Complementary Techniques
ANSTO school

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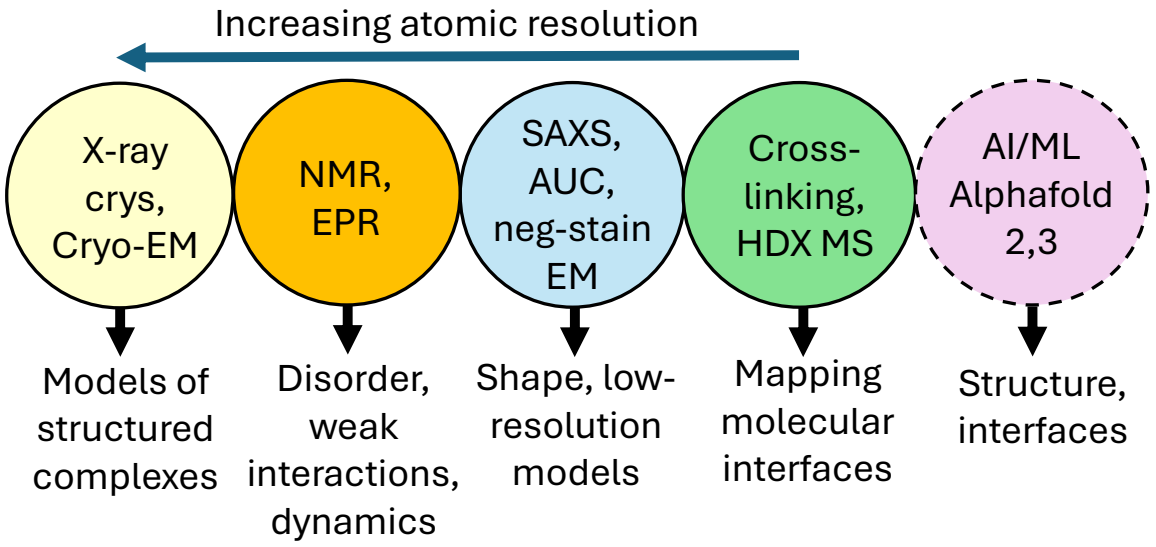
Structural Biology Techniques as applied to proteins and other macromolecules

Increasing complexity (assemblies, disorder, multiple conformational states) require a broad range of experimental approaches.



AUC, analytical ultracentrifugation
HDX-MS, hydrogen-deuterium exchange mass spectrometry
NMR, Nuclear Magnetic Resonance spectroscopy
EPR, Electron Paramagnetic Resonance spectroscopy

What can we do with solution NMR?

- **Three-dimensional structure** (<30 kDa): small proteins especially with flexible regions.
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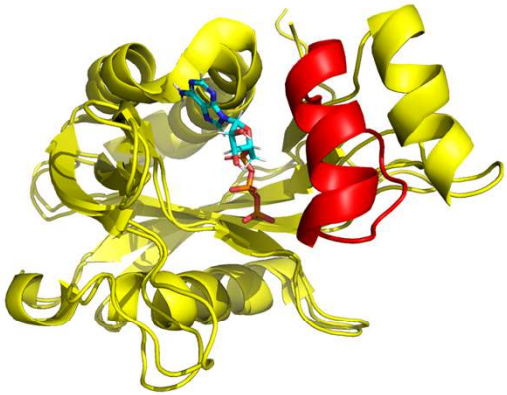
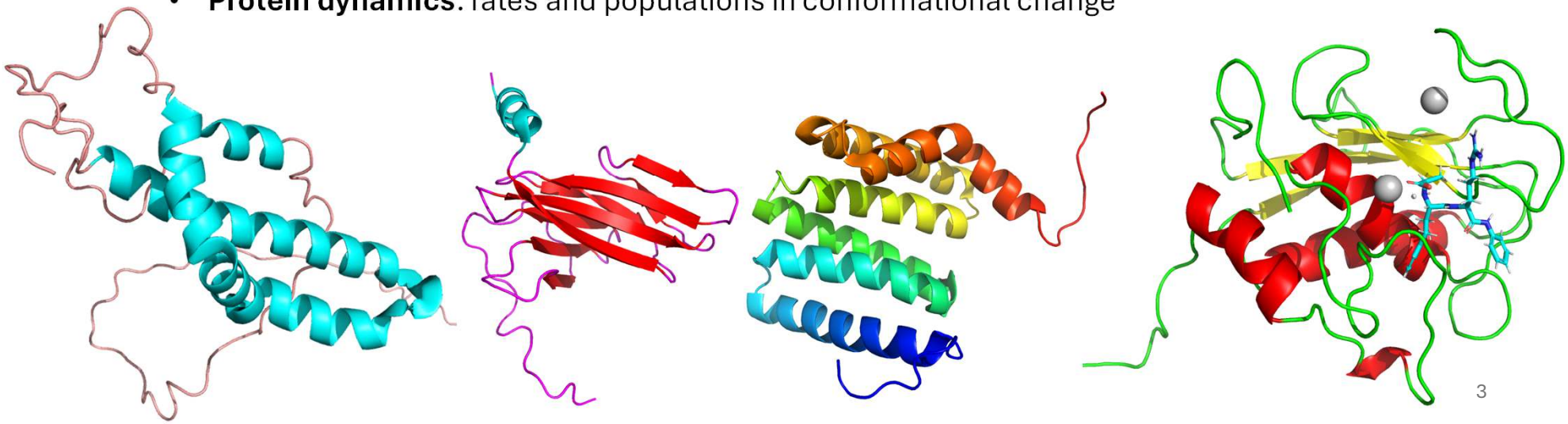
- **Ligand binding or measure interactions**: define binding sites, screening for drug leads.

- **Protein Disorder**: characterizing transient structures, understanding phase separation.

- **Protein dynamics**: rates and populations in conformational change



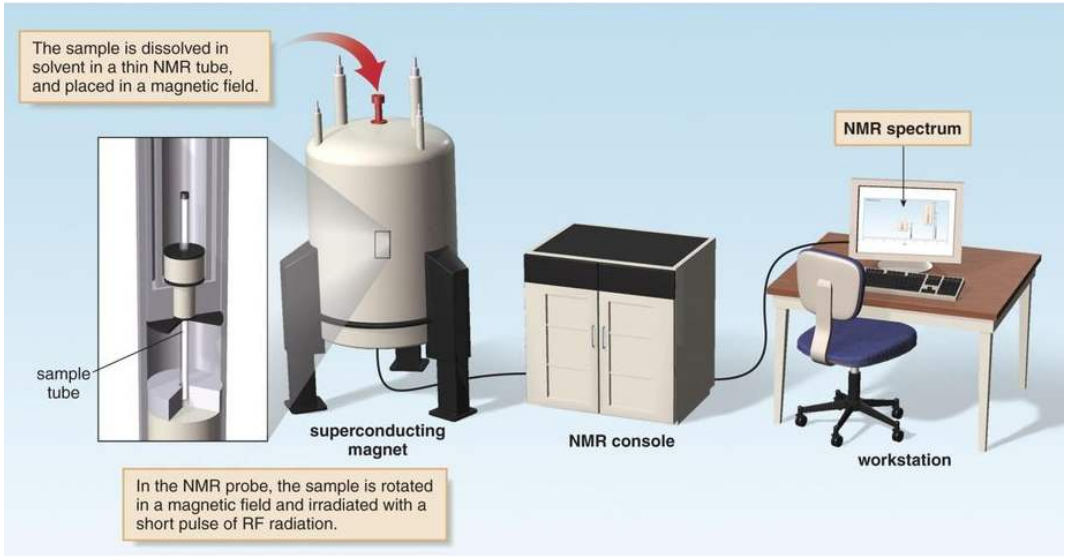


NMR spectroscopy, what do you need to know?

An NMR active nucleus resonates in a magnetic field with:

a) a specific characteristic frequency (chemical shift) determined by its chemistry and molecular environment

b) a characteristic line-width determined by molecular tumbling and internal dynamics



NMR active nuclei: ^{1}H,^{13}C,^{15}N,^{19}F,^{31}P these are spin-1/2 and give simple spectra.

Chemical shift (δ ppm), the position of a resonance in a spectrum

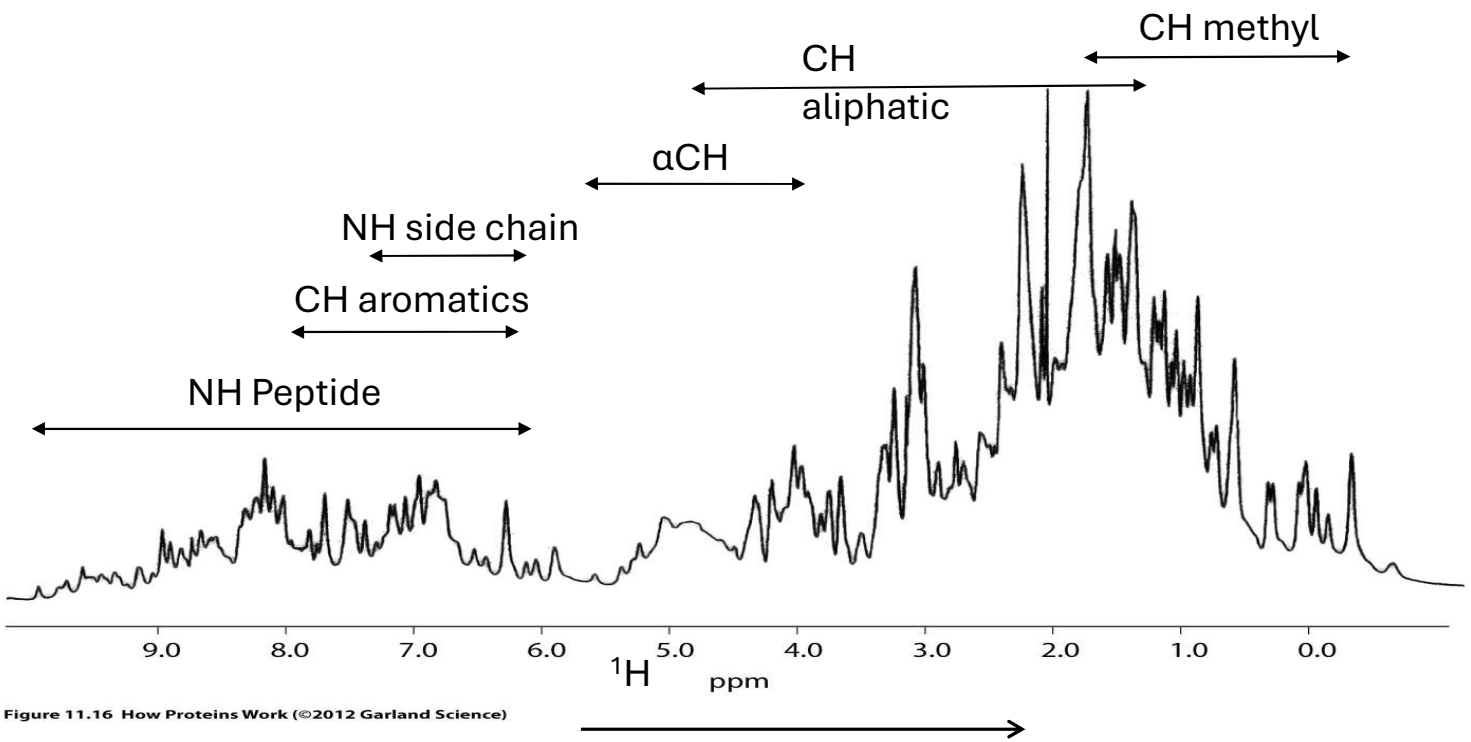


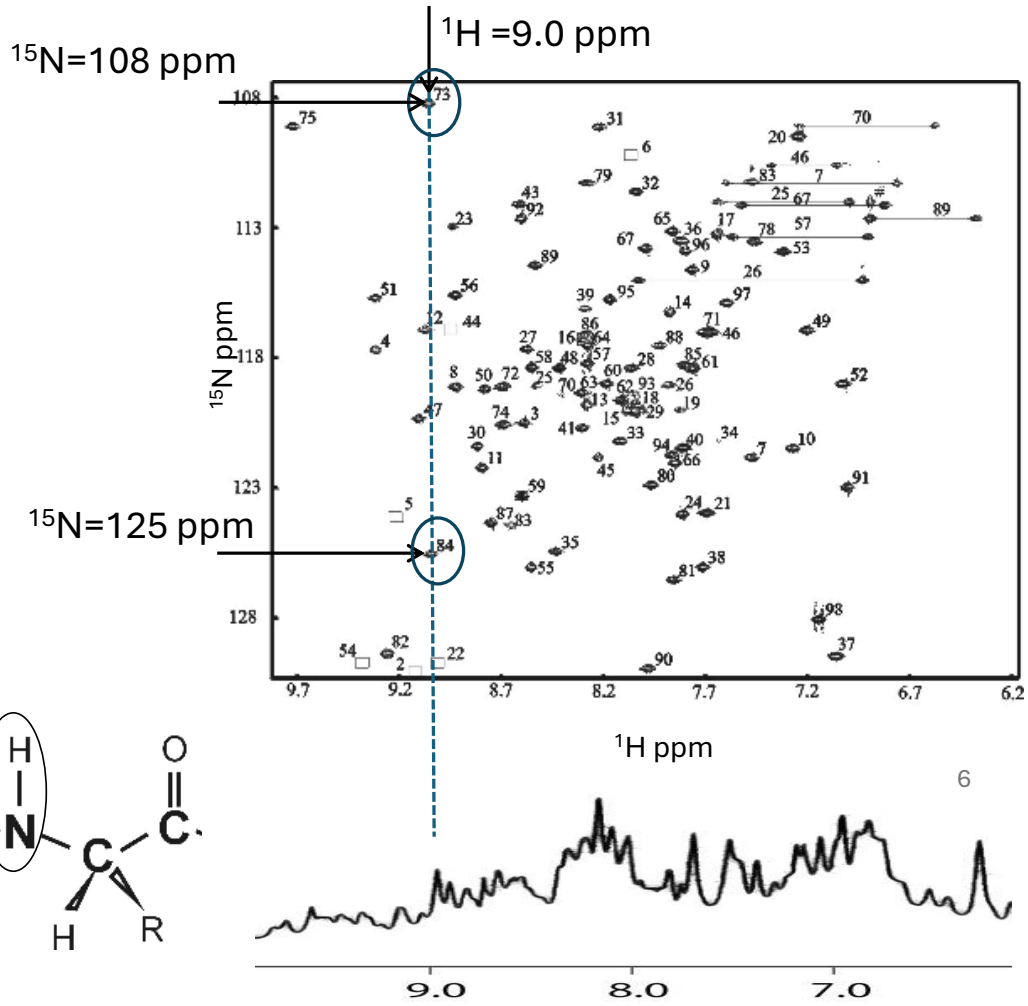
Figure 11.16 How Proteins Work (©2012 Garland Science)

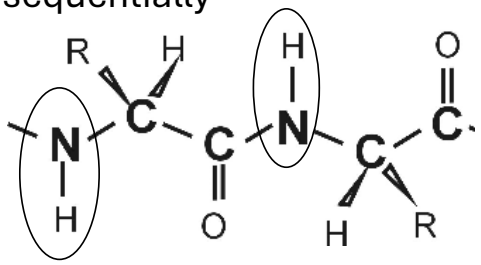
The nucleus is progressively more shielded from its electron cloud

- A typical ^{1}H NMR spectrum of a well-folded protein with a well-defined structure has a <u>well-dispersed spectrum</u>
- Despite well-dispersed many resonances are not resolved.

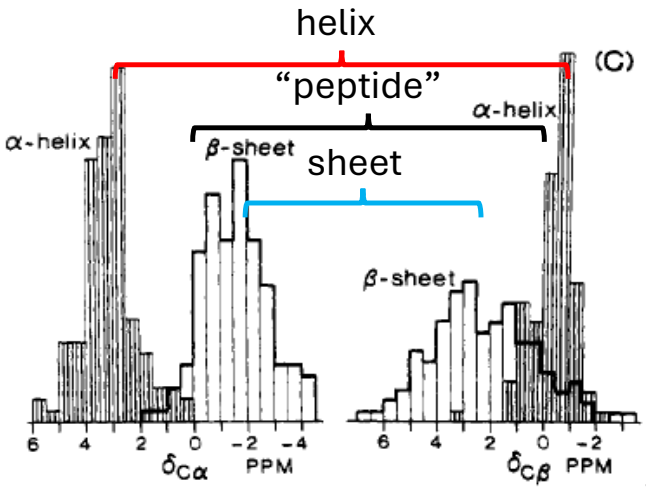
Chemical shift, in more than one dimension

- Multidimensional means <u>two or more frequency ranges</u> (dimensions) – here one is ^{1}H, the other ^{15}N.
- This spectrum, the **^{15}N,^{1}H HSQC**, has only resonances that arise from ^{1}H <u>covalently</u> attached to ^{15}N.
- The advantage is we can resolve overlapping resonances.
- The disadvantage is the protein has to be labelled with the rare isotopes ^{15}N and ^{13}C
- In the example peaks are labeled for each amide of the sequence. We apply methods to sequentially assign resonances (triple resonance NMR).





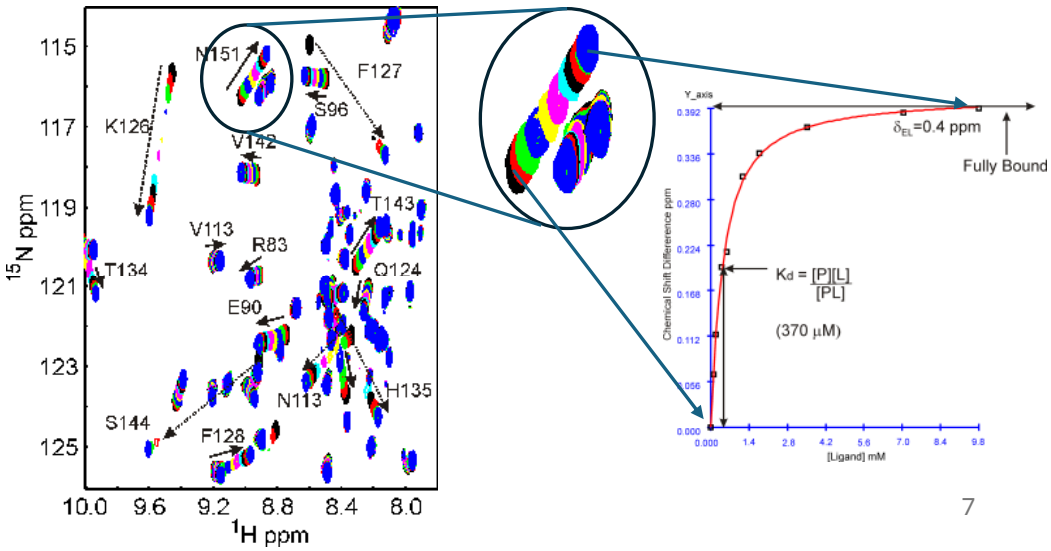
Practical points for chemical shift.



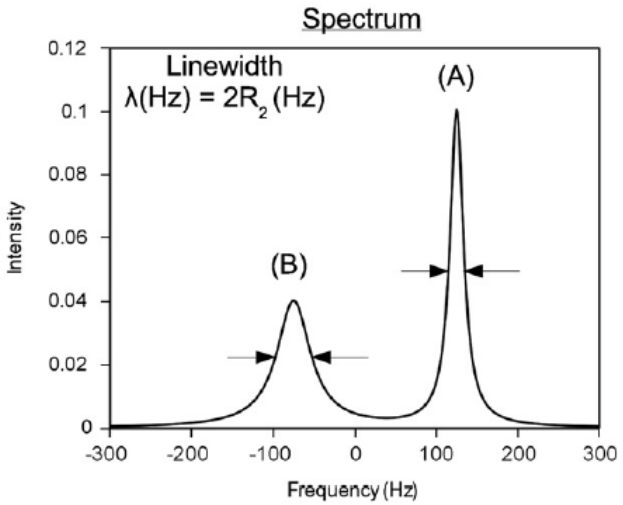
Spera and Bax JACS 113 p5491

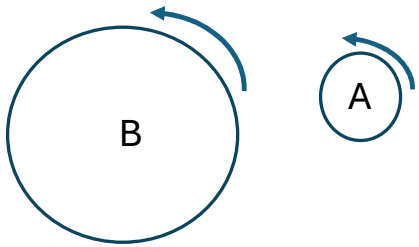
- The chemical shifts of resonances, especially the $^{13}C\alpha$ and $^{13}C\beta$, are strongly dependent on secondary structure, even if the structure is transient.

- Chemical shift is sensitive to binding of ligands of almost any K_D (nanomolar to molar). Ligand titration can be used to calculate K_D (>1 µM) and to map ligand-binding sites or change to conformation.



line-width (λ Hz or s^{-1}), a measure of molecular motion





<u>Line-width</u> (λ) full peak-width at half-peak height. Quantified as R_2 (spin-relaxation rate, Hz or s^{-1})

$\lambda_{(\text{half height})} = 1/(\pi T_2)$ (spin relaxation time, s)

$1/T_2 = R_2$ (s^{-1}) (Rate constant)

The natural line-width is mostly influenced by <u>molecular size</u>.

The R_2 for ^{1}H, ^{13}C or ^{15}N can be measured.

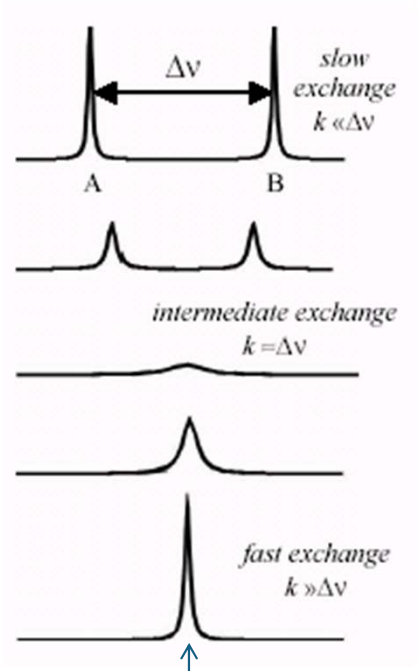
Resonance (A) has a line width (λ) of 25 Hz;
therefore T_2 is 13 ms and R_2 is 78 s^{-1}

Resonance (B) has a line width 50 Hz;
therefore T_2 is 6 ms and R_2 of 157 s^{-1}

We would say B <u>relaxes faster</u> than A or the signal for B disappears faster than the signal for A.

Proteins are flexible, how does internal motion effect line-width?

Conformational change, ligand binding and internal motion influences line width. Line width is the sum of the intrinsic molecular tumbling and this extra motion (chemical exchange).



A nucleus somewhere on the protein that experiences "extra motion".

Consider a protein that can exist in two states eg folded helix (A) /unfolded helix (B), apo (A) /ligand-bound (B).

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B$$

What is observed depends on the **<u>rate of exchange</u>** ($k_{ex} = k_1 + k_{-1}$) between the two states, A and B. Three extremes:

<u>Fast</u>: the line width is typical of the molecule's size; cannot distinguish A and B. **k_{ex} is usually faster than microsecond timescale.**

<u>Slow</u>: two distinct resonances are observed, but line width of each is typical of the molecule's size. Remember the sample is homogeneous. Look back at fast – its chemical shift is at the mid-point. If A ≠ B, the chemical shift will be weighted. **k_{ex} is usually millisecond to longer than seconds.**

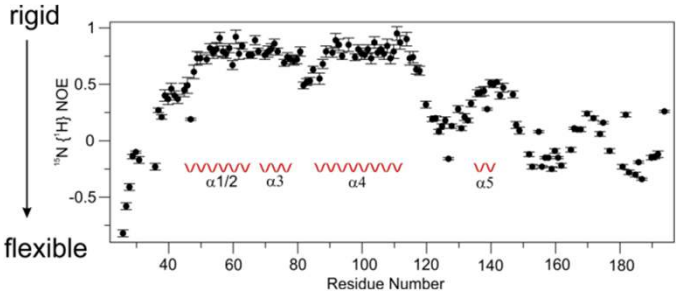
<u>Intermediate</u>: one or two resonances, but note the wide lines so large R_2. (The extra line-width is called R_{ex} (chemical exchange) and is a probe into "functional" motion). **k_{ex} is usually micro to milliseconds.**

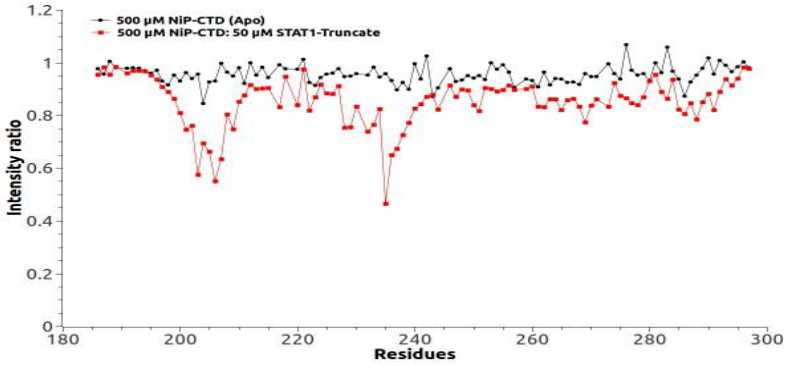
Practical points for line-width.

- Exchange rates (k_{ex}), relaxation rates (e.g R_2, R_1, het-NOE), populations of conformational states can be quantitated.
- Transient structure can be observed as line-broadening complementing chemical shift.
- Experiments can also be designed to take advantage of "broadening" resonances to probe interactions and conformation.

1. Transfer of saturated magnetization from one molecule to an interacting molecule: signal broadening maps a binding site.

2. Covalently attach a broadening agent (paramagnetic or spin-label) and monitor line broadening that signifies an interaction.



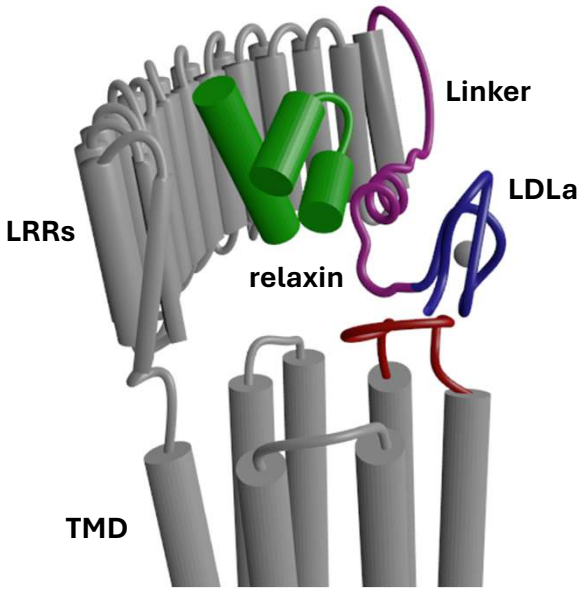


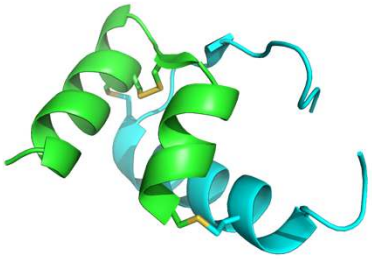
Mechanism of activation of RXFP1 (a GPCR)

1. **LDLa and linker interact with Transmembrane Domain (TMD)**

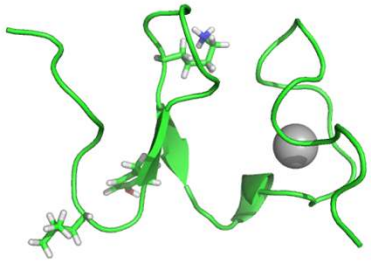
2. **Relaxin binds to:**
   - LRRs
   - Linker

3. **Relaxin binding stabilizes linker helix:**
   - Reorients LDLa and linker
   - Active state of receptor promoted





relaxin

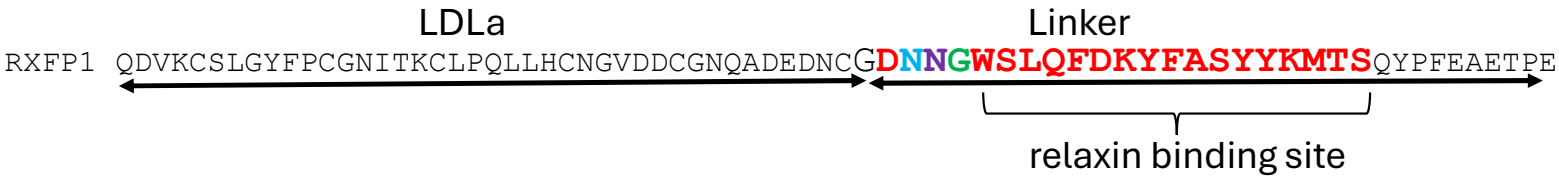


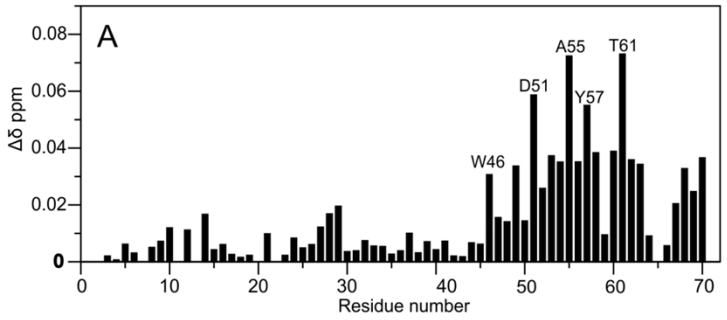
LDLA module

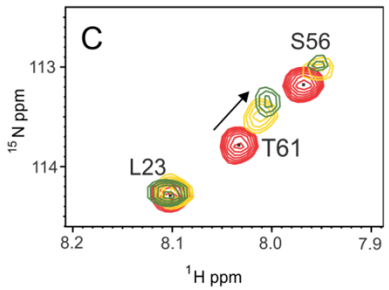
SIGNALLING

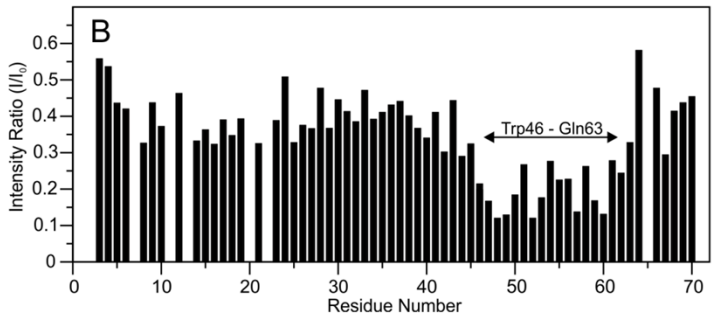
Sethi et al. *Nat. Commun.* 2016
Hopkins et al *J. Biol. Chem.* 2007

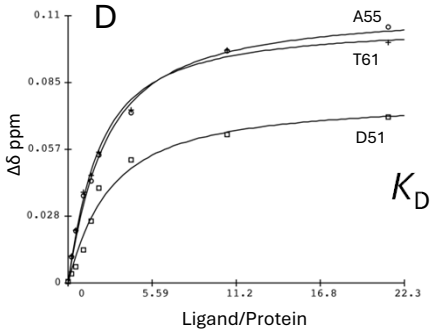
The binding site of relaxin on the LDLa-Linker.







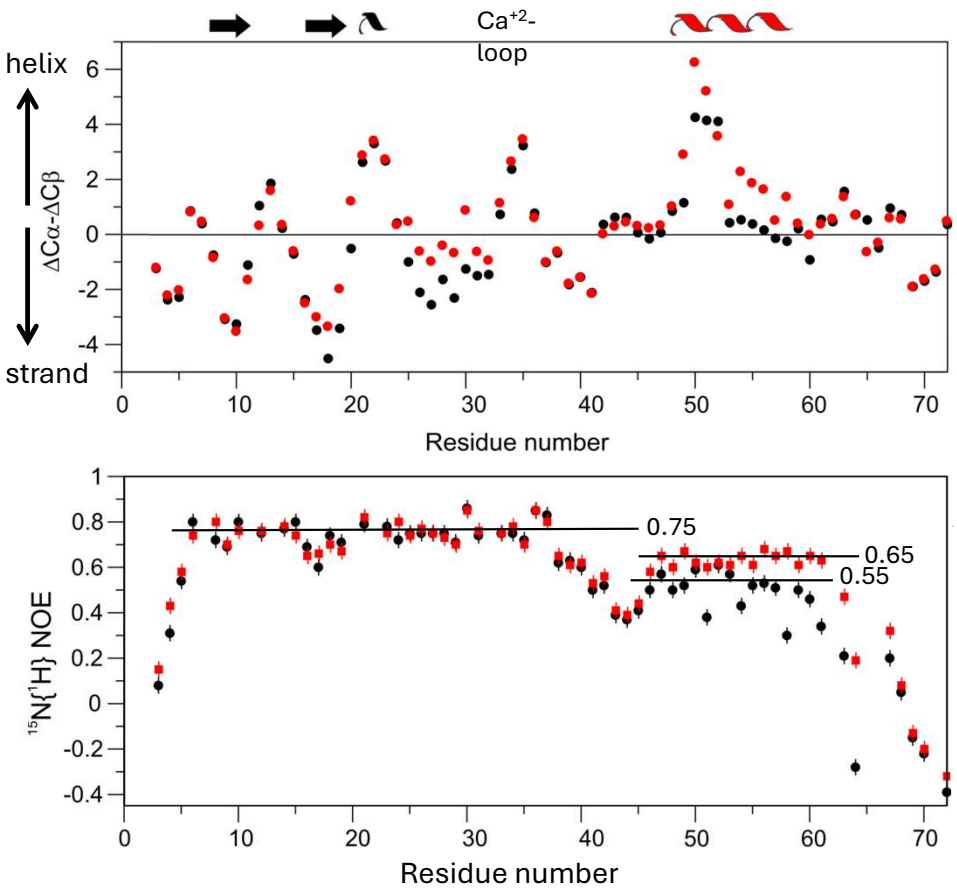


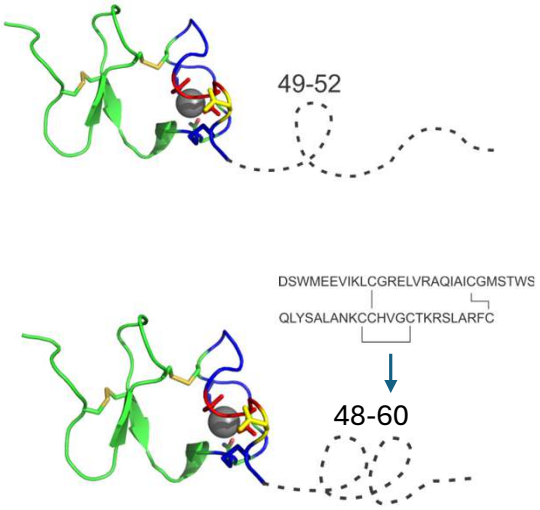


- Chemical shift and line-broadening show where relaxin binds
- Conformational change?

K_D 90 μM

A helix is stabilized in the linker on binding relaxin.

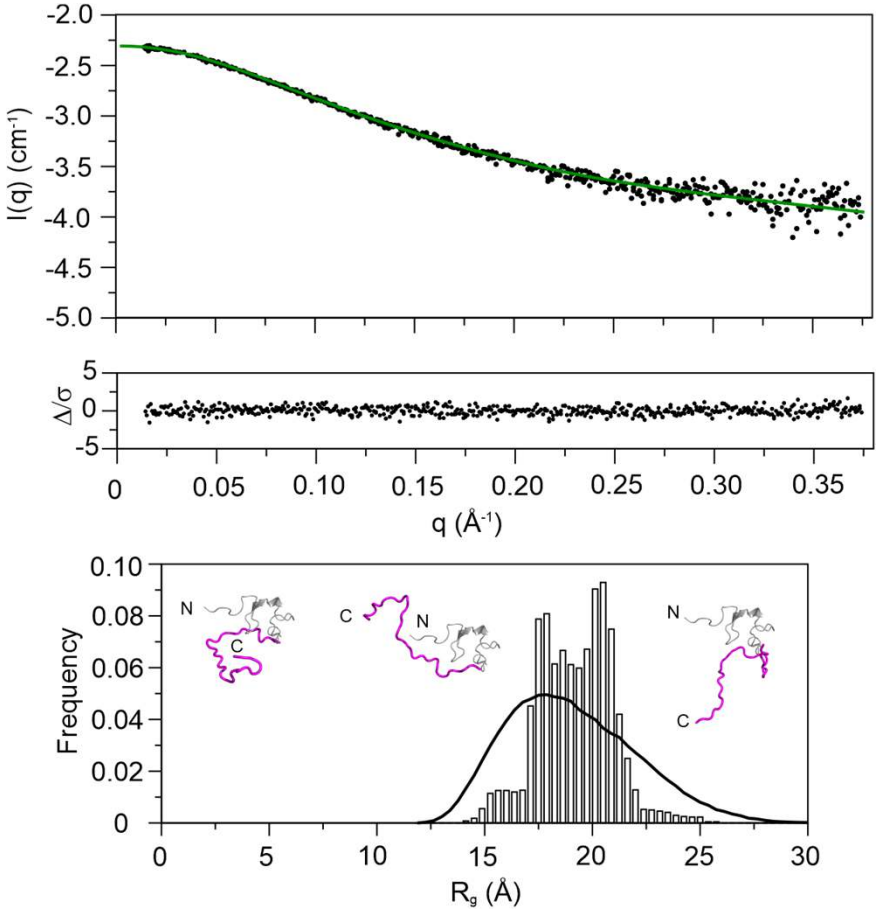




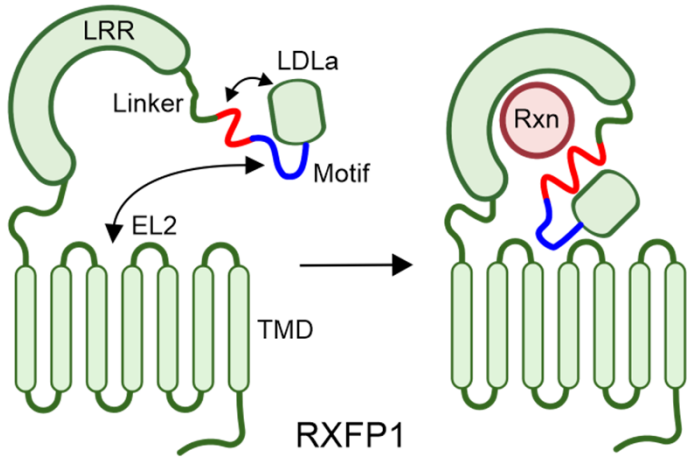
Is this the structure? Where is the linker in relation to the LDLa module?

- No relaxin
- With relaxin

Small Angle X-ray Scattering of the LDLa-linker

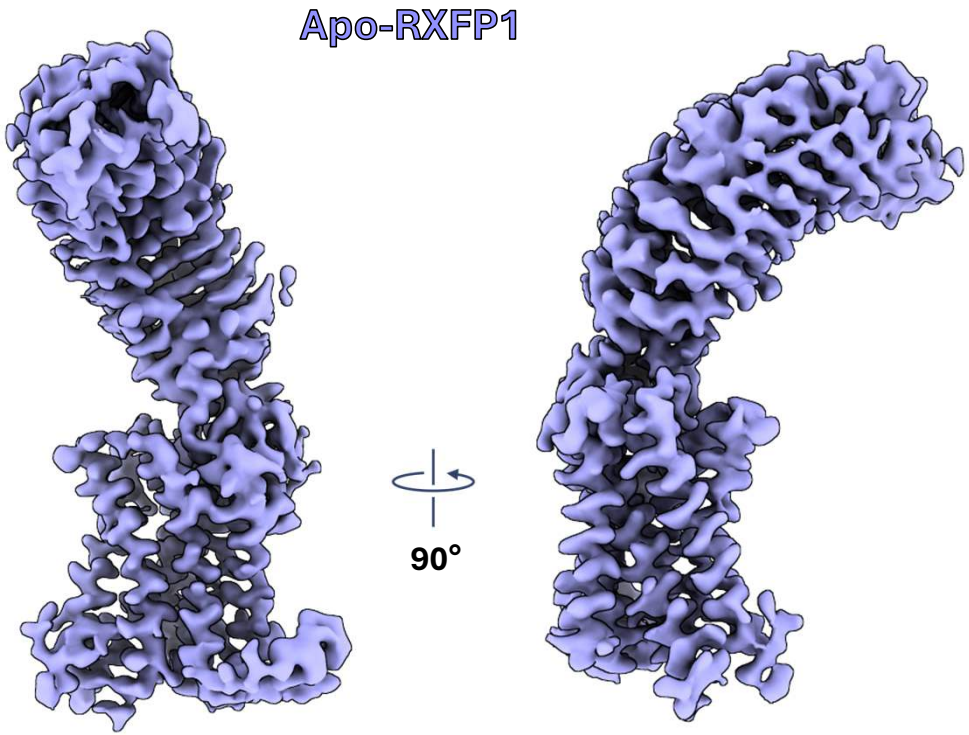


- EOM analysis shows the linker folds back on the LDLa module
- We propose the activation mechanism requires relaxin and the LDLa module to stabilize the linker conformation. Are we right?

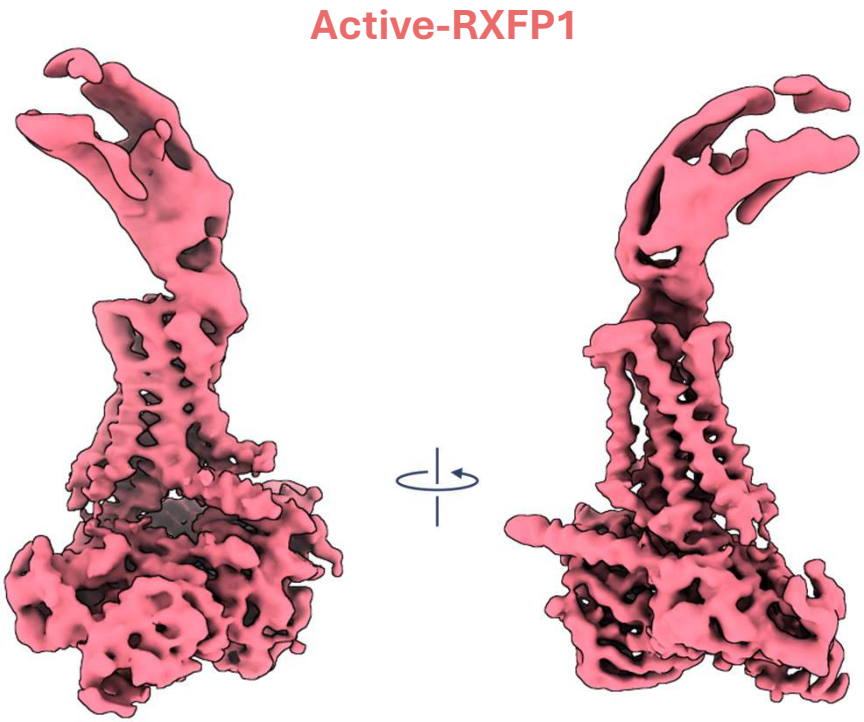


cryoElectron Microscopy of the Apo and Active full-length relaxin receptor RXFP1: LDLa-linker is missing!

Is there another method to test LDLa-linker/relaxin interaction?

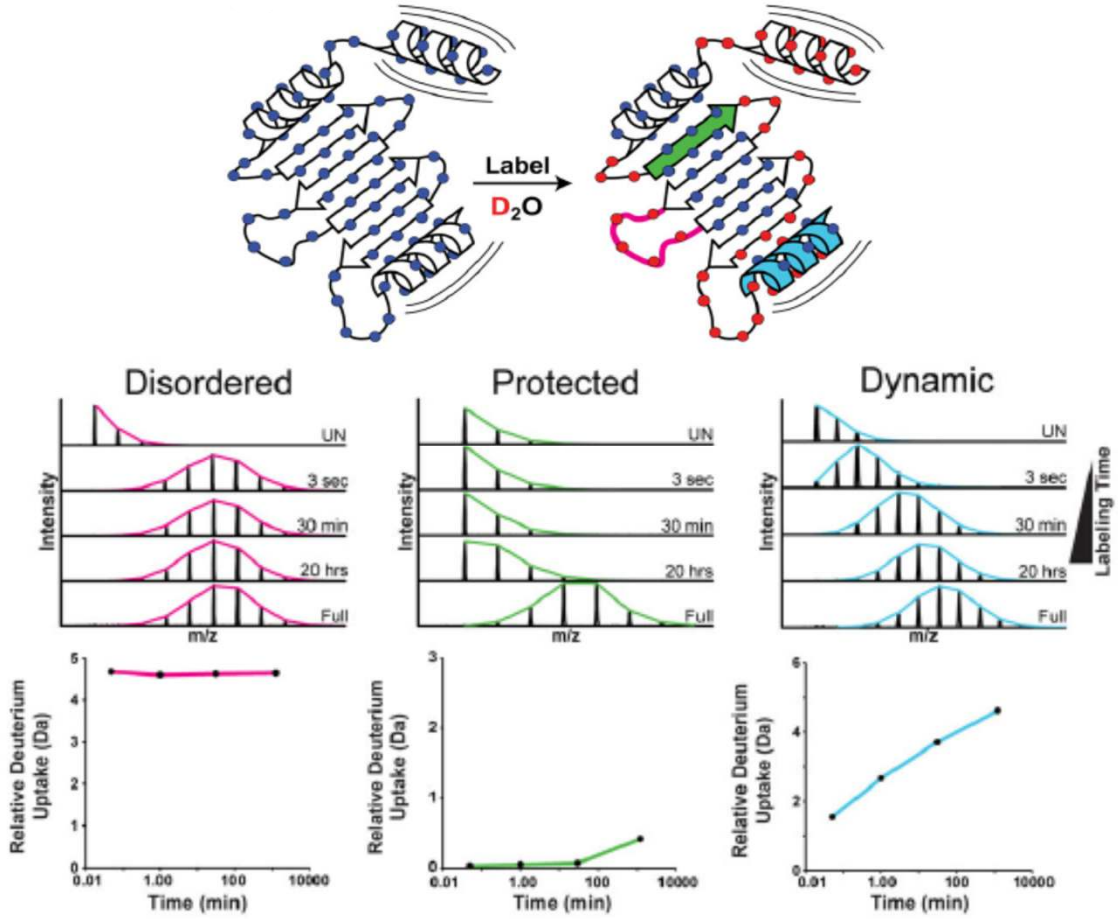


Tiffany Myint, unpublished



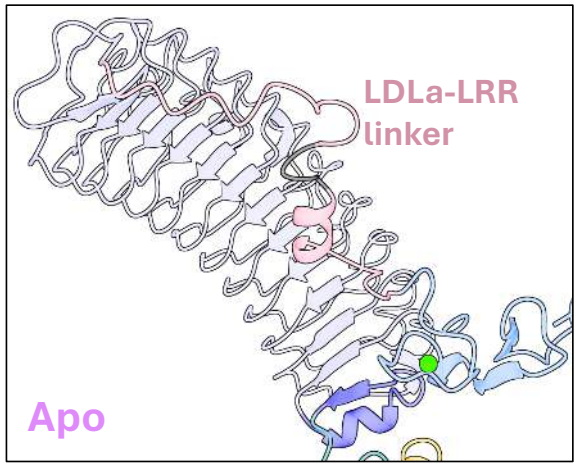
Kruse Lab, Harvard

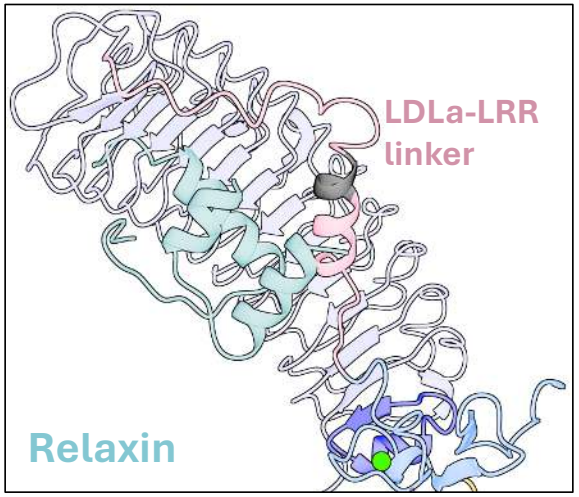
Hydrogen-deuterium Mass Spectrometry

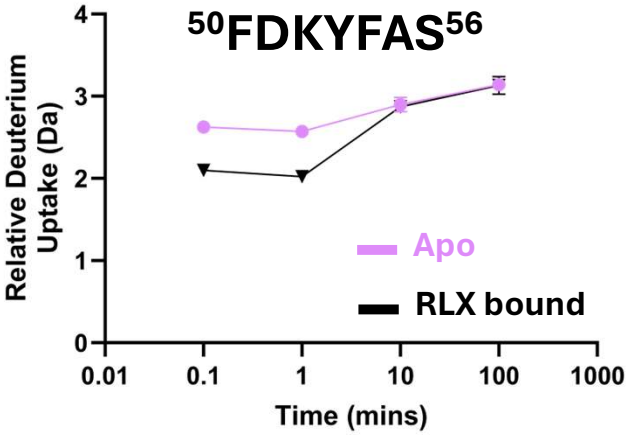


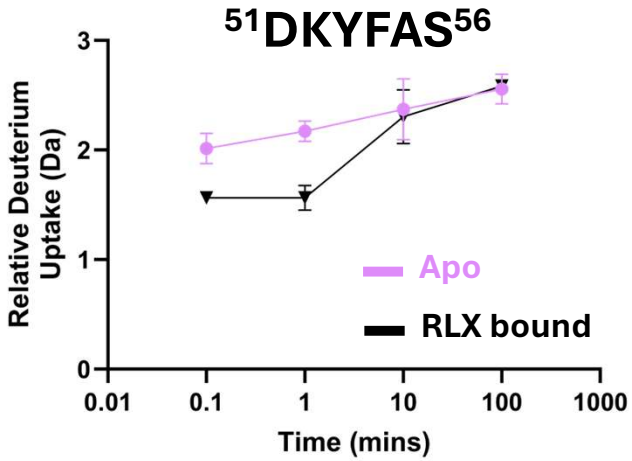
- HDX measures the peptide NH
- HDX measures hydrogen bond strength (protection).
- Exchange requires breaking of the hydrogen bond (unfolding)
- Dissolve protein in ^2H_2O and over time take a sample, digest (pepsin) and monitor change in mass of peptide due to ^2H replacing ^1H.

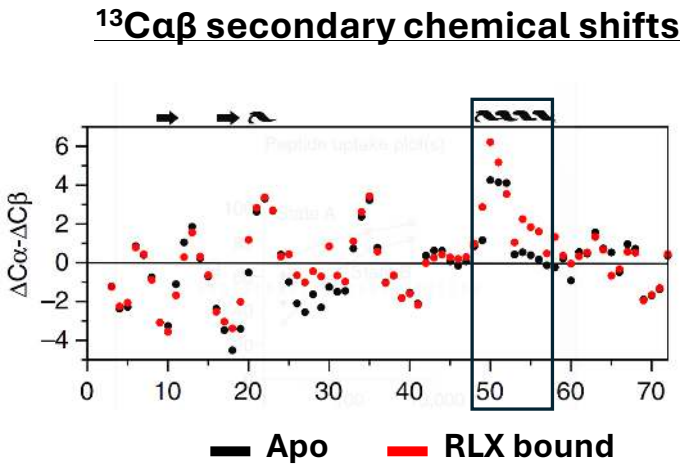
HDX-MS of apo & relaxin-bound full-length RXFP1 support stabilization of linker helix











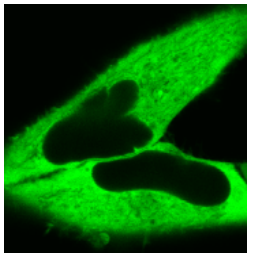
Brad Hoare, unpublished

Host-virus Interactions of the multifunctional P protein from Rabies

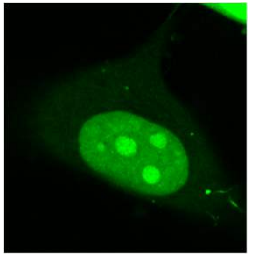
- Rabies virus, small genome (12 kb), 5 proteins (N,P,L, M,G)
- P protein is highly multifunctional; 5 isoforms.
- Only full-length P1 is involved in replication. What do the others do (focus on P3)
- P1 a 66 kDa dimer; P3 55 kDa.
- What are the molecular mechanisms in host interactions?

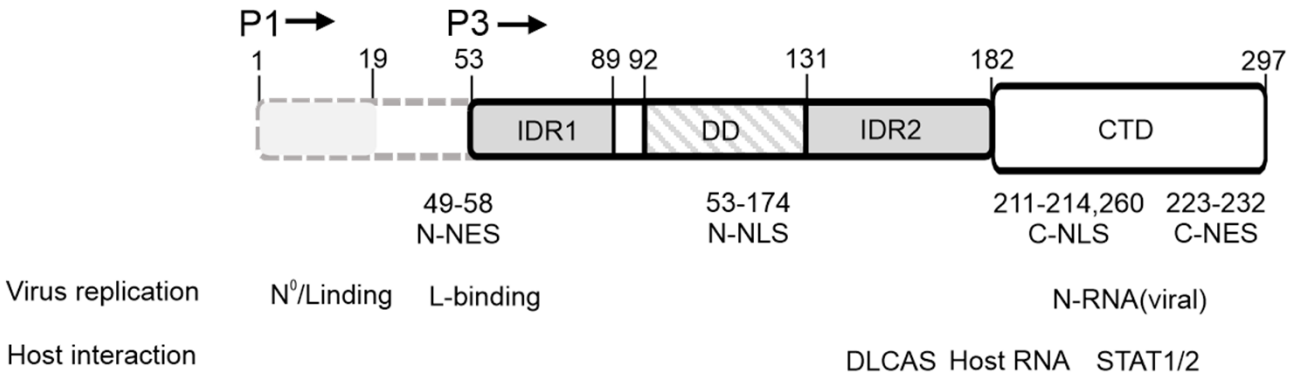
GFP-tagged P

P1, cytosolic

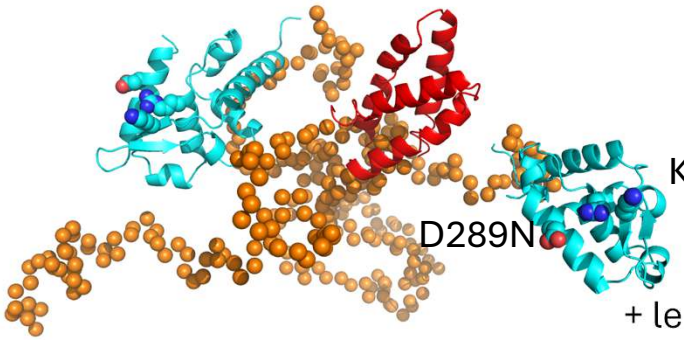


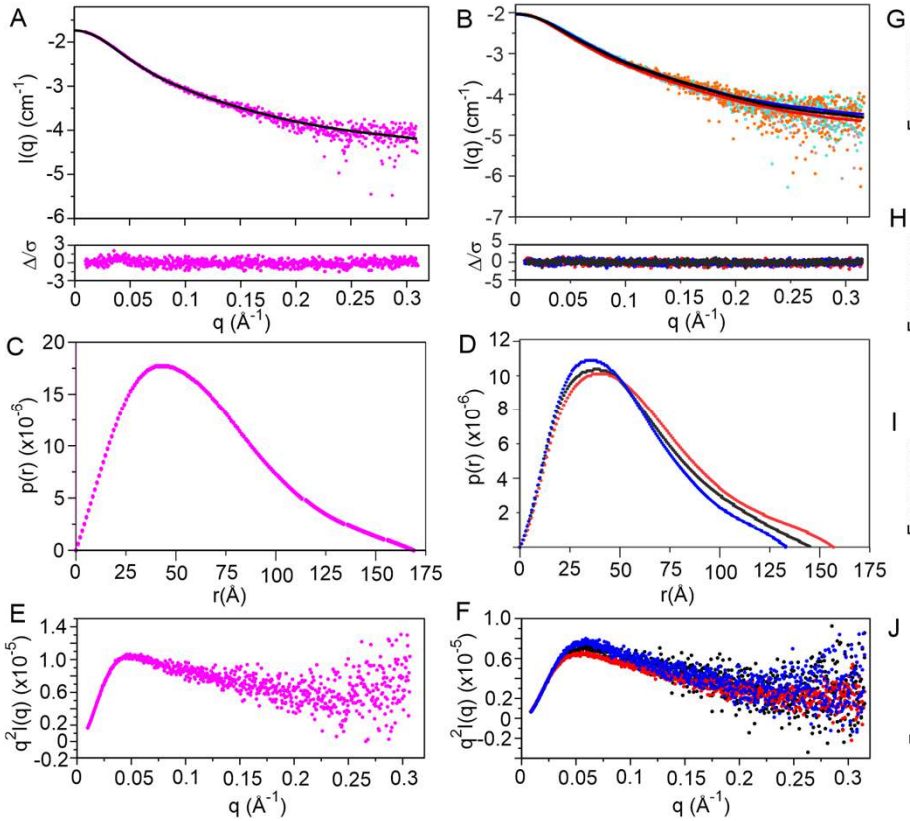
P3, nuclear, nucleous

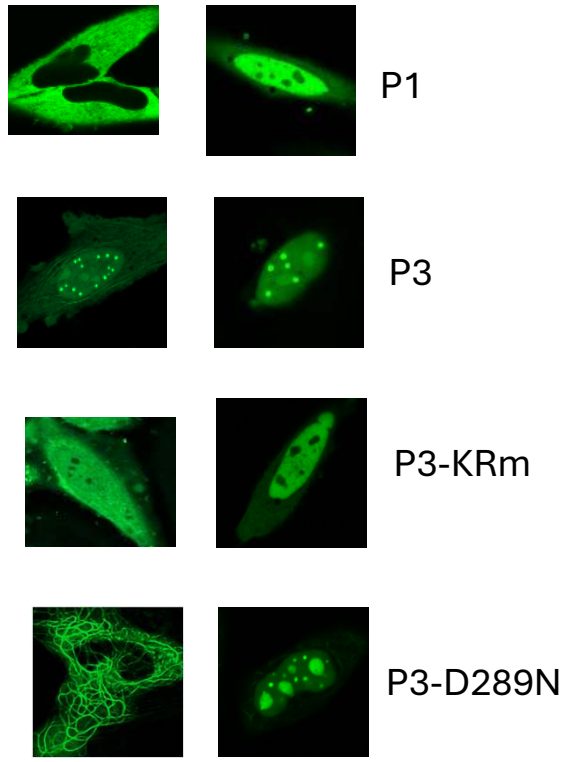




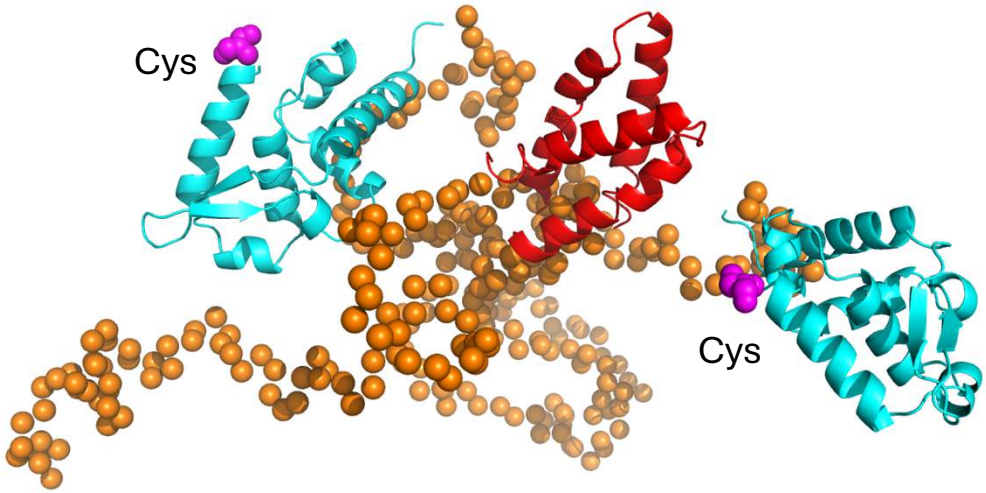
Are there molecular shape differences for P1 and the shorter isoform P3?

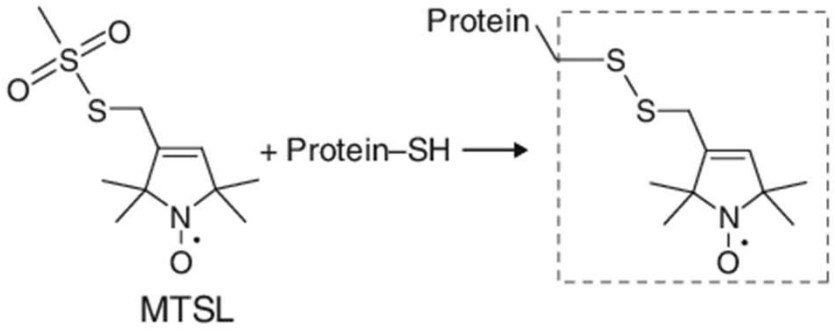






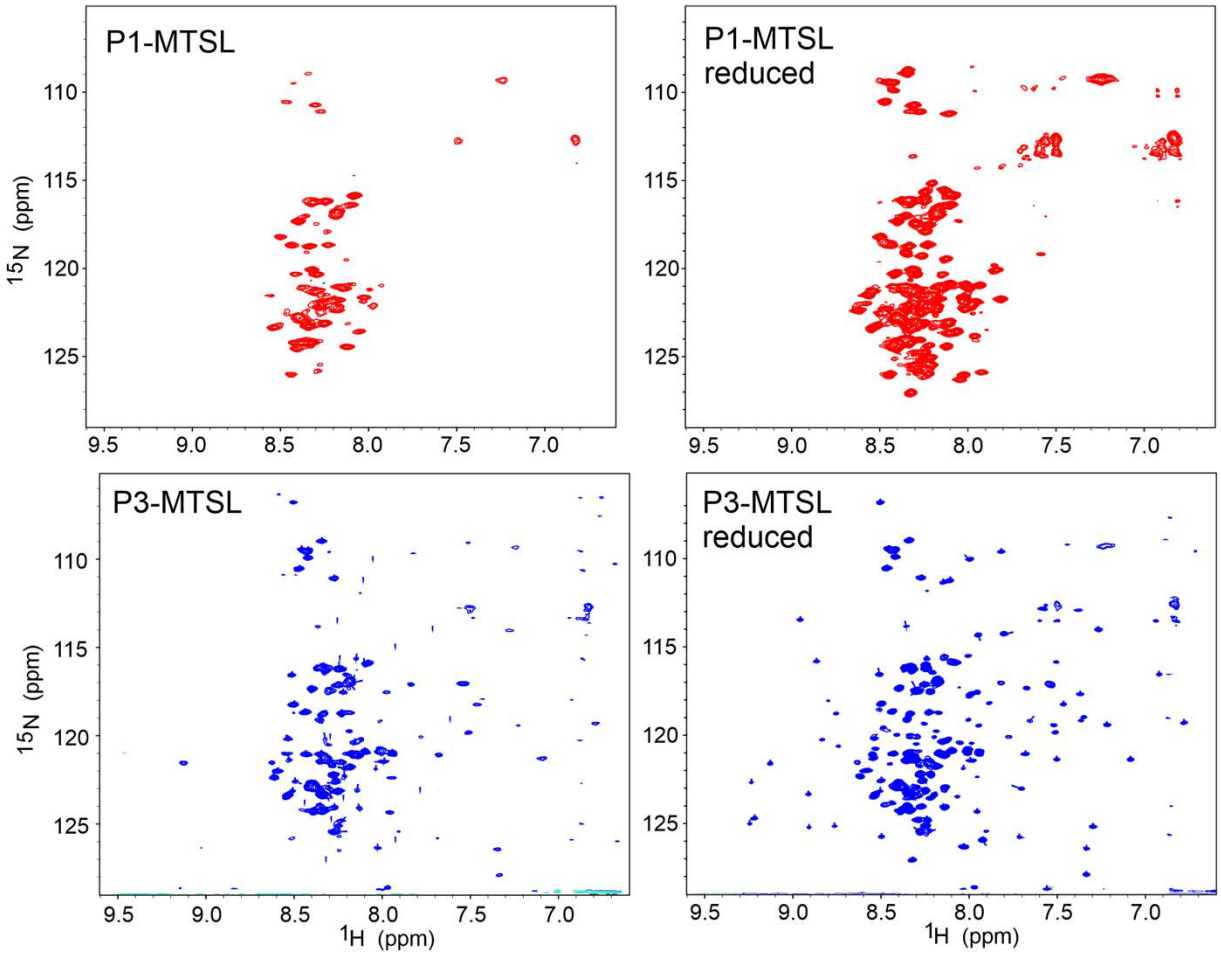
Measuring conformational change using NMR Paramagnetic Resonance Enhancement (PRE).

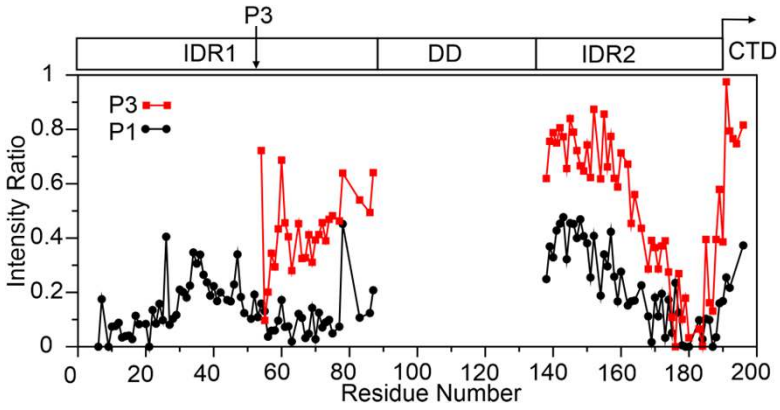




- Covalently attach a paramagnetic spin-label (e.g. MTSL) to a Cys.
- If the spin-label is near another nucleus we will see broadening of its resonance.
- Collect two spectra, one with the spin-label and another with the spin-label reduced (ascorbate)

Assessing molecular shape of P1 and P3 by PRE experiments.





All resonances of the IDRs are broadened in P1 and much less in P3 suggesting:

- P1 is mostly closed
- P3 is mostly open

Functional significance?

Acknowledgments

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Stephen Rawlinson