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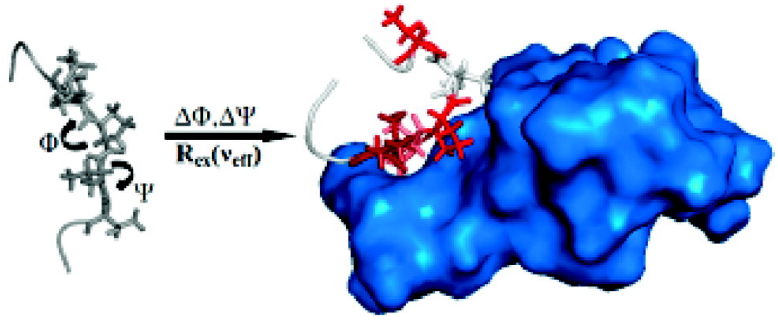

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Dynamics of Ligand Binding from ^{13}C NMR Relaxation Dispersion at Natural Abundance

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Conformational dynamics are drawing increasing attention in drug discovery due to their effect on the stability and diversity of protein−ligand complexes.[1] Both protein and ligand dynamics are important. Yet, the ligand has received less scrutiny, despite being the object of iterative modification in drug design. Accordingly, we apply here ^{13}C Nuclear Magnetic Resonance (NMR) relaxation dispersion measurements, at natural abundance, to a ligand in chemical exchange between free and protein-bound states. Specifically, we compare site-specific relaxation dispersion profiles of ligand ^{13}C nuclei in the presence and absence of its protein receptor. Profiles that differ are ^{13}C sites whose chemical shifts change upon protein binding. Fits of the profiles give the rate constants for the exchange of ligand between its free versus bound conformations. Because these experiments detect the ligand, they are applicable even in the absence of a protein structure. While previous ligand mobility studies have used natural abundance ^{13}C relaxation,[2−6] this study, to the best of our knowledge, is the first demonstration of ^{13}C relaxation dispersion at natural abundance on a ligand in protein-binding exchange.

Peptide−protein interactions of modest binding affinity often guide the early stages of drug design,[7] and we illustrate the dispersion methods on a 10-residue phosphopeptide that binds human Pin1 with $K_D \sim 10$ μM.[8] Pin1 is a 163 residue prolyl isomerase that recognizes phospho-Ser/Thr-Pro (pS/T-P) motifs in other signaling proteins relevant for cancer and Alzheimer's disease.[9] Our ligand (EQPLpTPVTDL) contains a Pin1 pT-P target site from the mitotic phosphatase, Cdc25, a Pin1 substrate.

Chemical exchange of ligands between their free and receptor-bound states can modulate the ligand chemical shifts, thus boosting the ligand $R_2 = 1/T_2$ relaxation rates by an amount R_{ex}.[10,11] Two-dimensional (2D) relaxation dispersion measurements profile R_{ex} versus spin-lock field strength in a site-specific manner.[12] Fits of the profiles yield the exchange rates and, under favorable exchange regimes, the underlying chemical shift differences and populations of the exchange coupled states.[13]

We explored ^{13}C relaxation dispersion since it is now widely accepted that ^{13}Cα, β chemical shifts reflect primarily backbone ϕ, ψ, and side chain χ_1 torsion angles.[14−20] Those ^{13}Cα sites showing relaxation dispersion, and, hence, chemical shift modulation, are candidate sites of ϕ, ψ, and χ_1 fluctuations on the μs−ms time scale. This dynamic interpretation of ^{13}Cα relaxation dispersion has been used in studies of ^{13}C-labeled proteins.[21−23]

While ^{13}C labeling is relatively facile for proteins, it is often impractical for ligands, in pharmaceutical research settings, due to cost or synthetic complexity. Recognizing this, we use natural abundance ^{13}C ($\sim$1%) for our dispersion measurements. Natural abundance also reduces ^{13}C−^{13}C scalar and dipolar couplings that can complicate relaxation pathways. Cryogenically cooled probes and high magnetic fields remove the historical need for ultrahigh ligand concentrations (>10 mM); our concentrations of 2 mM suffice (see, e.g., Figure S1, Supporting Information).

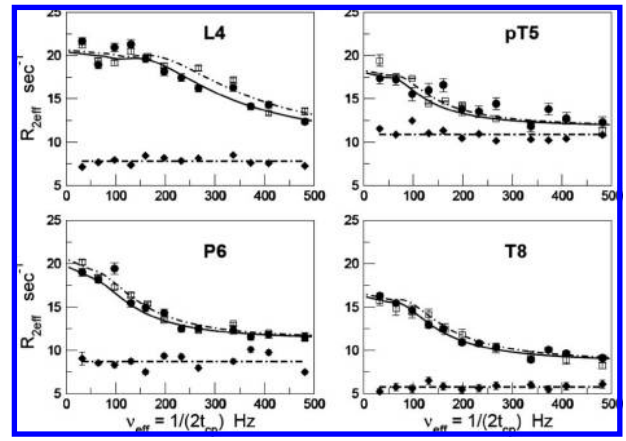

Figure 1. CPMG dispersion profiles for ligand ^{13}Cα positions with and without Pin1. The profile are R_{2eff} versus $1/(2t_{cp})$, where t_{cp} is the CPMG interpulse delay. Symbols are as follows: open squares (18.8 T), filled circles (16.4 T), filled diamonds (free ligand 16.4T), curves (fits to the Carver-Richards model[24]), flat dotted lines (average free ligand values).

We compared natural abundance ^{13}C dispersion measurements of the Cdc25 ligand in the presence and absence of U-^{15}N-(70%) ^{2}H Pin1. Samples were 90% H_2O/10% D_2O imidazole buffer, pH = 6.6, T = 278 K, 2 mM ligand, and 50 μM Pin1. We measured effective transverse ^{13}Cα relaxation rates, R_{2eff}, as a function of the Carr−Purcell−Meiboom−Gill[25,26] (CPMG) interpulse spacing t_{cp} using the 2D relaxation-compensated scheme of Loria et al.[12] Each spectrum took 2.6 h (64 scans per t_1, 42 complex t_1 points). The 70% protein deuteration mitigates proton dipolar relaxation and enhances the relaxation compensation. We obtained $R_{2eff}(1/2t_{cp})$ versus the CPMG field strength, $\nu_{eff} = 1/2t_{cp}$, profiles from $R_{2eff}(1/t_{cp}) = -1/T_{rlx} \ln\{I(1/t_{cp})/I_{ref}\}$.[27] $I(1/t_{cp})$ and I_{ref} are CH peak intensities with and without a CPMG interpulse delay of t_{cp}, and T_{rlx} = 62.4 ms. We recorded dispersions at 16.4 and 18.8 T using Bruker Avance systems equipped with TCI cryoprobes. Folding of Pin1 was confirmed by ^{15}N−^{1}H HSQC.

Figure 1 shows the dispersion profiles $R_{ex}(\nu_{eff}) + R_{2,0}$, where $R_{ex}(\nu_{eff})$ is the exchange contribution and $R_{2,0}$ is the nonexchange (ν_{eff}-independent) contribution. When Pin1 is present, we see significant dispersion for L4, pT5, P6, and T8; when Pin1 is absent (the isolated ligand), we do not. Hence, the dispersion reflects interaction with Pin1. This is reasonable since these residues encompass the pT-P site targeted by Pin1. Structural studies (cf. Figure 2) also show these residues at the protein−ligand interface.[28] Other residues show no dispersion (cf. Figure S2, Supporting Information).

To extract exchange parameters, we fit the four ^{13}Cα dispersion profiles to two-state exchange models: the Luz−Meiboom[29] model

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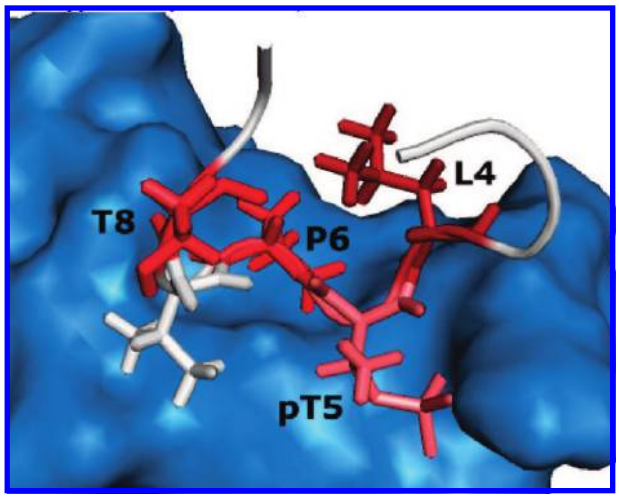

Figure 2. Cdc25 phospho-peptide bound to the WW domain of Pin1 based on Wintjens et al.[28] Red denotes sites showing ^{13}C relaxation dispersion upon introduction of full-length Pin1.

Table 1. ^{13}Cα Exchange Parameters for Cdc25 Phosphopeptide

Cα	k_{ex} (rad/s)	Δ_{ppm}	p_b	R_{ne} s^{-1}
L4	576 ± 67	1.64 ± 0.05	0.020 ± 0.002	9.7 ± 0.3
pT5	291 ± 120	0.63 ± 0.07	0.025 ± 0.001	11.7 ± 0.1
P6	489 ± 24	0.58 ± 0.02	0.027 ± 0.001	11.1 ± 0.1
T8	337 ± 29	0.74 ± 0.02	0.026 ± 0.002	8.6 ± 0.1

and the Carver−Richards[24] model. The Luz−Meiboom model has three fitting parameters: the exchange rate constant k_{ex}, the nonexchange relaxation $R_{2,0}$, and an aggregate parameter $\Phi = p_b(1 - p_b)*(\delta\omega)^2$, where p_b is the minor species fraction and $\delta\omega$ is the chemical shift difference (in rad/s) between the two states. The Luz−Meiboom model assumes fast exchange ($k_{ex} > |\delta\omega|$). The Carver−Richards model applies for arbitrary exchange time scales and resolves p_b from $\delta\omega$, resulting in four fitting parameters: k_{ex}, $R_{2,0}$, p_b, and $\delta\omega$. For all but pT5 ^{13}Cα, global fits of the 16.4 and 18.8T dispersions gave significantly lower residual χ^2 errors using the Carver−Richards model (p-values < 0.05; see Supporting Information). Table 1 gives the fitted Carver−Richards parameters, and Figure 1 overlays the fitted dispersion curves on the raw data. These parameters indicate a conformational exchange process for the four ^{13}Cα nuclei: $k_{ex} \approx 300-600$ s^{-1} and minor populations of ∼2−3%. The k_{ex} values are typically $\leq |\delta\omega|$, indicating intermediate to slow exchange on the chemical shift time scale.

We have not identified any minor state resonances in the 2D ^{13}C−^{1}H spectra, presumably because of their low population and broad line widths. Nevertheless, the ^{13}C dispersion of the major state sheds light on the low-populated ("invisible"[13,27,30]) state.

The values in Table 1 suggest the dispersion reflects the exchange of ligand between its free and protein-bound states. This raises the question of how binding alters the ligand ^{13}Cα shifts. In principle, both environmental factors (e.g., electric fields, hydrogen bonds) and local torsion angles (ϕ, ψ, χ_1) affect the ^{13}Cα shifts. However, overwhelming evidence[14,15,17,18] suggests the torsion angles effects dominate. Thus, our ^{13}Cα relaxation dispersion (shift modulation) most likely reflects ϕ, ψ, and χ_1 torsions toggling between angles of the free peptide with those enabling intermolecular contacts with Pin1. However, as we cannot totally exclude the presence of environmental effects, we are pursuing Pin1 dispersion experiments to be more definitive.

In summary, we have demonstrated that natural abundance ^{13}C relaxation dispersion is a viable means for exploring μs−ms ligand dynamics related to protein binding. Deeper analyses of the ^{13}C shift changes may provide insights into the minor state conformation(s).[18] Similar dispersion measurements should be applicable to side chain ^{13}C. Extension to tight binding ligands should be feasible if the protein is well deuterated and sufficiently soluble. The μs−ms dynamics probed here, along with the ps−ns motions probed by previous methods,[5,6,31,32] enable ligand flexibility−activity studies over a broad time scale.

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Supporting Information Available: NMR spectra, parameter fitting procedures, relaxation rates for all residues. The material is available free of charge via the Internet at http://pubs.acs.org.

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