffer from poor NMR spectral quality, which has limited the number of IDPs subjected to experimental NMR relaxation studies despite their potentially high value. Recent success applying both NMR spectroscopy[6,7] and computational modeling[8,9] to IDPs has produced valuable hypotheses regarding the nature of the IDP free-energy landscape and folding-upon-binding transition. Because these transitions almost always include a change in backbone conformation, the assessment of spin relaxation as an experimental measure of backbone conformational dynamics could provide potent mechanistic information if suitably well-resolved data sets were routinely available.

$^{13}$C-detected NMR spectroscopy of biomolecules in solution has recently become a powerful complement to conventional $^{1}$H-detected spectroscopy.[10,11] The advantages of carbon detection are especially prominent for intrinsically disordered proteins due to its ability to overcome limited $^{1}$H chemical shift dispersion.[12−16] Extensive purely heteronuclear and triple resonance assignment methodologies built around detection on the backbone carbonyl or C$^{\alpha}$ are now available.[11,15] The potential to characterize protein dynamics in a $^{13}$C-detected format has also been demonstrated in multiple reports of $^{13}$C $T_1$ and $T_2$ relaxation measurement[17,18] and one report of $^{13}$C$\alpha$-detected $^{1}$H−$^{13}$C$\alpha$ NOEs.[19]

We have previously reported $^{1}$H$^{N}$-start triple resonance experiments built from the conventional $^{15}$N,$^{13}$C-CON-IPAP experiment that perform well with "stock" Bruker $^{1}$H-inner coil TCI cryoprobes.[15] These experiments have been applied to the C-terminal region of the RNA polymerase II (RNAPII) C-terminal domain phosphatase, FCP1, which is a protein that promotes recycling of the polymerase into a new mRNA biogenesis cycle.[20] FCP1 is recruited to the RNAPII holoenzyme through interactions with the C-terminal winged-helix domain of RAP74, which results in a stable protein−protein complex featuring the formation of an $\alpha$-helical structure for the final 16 residues in the FCP1 sequence.[21] Utilizing $^{13}$C-direct detected NMR, we have shown that the residues comprising the RAP74 binding surface of FCP1 demonstrate helical tendencies in the unbound state.[22] Subsequent molecular dynamics calculations performed in our laboratory suggest that the FCP1 helix is less well ordered in complex than previously thought.[23] Collectively, these results lead to the hypothesis that FCP1 loses relatively little conformational entropy upon binding RAP74 and that the associated coupled folding−binding transition may yield a

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bound-state ensemble that is still highly dynamic. To date, NMR spin relaxation studies of neither the RAP74 winged-helix domain nor the FCP1 C-terminal domain have been reported, leaving a pressing need for experimental validation of these hypotheses.

In this Letter, we report $^{1}H^{N}$-start experiments[13] built from conventional $^{15}N$,$^{13}C$-CON pulse programs[11] that readily measure $^{15}N$ $T_1$ and $T_2$ relaxation rates on standard $^{1}H$-inner coil cryogenically cooled probes. Application of these new pulse sequences confirms that FCP1 remains nearly as disordered in complex with RAP74 as it is when unbound, except in the direct binding region. Furthermore, only a single 10 residue motif in RAP74 loses conformational freedom in the complex. The FCP1−RAP74 complex is one of many described in the literature as "fuzzy", meaning qualitatively that significant disorder has been hypothesized to prevail even in the bound state, as is quantitatively demonstrated here.

Recombinant FCP1 (representing residues 879−961 of the human sequence) and RAP74 (residues 426−517 of the human sequence) were prepared as described previously and concentrated to 1.0 mM for spin relaxation measurements.[22] All NMR spectra were collected at 25 °C on a Bruker Avance III spectrometer, equipped with a TCI cryoprobe and operating at 11.7 T static magnetic field strength. Collection of quantitative $^{15}N$ spin relaxation parameters for FCP1 required the development of $^{13}C$-detected pulse programs that we name CON(T1)-IPAP and CON(T2)-IPAP (Figure 1). A description of the magnetization transfer through these two pulse sequences is provided in the Supporting Information. Spin relaxation measurements of $^{15}N$-labeled RAP74 were performed using standard proton-detected methods. Following acquisition, all spectra were processed in NMRpipe[24] and analyzed in SPARKY (SPARKY3.113; T.D. Goddard and D.G. Kneller, University of California, San Francisco, CA). Standard triple resonance NMR techniques were used to confirm previously reported[25,26] backbone resonance assignments of RAP74 both free and bound to FCP1. The acquired RAP74 relaxation data were fit using the Lipari−Szabo model-free formalism[27] as implemented in the program ModelFree 4.20,[28] with diffusion tensor fitting performed using the quadric method.[29] The coordinates from the crystal structures of apo-RAP74 (1I27)[30] and the RAP74-FCP1 complex (1J2X)[21] were used as structural references for axially symmetric diffusion tensor determination.

Backbone dynamics measurements provide powerful insight into the biological function of globular proteins in solution but require well-resolved NMR resonances for quantitative analysis, which hinders their routine application to intrinsically disordered proteins. The novel carbon-detected $^{15}N$ relaxation experiments presented here are readily applied to disordered proteins, which tend to have excellent chemical shift dispersion in the $^{15}N$,$^{13}C$-CON-IPAP experiment (Figure 2A). Far from being uniform and featureless, the plots of $T_1$ (Figure 2B) and $T_2$ (Figure 2C) as a function of residue number in FCP1 include considerable variations from the baseline, as has been seen for other IDPs with well enough dispersed $^{1}H$,$^{15}N$-HSQC spectra to enable spin relaxation measurement.[12,31−33] For example, the region spanning residues 900−915 contains four gly, four ala, and three ser residues; collectively, this sequence bias imparts greater flexibility evidenced by a simultaneous increase in $T_1$ and $T_2$, relative to the global baseline. Although FCP1 does not adopt a stable globular structure, the $R2/R1$ ratio at each residue can be converted into an apparent correlation time $\tau_c$. The value of $\tau_{c,app}$ remains essentially

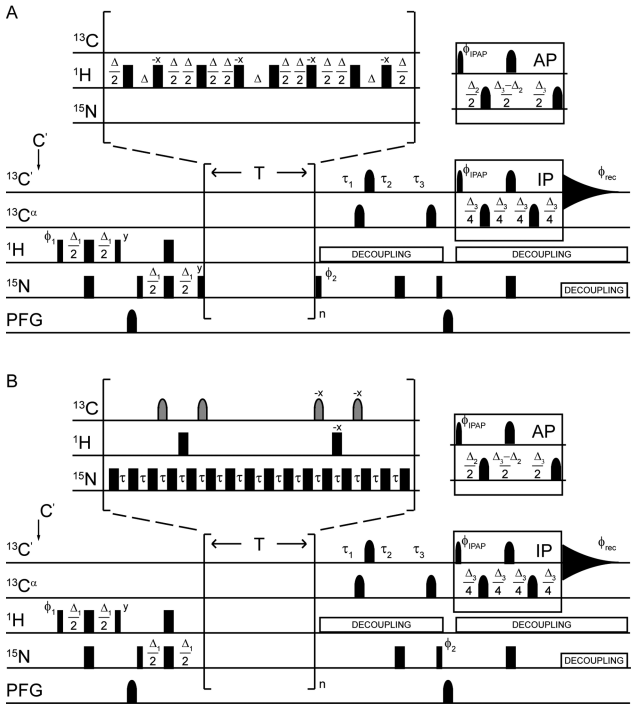


**Figure 1.** Pulse sequences for the (A) CON(T1)-IPAP and (B) CON(T2)-IPAP experiments. The delays are $\Delta$ = 5 ms, $\Delta_1$ = 4.6 ms, $\Delta_2$ = 9.0 ms, and $\Delta_3$ = 25 ms. $^{15}N(t_1)$ chemical shift evolution is performed using a semiconstant time period with delays $\tau_1 = (\Delta_3 + t_1)/2$, $\tau_2 = (1 - \Delta_3/t_{1max})t_1/2$, and $\tau_3 = (1 - t_1/t_{1max})\Delta_3/2$. Pulses are applied at the frequency indicated on the left of each line, with narrow and wide rectangles or shapes representing 90 and 180° pulses, respectively. The insets in panels A and B show an expansion of the incremented relaxation delay periods and include gray pulses on $^{13}C$ to indicate band-selective $^{13}C'$ and $^{13}C^{\alpha}$ inversion pulses for refocusing of $^{15}N-^{13}C$ coupling. In both cases, the phase cycle is $\varphi_1$ = $x$, $-x$; $\varphi_2$ = $y$, $y$, $-y$, $-y$; $\varphi_{IPAP}(IP)$ = $x$, $x$, $x$, $x$, $-x$, $-x$, $-x$, $-x$; $\varphi_{IPAP}(AP)$ = $-y$, $-y$, $-y$, $-y$, $y$, $y$, $y$, $y$; and $\varphi_{rec}$ = $x$, $-x$, $-x$, $x$, $x$, $-x$, $-x$, $x$. Quadrature detection in the indirect dimension was obtained by States-TPPI incrementation of $\varphi_2$.

constant from residues 890 to 940, with means of 3.3 ± 0.4 and 3.0 ± 0.6 ns for the apo- and RAP74-bound states, respectively. While the RAP74 binding region of FCP1's dynamics in the unbound state are generally consistent with those from the rest of the apo-FCP1 chain, in the complex, $\tau_{c,app}$ increases to 12 ± 2 ns in the FCP1(944−960) binding region.

The aberrant dynamics of the FCP1 binding region in complex with RAP74 are especially valuable for the information that they provide regarding the persistence of disorder in the bound state, as is often described through the metaphor of a "fuzzy" complex.[2] If the complex between FCP1 and RAP74 were rigid, the average $T_1$ and $T_2$ in both proteins would be dictated by the same global rotational tumbling time, and thus, the baselines would match. In contrast to this prediction, the average $T_1$ in FCP1(944−960) is significantly higher than the average $T_1$ of residues in the globular domain of RAP74 (see Figure 3B); the opposite is true for $T_2$, where the average value is lower than expected for FCP1(944−960) in the complex. An elevated $T_1$ baseline for FCP1(944−960), relative to that of RAP74, is therefore indicative of large-amplitude local dynamics for the FCP1 backbone in this region. This interpretation is consistent both with MD simulations[23] and with the very high B-factors observed crystallographically[21] for an FCP1 peptide in


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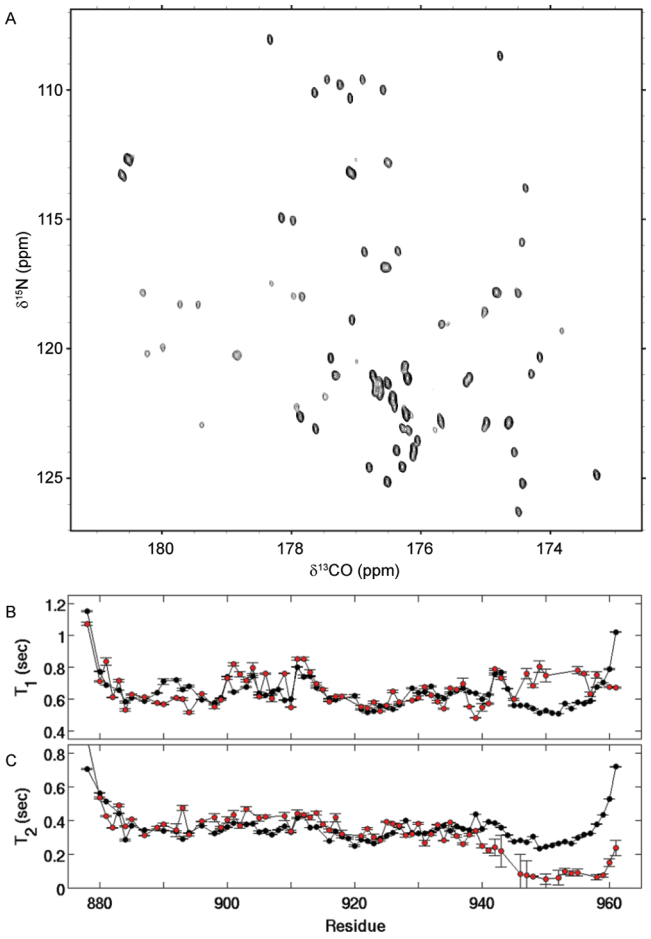


**Figure 2.** (A) $^{15}$N,$^{13}$C-CON(T2)-IPAP spectrum of FCP1 in complex with RAP74 collected with a relaxation delay of $T$ = 16 ms using the pulse program shown in Figure 1B. Fitted values of (B) $T_1$ and (C) $T_2$ as a function of residue number are shown with error bars representing the uncertainty from numerical fitting to a single-exponential decay. In both panels, apo-FCP1 is shown in black, while FCP1 bound to RAP74 is shown in red.

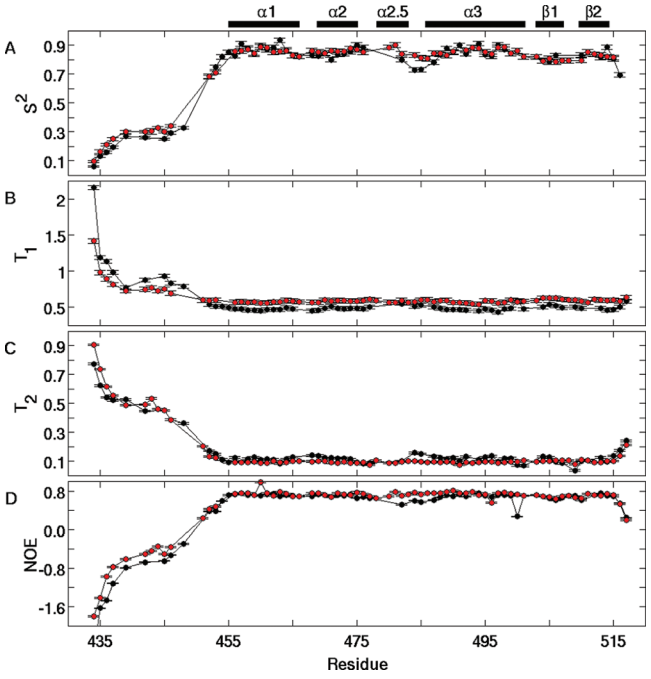


**Figure 3.** (A) Backbone order parameters for RAP74 generated from Lipari–Szabo model-free analysis of the (B) $^{15}$N-$T_1$, (C) $^{15}$N-$T_2$, and (D) [$^1$H–$^{15}$N]-NOE of the protein in the unbound (black) and FCP1 bound (red) states. For the order parameters, error bars represent uncertainties output from fitting in Modelfree-4.20; error bars on the relaxation rates and NOEs reflect uncertainty from fitting to a single-exponential model and from averaging over two replicate experiments, respectively.

complex with RAP74. Such dynamics should also tend to elevate the $T_2$ baseline, indicating that the very low values observed here may be caused by chemical exchange on an intermediate time scale. More quantitative evaluation of these dynamics will require future studies of NMR relaxation dispersion in this system.

The spin relaxation data validate the hypothesis that the FCP1 chain is highly disordered both free in solution and while in complex with RAP74. To complete the characterization of backbone dynamics in this system, we next collected $^{15}$N spin relaxation data for apo-RAP74 and for RAP74 in complex with FCP1 (Figure 3B–D). Analysis of the data using the quadric method indicates that RAP74 tumbling is moderately anisotropic in solution, as reported by $D_{||}/D_{\perp}$ = 1.20 and 1.17 for apo-RAP74 and the complex, respectively. Within the context of the axially symmetric diffusion tensor, apo-RAP74 was best fit to $\tau_{iso}$ = 6.8 ns, which is a little higher than expected for a domain of this size. On the basis of prior experience, we attribute this to the presence of a 20 residue disordered tail at the domain's N-terminus. NMR pulsed-field gradient diffusion measurements yielded a translational diffusion coefficient of $1.14 \times 10^{-10}$ m$^2$/s for apo-RAP74 (data not shown), which is also consistent with the expected result for a species with RAP74's monomer weight. Consistent with the addition of FCP1's mass to the domain, $\tau_{iso}$ increases to 8.6 ns in the complex.

Because RAP74 is globular, it is meaningful to calculate generalized order parameters ($S^2$) representing its backbone flexibility. Plotting $S^2$ as a function of residue number (Figure 3A) indicates that the majority of sites in RAP74 experience no change in backbone dynamics upon complex formation. Notably, the one region of difference is helix 2.5 and the loop connecting it with helix 3, which become more ordered when FCP1 is bound. Residues in this region of RAP74 have previously been proposed to mediate interactions with CK2 phosphorylated FCP1,[34] suggesting that phosphorylation acts to tighten binding by strengthening pre-existing interactions. Similarly to the independence of the disordered extension of FCP1 from the bound region, the dynamics of the long N-terminal disordered region of RAP74 are significantly greater than those of the globular domain and largely unaffected by the addition of FCP1.

The data discussed above are summarized pictorially in Figure 4. In this image, the ribbon diagram of RAP74 is shown in white for regions that experience no change in dynamics, while the portion including helix 2.5 that becomes more rigid upon complex formation is shown in blue. The N-terminal extension of RAP74 is not shown for clarity. Similarly, the ribbon diagrams representing FCP1 have been colored pink for those residues that lose flexibility in the complex and white for those that do not experience a change in dynamics. In order to best represent our working model of the FCP1–RAP74 complex, we have superimposed five snapshots of FCP1, spaced one per 50 ns, from our laboratory's previously


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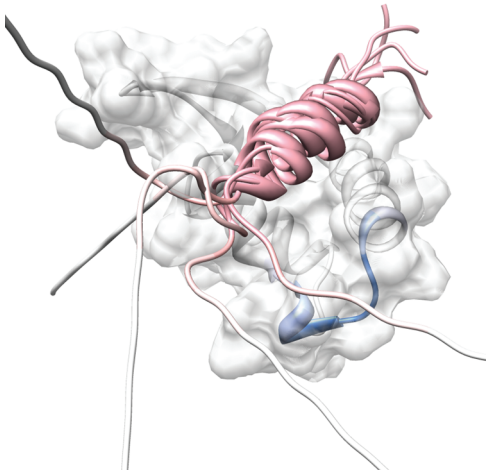

**Figure 4.** A ribbon diagram of the FCP1−RAP74 complex depicting the change in conformational dynamics experienced by both proteins upon complex formation. RAP74 is depicted as a backbone ribbon diagram embedded within a transparent van der Waals surface. The RAP74 ribbon is represented in blue for sites that become more ordered in the complex and white for residues with no statistically significant change in backbone order parameter. The ribbon representation of FCP1 is colored pink for sites that become more ordered in the complex and white for residues that experience no significant change in backbone spin relaxation rates. Models of FCP1 are taken from snapshots spaced every 50 ns through a MD trajectory of the FCP1−RAP74 complex[23] (which modeled FCP1 944-61) with 10 extra residues added to the N-terminus in an extended conformation. Contact between this region of FCP1 and RAP74 is suggested by the increase in the order parameter for the helix 2.5 loop of RAP74 and the persistence of increased order in FCP1 past the end of the canonical $\alpha$-helix.

published molecular mechanics simulation of the system.[23] As seen in Figure 2, the region of FCP1 that becomes more conformationally restricted extends several residues past the end of the crystallographically observed $\alpha$-helix that begins with S944. Therefore, the members of this bundle have had 10 nonsimulated residues added to their N-terminus in order to show better the general direction in which the remaining 64 residues of the chain would project from the complex. Several of the modeled FCP1 chains make contact with the helix 2.5 region of RAP74 that becomes more ordered in the complex. Putting this data into context, it is now known that the regions of IDPs remaining disordered in complex are often important for function. Acknowledgment of this trend has resulted in discussion of the phenomenon termed "fuzziness" in IDP complexes.[2] Proposed CK2 phosphorylations on residues S942 and S944 of FCP1, which have been shown to increase the RAP74 binding affinity in vitro,[34] could reasonably restrict the available conformations of FCP1 near helix 2.5, thus stabilizing the interactions that we observe. If so, this suggests a functional role for the retention of FCP1 disorder proximal to RAP74 in allowing post-translational modification to modulate affinity. This hypothesis is further supported by the enhancement of RAP74 affinity observed for an alternative peptide in the central region of FCP1 following its phosphorylation on analogous serines.[35]

In summary, application of the carbon-detected $^{15}$N spin relaxation experiments that we report here to investigations of the conformational dynamics of FCP1 enhance our understanding of IDP conformational sampling both free in solution and when bound to globular partners. For globular proteins, $^{15}$N backbone spin relaxation measurement has become commonplace in constructing binding mechanisms and rationalizing measured thermodynamics. However, significant changes in dynamics are rarely observed upon protein−protein complex formation; consider the highly local response of RAP74 to FCP1, for example. In contrast, intrinsically disordered proteins are all but obligated to undergo a change in backbone conformation upon complex formation, even in the most dynamic of "fuzzy" complexes, making the assessment of backbone spin relaxation imperative. For example, recent work by Kriwacki and colleagues demonstrates that dynamic associations mediated by the D2 subdomain of the IDP p27 modulate the affinity of association with a variety of Cdk/cyclin complexes that are present at temporally distinct stages of cell cycle progression.[36]

Here, we have shown that very few FCP1 residues experience a change in dynamics upon complex formation but that the change in dynamic behavior for the 20 residues of the binding interface is dramatic. Furthermore, post-translational modifications of S942 and S944 target exactly the region of FCP1 that forms a structural and dynamic boundary between the RAP74 binding region (944−960) and the disordered spacer that connects this region with FCP1's N-terminal globular domains. This weakly ordered buffer zone, which loosely includes residues 938−944 of FCP1, remains predominantly disordered in the complex. In analogy to the use of p27 bound-state dynamics in modulating affinity for various cyclins, altering the bound-state dynamics of FCP1 through post-translational modification may modulate the RAP74 affinity.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Expanded NMR methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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