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1 Methods

1.1 Preparation of Proteins

Full length human Cdk2 and truncated human cyclin A (residues 173 – 432) were expressed in *E. coli* with an N-terminal 6×His affinity tag after sub-cloning into pET28a (Novagen, Madison, WI) using established procedures.[1] Isotope-labeled [^{2}H/ ^{13}C]-p27 (residues 22-105 of human p27^{Kip1}, known as the kinase inhibitory domain; hereafter referred to as p27) was expressed in a MOPS-based minimal medium and purified using established procedures.[2] Protein concentrations were determined by UV absorbance at 280 nm using a molar extinction coefficient of 15,470 $\text{M}^{-1}\,\text{cm}^{-1}$ for p27, 36,900 $\text{M}^{-1}\,\text{cm}^{-1}$ for Cdk2, and 34,840 $\text{M}^{-1}\,\text{cm}^{-1}$ for cyclin A.[1] Protein complexes, including [^{2}H/ ^{13}C]-p27/Cdk2, [^{2}H/ ^{13}C]-p27/cyclin A, and [^{2}H/^{13}C]-p27/Cdk2/cyclin A, were prepared by mixing and overnight incubation of equimolar ratios of the respective components.

1.2 NMR Spectroscopy

For DNP hyperpolarization, 8.8 µL of 2.5 mM [^{2}H/ ^{13}C]-p27 stock solution was mixed with 1.0 µL of 150 mM tris[8-carboxy-2,2,6,6-tetra(hydroxyethyl)-benzo-(1,2-d:4,5-d)-bis(1,3)-dithiole-4-yl]methyl sodium salt (OXO63, Oxford Instruments, Abingdon, UK) and 0.2 µL of 50 mM gadolinium diethylenetriaminepenta acetic acid (Gd-DTPA, Sigma-Aldrich, St. Louis, MO). The mixture was irradiated on ^{13}C nuclei in a HyperSense DNP polarizer (Oxford Instruments). Hyperpolarization occurred for 4 h at a temperature of 1.4 K, using microwaves of 93.974 GHz frequency and 60 mW power. Subsequently, the mixture was diluted into buffer containing 20 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (TRIS-HCl), pH 7.2, 300 mM NaCl, 5 mM dithiothreitol (DTT). The leading 450 µL of the [^{2}H/^{13}C]-p27 solution was injected into a 5 mm NMR tube pre-installed in a broadband observe (BBO) probe head of a 400 MHz NMR spectrometer (Bruker Biospin, Billerica, MA) using pressurized nitrogen gas in a previously described sample injection device.[3] In the case of the [^{2}H/^{13}C]-p27/Cdk2/cyclin A ternary complex, the pre-installed NMR tube contained 50 µL of 200 µM unlabeled Cdk2/cyclin A.

Following injection, NMR spectra were acquired using the pulse sequence $\tau_{stab} - [p_s - G_z - p_s \;\; G_x - p_s - G_y - \alpha_n - \text{acq} - G_2]_{\times 5}$, where τ_{stab} = 200 ms is the time allowed for sample stabilization, p_s a selective pulse of EBURP-1 shape (20 ms, centered on ethylene glycol resonance at 65.3 ppm), $G_{x,y,z}$ are pulsed field gradients along the axis indicated, and G_2 is a randomized pulsed field gradient to remove coherence before starting the subsequent scan. The excitation pulse, a variable small flip angle pulse (α_n = [26.6°, 30°, 35.3°, 45°, 90°]) was adjusted to – apart from spin relaxation – evenly distribute signal from the initial hyperpolarization in all 5 scans. A total of 6400 complex points were acquired per scan, using an acquisition time of 253 ms. During acquisition, ^{1}H and ^{2}H decoupling were applied. Additional delays were added before and after acquisition, so that the scan repetition delay was 343 ms. Chemical shifts were referenced against the ^{13}C resonance of ethylene glycol in the sample, which was determined using an external standard containing 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS).

Data was Fourier transformed using Topspin 3 software (Bruker), with application of 20 Hz line broadening and using zero-filling to 16384 points. Further data analysis was performed using Matlab (MathWorks, Natick, MA). Signal changes in the spectra from the same data set were quantified by smoothing the spectra with a moving average over 33 data points, followed by fitting of a single exponential function $a \cdot \exp(-rt)$ with fit parameters a and r to the obtained intensities from spectra 2 5, at each chemical shift position.

Due to the presence of a concentration gradient during sample dissolution,[3] and since only the leading 450 L of the total volume of the dissolution solvent of 4 mL is injected into the NMR spectrometer, the overall dilution factor of hyperpolarized p27 samples was determined using reference experiments.[4,5] A dilution factor of 140 for the hyperpolarized sample, in combination with the above sample concentrations for p27, Cdk2 and Cyclin A, resulted in a nominal concentration of 20 µM for all three proteins in the NMR spectrometer.

For 1D ^{13}C NMR experiments without hyperpolarization, samples of [^{2}H/^{13}C]-p27, [^{2}H/^{13}C]-p27/Cdk2, [^{2}H/ ^{13}C]-p27/cyclin A, and [^{2}H/^{13}C]-p27/Cdk2/cyclin A, respectively, were prepared by mixing 150 µM of [^{2}H/^{13}C]-p27 with equimolar amounts of the required components. All 1D ^{13}C spectra without hyperpolarization were measured on a 600 MHz NMR spectrometer (Bruker) equipped with a QCI cryoprobe.

2 Supporting Figures and Tables

2.1 Spectra of Hyperpolarized p27 and p27–Cdk2/Cyclin A Complex

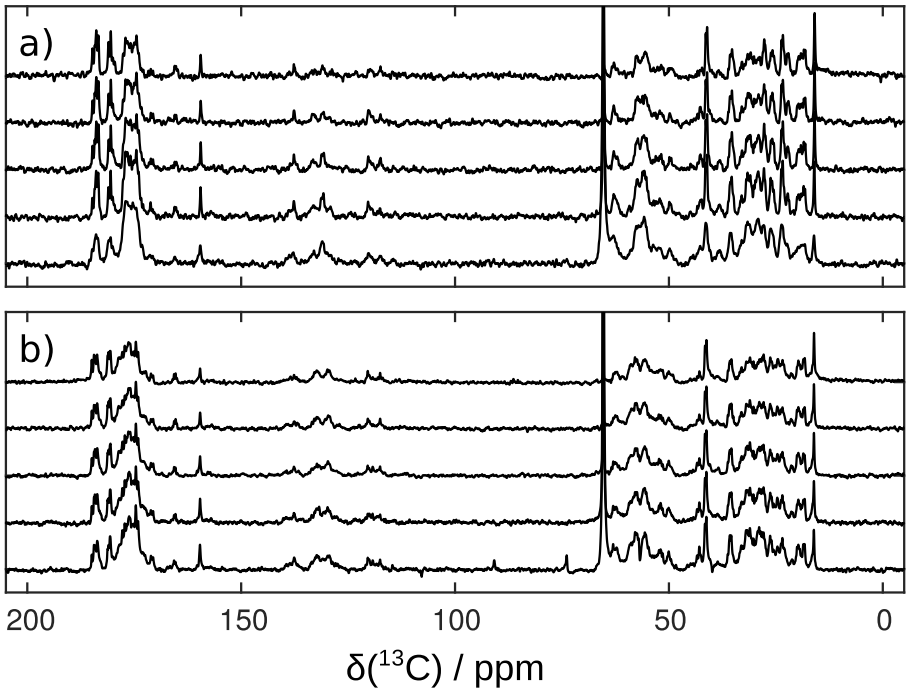

Figure S1: a) Series of spectra acquired following injection of hyperpolarized p27 into the NMR spectrometer, with the first spectrum shown as the lowermost trace. Hyperpolarized p27 was admixed with 50 µL dissolution buffer already present in the NMR tube. b) Series of spectra as in (a), but with admixing of Cdk2/cyclin A in the NMR spectrometer. Sample and experimental conditions are indicated in the Methods section.

2.2 Spectra of Non-Hyperpolarized p27 and Complexes

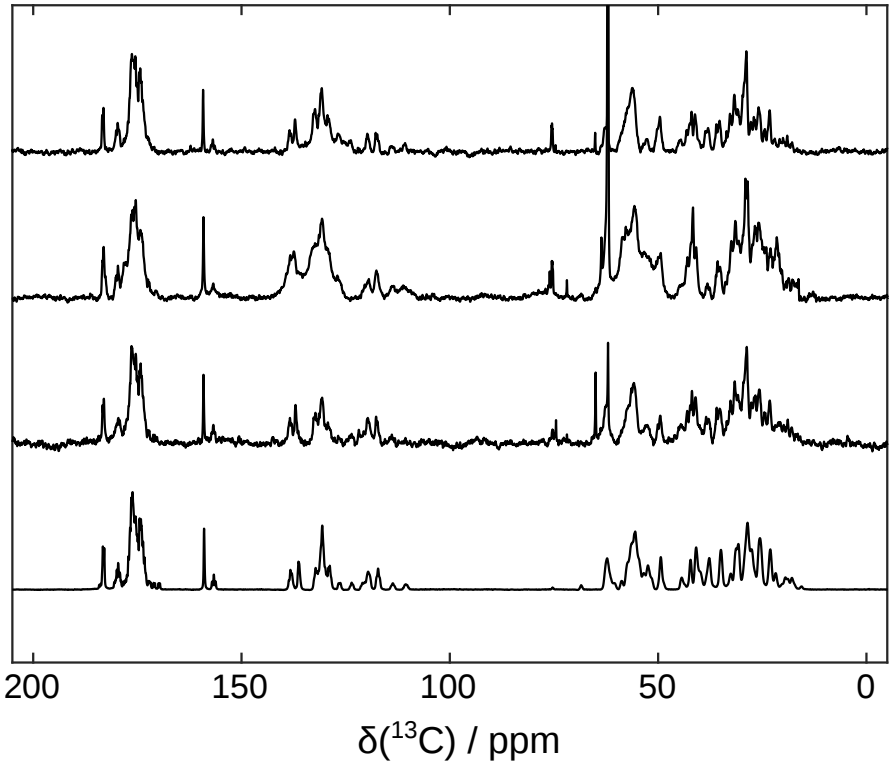

Figure S2: ^{13}C NMR spectra of, bottom to top, p27, p27 with Cdk2, p27 with Cdk2/cyclin A, p27 with cyclin A. Protein concentrations were nominally 150 µM p27, 100 µM p27 and Cdk2, 150 µM p27 and Cdk2/cyclin A and 140 µM p27 and cyclin A. All samples were prepared in a solution containing 20 mM sodium phosphate, pH 6.5, 200 mM NaCl, 10%(v/v) D_2O and 10 mM dithiothreitol-d10. Samples were prepared by mixing 150 µM ^{2}H, ^{13}C-p27 with equimolar amounts of the required components and incubated overnight. The samples have different final concentrations and signal intensities due to differences in the solubility and size of the respective complexes. All spectra were measured on a 600 MHz (14.1 T) NMR spectrometer equipped with a QCI cryoprobe (Bruker Biospin, Billerica, MA) with ^{1}H decoupling (WALTZ-16 with a field strength of 1.3 kHz). Signal averaging was for 4048, 21504, 35840, and 21504 scans for the spectra from bottom to top, using a delay between scans of 3 s and a 90° excitation pulse. Spectra were calibrated indirectly based on ^{1}H chemical shift of DSS that was added to the p27 sample, and were scaled to equal intensity in the ^{13}CO region.

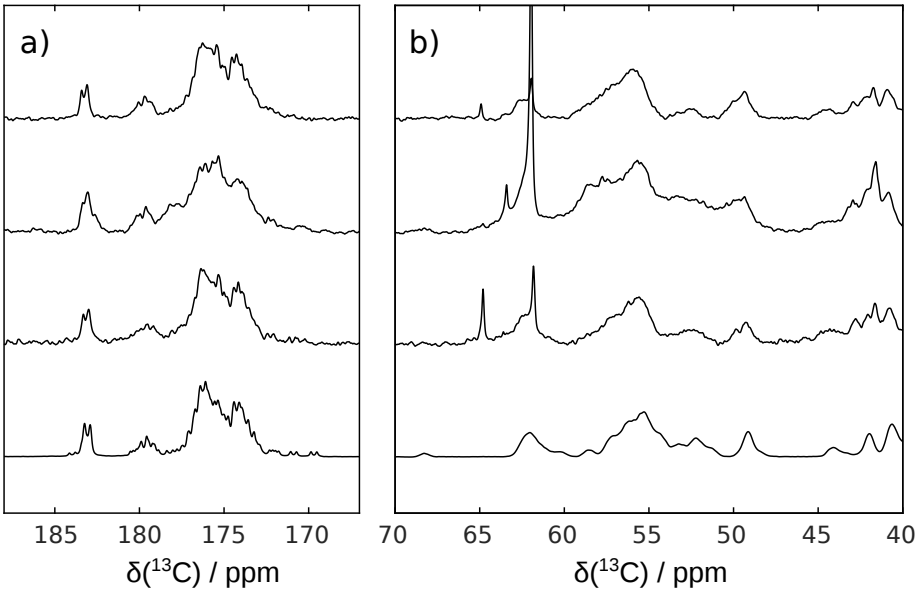

Figure S3: ^{13}CO (a) and $^{13}C_{\alpha}$ (b) region of the spectra from Figure S2. Spectra are, from bottom to top, p27, p27/Cdk2, p27/Cdk2/cyclin A, p27/cyclin A. Sample conditions and experimental parameters are indicated in the caption to Figure S2

2.3 Calculated Relaxation Rates

To understand the decay curves shown in Figure 3, expected relaxation parameters (as a function of rotational correlation time, τ_c) were calculated using molecular parameters.[6–8] In the case of ^{13}CO, the dominant relaxation mechanism is due to chemical shift anisotropy (CSA) (Figure S4a), whereas for $^{13}C_\alpha$, relaxation due to dipole–dipole (DD) interaction with the α deuterium dominates (Figure S4b). Residual protons in the deuterated aliphatic sites affect ^{13}CO only weakly (Figure S4c), whereas for protonated $^{13}C_\alpha$, the decay rate constants would be too large to still observe a signal (Figure S4d). Residual protonation is therefore not further considered. The resulting relaxation rates were used for comparison with the experimentally determined signal decay rates, as described in the main text.

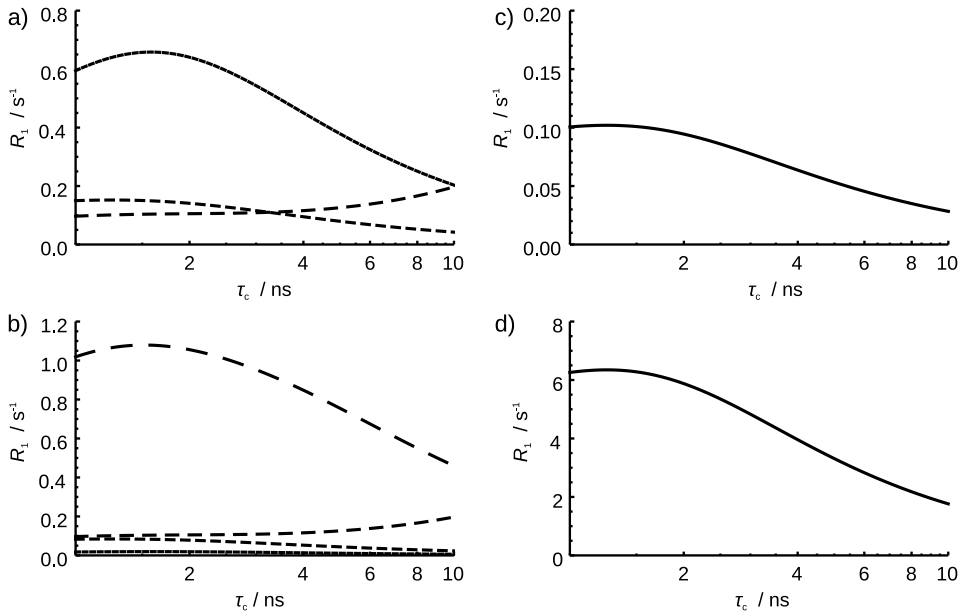

Figure S4: Calculated R_1 relaxation rates as a function of rotational correlation time τ_c. a) R_1[^{13}CO] due to chemical shift anisotropy, dipole–dipole interaction with amide proton, and with $^{13}C_\alpha$, for lines with shortest to longest dashing, respectively. b) R_1[$^{13}C_\alpha$] due to chemical shift anisotropy, dipole–dipole interaction with amide proton, with ^{13}CO or $^{13}C_\beta$ (using same distance), and with α deuterium for lines with shortest to longest dashing, respectively. c) R_1[^{13}CO] due to dipole–dipole interaction with α proton. d) R_1[$^{13}C_\alpha$] due to dipole–dipole interaction with α proton. Parameters for the calculation were ^{1}H Larmor frequency of 400 MHz, $\Delta\sigma_{CO}$ = –125 ppm, $\Delta\sigma_{C_\alpha}$ = 21.5 ppm, r_{COHN} = 0.203 nm, r_{COH_α} = 0.217 nm, r_{COC_α} = $r_{C_\alpha C_\beta}$ = 0.153 nm, $r_{C_\alpha H_\alpha}$ = 0.109 nm, $r_{C_\alpha HN}$ = 0.224 nm, $r_{C_\alpha D_\alpha}$ = 0.105 nm.[6,7]

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