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Toward Flexibility−Activity Relationships by NMR Spectroscopy: Dynamics of Pin1 Ligands

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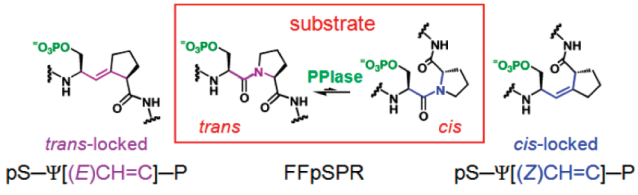
Flexible ligands are common starting points in drug discovery. Such ligands often change their conformational flexibility upon receptor interaction. Mapping these changes can help guide the manipulation of ligand flexibility in molecular design. To gain this insight, we need methods that are sensitive to (1) ligand motions related to receptor activity such as binding or catalysis and (2) ligand modifications that alter the activity-related motions.

Here we demonstrate an approach that satisfies these requirements: ligand flexibility−activity studies by Nuclear Magnetic Resonance (NMR). These studies apply dynamic NMR experiments to a *series* of related ligands that target a common receptor and compare the site-specific changes in ligand dynamics stimulated by receptor interaction. The comparisons reveal how variations in ligand structure can perturb ligand motions important for activity and, thus, provide site-specific information for changing ligand mobility (e.g., rigidification). This systematic approach for articulating activity-related ligand dynamics is a critical step toward establishing flexibility−activity relationships (FAR), to complement standard structure−activity relationships (SAR) in iterative design.

We illustrate these NMR FAR-related methods by studying ligands with motions that are (1) intrinsic to protein interaction and (2) sensitive to structural modifications. Specifically, we examine three structurally similar but dynamically differentiated ligands of human Pin1 (Scheme 1). Pin1 is a mitotic regulator that accelerates the cis−trans isomerization of phospho-Ser/Thr-Pro motifs of other signaling proteins.[1,2] The ligands include a phosphopeptide substrate, Ac-Phe-Phe-pSer-Pro-Arg-NH_2 (FFpSPR), and two inhibitor analogues. The analogues replace the core peptidyl-prolyl linkage (pSP) by alkene isosteres, resulting in cis-locked (Ac-Phe-Phe-pSer-Ψ[(*Z*)CH=C]-Pro-Arg-NH_2) or trans-locked (Ac-Phe-Phe-pSer-Ψ[(*E*)CH=C]-Pro-Arg-NH_2) peptidic ligands that are competitive Pin1 inhibitors.[3,4]

Scheme 1. Three Ligands Show the Core Phospho-Ser-Pro Motif for Trans (in Purple) and Cis (in Blue)



We exploit the sensitivity of aliphatic ^{13}C chemical shifts to local conformation.[5−8] Receptor interactions can dynamically alter ligand conformation, modulating the ligand ^{13}C shifts, thus causing ^{13}C relaxation dispersion. To map ligand dynamics site-specifically, we use NMR ^{13}C relaxation-dispersion measurements at natural ^{13}C abundance (∼1%). These experiments can map millisecond exchange dynamics for ligand CH moieties[9,10] by measuring transverse ^{13}C relaxation during a Carr−Purcell−Meiboom−Gill (CPMG) pulse train.[11,12] Critically, the dispersion can detect ligand populations as low as 1%.[13,14] Thus, bound-state information becomes available for ligands exchanging between the major free and the minor receptor-bound states.

The dispersion experiments detect the modulation of the ^{13}C chemical shifts caused by exchange dynamics. The modulation enhances the ^{13}C transverse relaxation (line broadening), which becomes dependent on ν_{eff}, the frequency of 180° pulsing during CPMG spin-lock. 2-D $^{13}C-^{1}H$ methods[15−17] quantify this dependence for each protonated ^{13}C nucleus via a relaxation dispersion profile, $R_{2,eff}$ vs ν_{eff}. $R_{2,eff}$ is the ^{13}C transverse relaxation rate during the compensated CPMG spin-lock,[15] and $\nu_{eff} = 1/(2t_{cp})$, where t_{cp} separates consecutive 180° pulses. ^{13}C sites with dynamics that modulate the ^{13}C chemical shift yield dispersion profiles with $R_{2,eff}$ decreasing with higher ν_{eff}. Model fits of these profiles[18,19] give thermodynamic and kinetic parameters for the underlying exchange dynamics. Flat profiles mean either a lack of exchange dynamics or exchange rates that are extremely fast or slow on the chemical shift time scale. In the latter case, changing the static field, temperature, or receptor−ligand ratio can nudge the exchange rates to enhance dispersion.

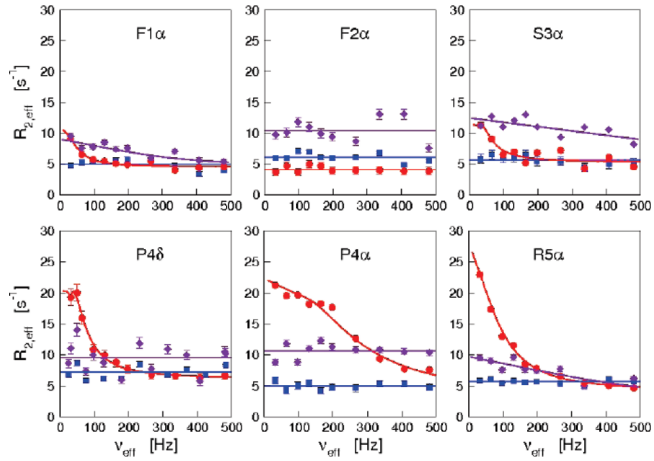


Figure 1. Natural abundance ^{13}C CPMG dispersion profiles for the $^{13}C\alpha$ and P4-$^{13}C\delta$ nuclei of substrate FFpSPR (●), cis-locked (■), and trans-locked (◆) inhibitors with 1% Pin1-PPIase at 295 K. Profiles are for 1 mM ligand at 18.8T for $^{13}C\alpha$ and 16.4T for P4-$^{13}C\delta$. Two-state exchange fits for FFpSPR $^{13}C\alpha$ sites F1α, S3α, P4α, R5α (red curves), and trans-locked $^{13}C\alpha$ sites F1α, S3α, R5α (purple curves) reflect 16.4T and 18.8T data. The P4-$^{13}C\delta$ fit (red curve) reflects just 16.4T data. Horizontal lines are average $R_{2,eff}$ values at 18.8T for sites showing no dispersion.

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Pin1 consists of a peptidyl-prolyl isomerase (PPIase) domain, connected via a flexible linker to a WW domain docking module. We used a truncated Pin1 construct (Pin1-PPIase) that omits the WW domain. 2-D $^{1}H-^{1}H$ EXSY experiments[20] on FFpSPR confirmed the cis−trans isomerase activity of Pin1-PPIase, and fits of Pin1-PPIase $^{15}N-^{1}H$ chemical shift perturbations during ligand titrations gave binding affinities of $K_{d,FFpSPR} = 203 \pm 46\ \mu M$, $K_{d,CIS} = 7 \pm 3\ \mu M$, and $K_{d,TRANS} = 78 \pm 23\ \mu M$ (Figures S2 and S3 in the Supporting Information). These affinities are consistent with K_i values using full-length Pin1.[4] We then measured natural abundance ^{13}C dispersion profiles for all three ligands at 1 mM concentration, in the absence and presence of 1% Pin1-PPIase (Figure 1). Measurements were at 16.4 and 18.8 T, using Bruker Avance systems with cryogenically-cooled probes and previously described pulse schemes.[9,10]

With 1% Pin1-PPIase, the substrate FFpSPR showed strong dispersion at pS3-Cα, P4-Cα, the side-chain P4-Cδ, and R5-Cα. The isolated substrate gave flat $R_{2,eff}$ vs ν_{eff} profiles, i.e. no dispersion (Figure S4 in Supporting Information). Thus, the strong dispersion represents exchange dynamics stimulated by Pin1-PPIase. The dispersion reflects all sources of ^{13}C shift modulation, including binding and isomerization, and thus involves exchange among four states (free and bound states of cis and trans). But closed-form analytical expressions for CPMG dispersion beyond two states make some assumptions of fast exchange.[21] Here, such assumptions were discouraged by the decrease in FFpSPR dispersion at lower temperature (Figure S5 in Supporting Information). Thus, we fit the FFpSPR dispersion to the general two-state model to yield *apparent* two-state parameters (Table 1). These parameters allow comparison of exchange behavior across the three ligands and include the nonexchange relaxation, $R_{2,0}$, the minor state population p_{minor}, the exchange rate constant k_{ex}, and the chemical shift difference Δ_{ppm} between the states. The Δ_{ppm} are reasonable compared to previous peptide studies[22] and chemical shift databases,[23] although their apparent nature cautions against a more detailed interpretation.

Table 1. FFpSPR ^{13}C Exchange Dynamics Parameters at 295 K

site	k_{ex} (r/s)	Δ_{ppm}	p_{minor}	$R_{2,0}$ (s^{-1})
F1-Cα[a]	220 ± 40	0.185 ± 0.003	0.055 ± 0.004	4.56 ± 0.06
S3-Cα[a]	59 ± 8	0.33 ± 0.02	0.10 ± 0.02	5.37 ± 0.13
P4-Cα[a]	832 ± 64	1.08 ± 0.05	0.03 ± 0.001	3.38 ± 0.45
P4-Cδ[b]	85 ± 9	0.45 ± 0.02	0.17 ± 0.04	6.21 ± 0.05
R5-Cα[a]	554 ± 13	0.25 ± 0.02	0.18 ± 0.002	4.17 ± 0.05

[a] Parameters based on 18.8 and 16.4 T data. [b] Based only on 16.4 T data.

The two-state models fit the individual FFpSPR dispersion profiles well but did not admit a global fit (i.e., all sites sharing the same k_{ex} and p_{minor}). This is most likely because each profile simultaneously reflects binding and isomerization, with the relative contribution varying from site to site. For example, the side-chain P4-Cδ exchange parameters ($k_{ex} = 85 \pm 9\ s^{-1}$, $p_{minor} = 17 \pm 4\%$) agreed well with $^{1}H-^{1}H$ EXSY measurements of the attached Hδ protons (Figure S2 in Supporting Information), which gave $k_{ex,CIS/TRANS} = 87 \pm 4\ s^{-1}$ and $p_{CIS} = 12 \pm 2\%$. EXSY probes exclusively the cis−trans isomerization; thus, the P4-Cδ exchange dynamics appear dominated by this process. By contrast, P4-Cα and F1-Cα appear to show a greater contribution of binding, owing to the faster k_{ex} (200−800 s^{-1}) and the corresponding $p_{minor} \approx 1\%$, which matches the population of the protein−ligand complex.

To obtain a global fit, we tried separating the time scales of binding versus isomerization by increasing the relative Pin1-PPIase concentration (up to 20%). The increases affected both ^{13}C dispersion and EXSY (Figure S6 and Table S1 in Supporting Information). But for concentrations > 2% at 295 K, S3-Cα was too broad for analysis and P4-Cα disappeared; thus, determining if sufficient time-scale separation had been achieved was not possible. Further protein increases should help but would demand greater protein deuteration than used here (∼50%) for selective ligand detection. While such studies are underway, we note that separating isomerization from binding is a challenge intrinsic to PPIase substrates and not to flexibility−activity studies in general. Indeed, binding rather than catalysis is often the main concern, and our initial studies of phosphopeptide ligands of the noncatalytic Pin1-WW domain suggest that ^{13}C dispersion can map ligand flexibility changes due exclusively to binding (Figure S7 in Supporting Information).

We then turned to the inhibitors. The pS−P linkages of the inhibitors are locked against cis−trans isomerization, and so their dispersion is more rigorously interpreted as a two-state binding exchange. The isolated inhibitors showed flat profiles (Figure S4 in the Supporting Information). Upon addition of 1% Pin1-PPIase, both inhibitors gave dispersions different from that of substrate (Figure 1). The trans-inhibitor gave dispersions for F1-Cα, pS3-Cα, and R5-Cα that were shallower and of smaller magnitude. The two-state fast exchange model[19] gave $k_{ex} = 2200-3300\ s^{-1}$ (Table S2 in Supporting Information). Assuming k_{ex} pertains to binding and using the $K_{d,TRANS} = 78\ \mu M$, we estimated an on-rate of $k_{on} = 2 \times 10^{7}\ M^{-1}\ s^{-1}$; this value is reasonable for a diffusion-limited complex formation.[24] By contrast, the cis-inhibitor showed no dispersion at 1% Pin1-PPIase, due to its slower binding exchange rate, consistent with its tighter binding affinity. We increased the rate by increasing the relative Pin1-PPIase concentration to 50%; however, this led to weak protein cross-peaks that overlapped ligand resonances and prevented analyses. Fortunately, the ligand P4-Cα cross-peak remained isolated and showed dispersion with $k_{ex} = 931 \pm 91\ s^{-1}$, $p_{minor} = 0.5 \pm 0.04$, and $\Delta_{ppm} = 0.34 \pm 0.02$ ppm (Figure S8 in Supporting Information). Notably, the cis-inhibitor P4-Cα Δ_{ppm} was significantly smaller than that of the substrate, suggesting altered exchange dynamics due to the local pS-P locking. Simulations of CPMG dispersions that kept $\Delta_{ppm} = 0.34$ ppm but used k_{ex} and p_{minor} values appropriate for 1% Pin1-PPIase gave nearly flat profiles, consistent with experimental observations (Figure S8 in Supporting Information). Thus, both inhibitors showed exchange dynamics consistent with their different μM binding affinities.

In all cases, ^{13}C dispersion reflects the modulation of the ^{13}C chemical shift, which in turn depends on the local conformation (ϕ, ψ, χ_1) and chemical microenvironment of the ^{13}C nucleus.[5−8] The dispersion behavior could reflect (i) the exchange of the microenvironment about a totally rigid ligand and (ii) ligand conformational (ϕ, ψ, χ_1) dynamics due to Pin1-PPIase binding, isomerization, or both. Protein-based experiments can explore the first cause[14,21] while the methods here explore the second. In this context, we note two observations. First, the exchange parameters differ between substrate and the inhibitors in a manner consistent with the expected loss of mobility due to pS-P locking. Second, the exquisite sensitivity of aliphatic ^{13}C shifts to local conformation is well-established[5−8] and heavily exploited in chemical-shift based protein structure determination.[23,25] Thus, to the extent that ^{13}C shifts reflect local conformation, our results suggest that ^{13}C relaxation dispersion can systematically explore how ligand modifications may perturb the ligand flexibility pertinent to receptor activity.

Figure 2 summarizes the relative magnitudes of the site-specific ^{13}C dispersion for all three Pin1 ligands with 1% Pin1-PPIase. For

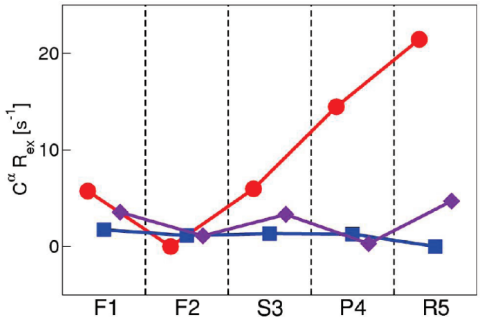

Figure 2. $^{13}C\alpha$ R_{ex} [$R_{2,eff}(\nu_{eff} = 32$ Hz) minus $R_{2,0}$] profile of substrate FFpSPR (●), cis-locked (■), and trans-locked (◆) inhibitors in the presence of 1% Pin1-PPIase. Ligand Cαs with higher R_{ex} values indicate enhanced functional dynamics. Locking the pS-P ω-bond alters the isomerization exchange dynamics at the C-terminus of substrate FFpSPR.

binding and catalysis, FFpSPR undergoes conformational reorganization at the pS-P core, indicated by the strong pS3 and P4 dispersion in FFpSPR. This was expected, since Pin1-PPIase targets pS-P motifs. Less expected was the strong dispersion of the C-terminal R5-Cα. The dispersion may reflect torsion angle excursions upon protein-complex formation, which help prime the slower catalytic event or provide postisomerization conformational relaxation. Computational studies on cyclophilin ligands support this latter notion.[26] P4-R5 instead of, or in addition to, pS-P locking could be an alternative inhibitor strategy. Multiple inhibitory strategies are useful, since some may be better suited for optimizing other critical drug-like properties, beyond inhibitory potency, such as oral bioavailability. The unanticipated R5-Cα mobility illustrates how flexibility−activity studies can broaden design options beyond those from static ligand models.

Differences between the locked inhibitors are also illuminating. Both of the pS-P locked inhibitors are more rigid than the substrate, yet the cis-inhibitor binds more tightly. Thus, *how* the ligand is rigidified is significant; in particular, we believe the cis-inhibitor is better preorganized to dock Pin1-PPIase.[27] This interpretation agrees with crystal structures suggesting preferential binding of cis conformers by the Pin1 PPIase domain.[28]

The flexibility−activity studies shown here are useful for early design stages, which often involve flexible ligands of micromolar affinity.[29−31] Our use of ^{13}C natural abundance shows compatibility with pharmaceutical research settings, in which ligand isotope enrichment is often impractical. For ligand concentrations lower than here (∼1 mM), due to low solubility or tight binding, ligand isotope enrichment and extensive protein deuteration would be needed for selective ligand observation. For uniformly ^{13}C enriched ligands, $R_{1\rho}$ instead of CPMG dispersion is preferable to reduce Hartmann−Hahn transfer artifacts.[32]

In summary, we have demonstrated an NMR-based approach that compares the changes in μs−ms flexibility for a series of ligands targeting a common receptor. The disclosed information includes (1) the locations and time scales of motion, (2) perturbations of those motions caused by ligand structural modifications, and (3) bound-ligand conformational constraints via ^{13}C chemical shift changes. Since the approach is ligand-based, it is applicable even when the 3-D receptor structure is not yet available. Understanding how to control and exploit flexibility in drug design is challenging, and multiple complementary methods will be needed. Thus, the approach described here is a first step toward developing systematic, experimental protocols for the development of FAR to complement SAR.

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Supporting Information Available: Examples of 2-D $^{13}C-^{1}H$ dispersion spectra; exchange parameters for the trans-locked inhibitor; figure of binding isotherms; dispersion for all three isolated ligands at 295 K; dispersion at multiple temperatures and protein concentrations; 2-D EXSY of the FFpSPR substrate; details of sample preparation, dispersion measurements, and model fitting. This material is available free of charge via the Internet at http://pubs.acs.org.

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