Advanced Applications of NMR:
Samples, Dynamics and Examples
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Where to find us!
• Samples
• Dynamics
• Examples
  – Ubiquitin: phosphorylation and novel conformer
  – LITAF: modeling patient mutations in a membrane protein
• Samples
• Dynamics
• Examples
  – Ubiquitin: phosphorylation and novel conformer
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Molecular weight range

<table>
<thead>
<tr>
<th>Molecular Weight</th>
<th>Full 3D Structure, Dynamics</th>
<th>Assignments, 3D fold recognition, internal dynamics, interactions, functional studies</th>
<th>Individual residues, complex interactions, docking, IDP, mobile linker motion</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 kDa</td>
<td>2D/3D</td>
<td>2D/3D</td>
<td>Methyl-TROSY</td>
</tr>
<tr>
<td>20 kDa</td>
<td>1H</td>
<td>1H, 15N, 13C</td>
<td>Selective / segmental labeling (Intein)</td>
</tr>
<tr>
<td>40 kDa</td>
<td>1H, 15N, 13C</td>
<td>1H, 15N, 13C</td>
<td></td>
</tr>
<tr>
<td>&gt;100-1000 kDa</td>
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</tbody>
</table>

“Complete Picture” “Site Specific”
Sample preparation

• Minimal Media
  – $^{15}$N ammonium chloride
  – and/or $^{13}$C glucose ($^2$H&$^{13}$C glucose)
  – D$_2$O

• Sample conditions
  – 550 μL of 100-500 μM in aqueous buffer
  – mid-range pH
  – 5 % by volume deuterated solvent (D$_2$O)

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NMR timescale of Protein dynamics

- Libration
- Vibration
- Sidechain rotation
- Individual Mobility
- ps
- \( T_1 \)
- \( T_2 \)
- \((H)\text{NN} \text{NOE}\)
- "fast"

- Ligand binding
- Conformational Exchange
- Catalysis
- Protein folding
- \( \mu s - ms \)
- \( 10^{-6} - 10^5 \)
- \( 10^1 s \)

- Segmental motion
- Allosteric regulation
- H/D-exchange
- ZZ-exchange
- CPMG Relaxation Dispersion
- Line-shape analysis

- return of excited system to equilibrium
- reorientation of nuclei in external magnetic field causes local field fluctuations

Relaxation

- Signal decay
- Fourier transformation
- Line width at half height

\[ \nu_{1/2} = \frac{1}{\pi T_2} \]

Relaxation is related to molecular motion

*Bloembergen, Purcell and Pound (Phys. Rev. 73, 679-712 (1948))
Inter-conversion between states

Inter-conversion on intermediate µs-ms timescale results in line broadening:
apparent $R_2$ increase

Observed for:
- Slow versus fast folding of proteins
- 2-state binding equilibrium
- Cis-trans proline isomerisation
- Segmental motions in proteins
- Monomer-oligomer equilibria in solution

rate constants from line shape

$$R_{ex} = p_1 p_2 \delta \nu^2 \tau_{ex}$$
p_1 and p_2 populations of states A, B
$\tau_{ex} = p_2/k_1 = p_1/k_1$ and $\delta \omega = |\omega_A - \omega_B|$

Slow exchange $K_{ex} << \delta \nu$
coalescence $K_{ex} \sim \delta \nu$
fast exchange $K_{ex} >> \delta \nu$

Conformational Exchange

Cross-relaxation: $^1$H$^{15}$N NOE

$^1$H$^{15}$N NOE is the ratio between steady-state and equilibrium magnetisation:

$$^1H-^{15}N\text{NOE} = 1 + \frac{\sigma_{XY}}{R_{XY}}\gamma_X$$

Dipole-dipole interactions cause cross-relaxation:

$$\sigma_{XY} = \frac{d_{XY}}{4}[-J(\omega_y - \omega_x) + 6J(\omega_y + \omega_x)]$$

Probes fast picosecond mobility

Auto-relaxation of a $^{15}$N nucleus

- Longitudinal relaxation rate $R_1 = 1/T_1$

$$R_{1}^{\text{iso}} = \frac{d_{\text{iso}}}{4} \{ J(\omega_{r} - \omega_{b}) + 3J(\omega_{b}) + 6J(\omega_{a} + \omega_{b}) \}$$

$$R_{1}^{\text{CSA}} = cJ(\omega_{b})$$

- Transverse relaxation rate $R_2 = 1/T_2$

$$R_{2}^{\text{iso}} = \frac{d_{\text{iso}}}{8} \{ 4J(0) + J(\omega_{r} - \omega_{a}) + 3J(\omega_{a}) + 6J(\omega_{a} + \omega_{b}) \}$$

$$R_{2}^{\text{CSA}} = \frac{c}{6} \{ 4J(0) + 3J(\omega_{b}) \}$$

with $d_{\text{iso}} = \frac{\hbar^2 y_{H}^2 y_{N}^2}{r_{HN}^6}$ and $c = (\sigma_{1} - \sigma_{2})/2 \gamma_{L}^2 B_{0}^2$ (Field dependent term!)

$T_2$ against $T_1$ – is a really useful plot...

Assume $\tau_e = 50$ ps
T₂ against T₁ – is a really useful plot...


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Ubiquitination

- Highly conserved
- Chain permutations
- Multiple functions

Ubiquitin Chain Diversity at a Glance

Masato Akutsu, Ivan Dikic and Anja Bremm

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Phosphorylation of Ubiquitin by PINK1

- Mutations in the Ubiquitin E3 ligase Parkin lead to propensity to develop autosomal juvenile Parkinsonism
- Parkin is activated by the Kinase PINK1
- Parkin has a UBL, and this is a target for PINK1 phosphorylation
- Phosphorylated ubiquitin can also activate Parkin
- PINK1 phosphorylates ubiquitin at S65 – what are the consequences?
Protein Fingerprint

NMR active nuclei report on structure and dynamics, sensitive to environment – instant information

In situ Phosphorylation

100 μM $^{15}$N Ubiquitin
350 μM PINK1
10 mM MgCl$_2$/ATP
Phosphorylation time course

~ 60 extra peaks!

100 μM 15N Ubiquitin
2.5 μM PINK1
10mM MgCl₂/ATP

Unphosphorylated Ub

Phosphorylated Ub


Two Conformations – Major 70%

Two Conformations – Minor 30%

Structural differences?

Stability differences?

What could be happening?

• Two Conformations

\[ \text{Major Species} \leftrightarrow \text{Minor Species} \]
Studying the H-bond network
Long Range HNCO experiment


Long Range HNCO experiment

Confirmed with NOESY

Wauer et al. (2015) EMBO J. 34 p307

Solvent Accessibility

Surface, loops, termini are exposed:

H O R H O R

---N-C-C-N-C-C-N-C-C-N-C---

R H O R H O

R O H R O H

---C-C-N-C-C-N-C-C-N-C-C---

O H R O H R


Solvent Accessibility Timescales

- Solvent exposed
- Ligand binding
- Segmental motion
- Conformational Exchange
- Protein folding

Timescales:

- $10^{-4}$
- $10^{-3}$
- $10^{0}$
- $10^{1}$ s

H-H exchange (NMR)  
H-D exchange (NMR/MS)

Solvent Accessibility

H-D exchange = m-h-days
H exchange = ms

Graph showing ratio of exchange over exchange time in ms for various residues (Leu8, Gly10, Gln49, Thr12, Lys63) for WT-Ubiquitin.
Solvent Accessibility – H exchange

PhosphoUb

Solvent Accessibility – H exchange

PhosphoUb
Cleanex
Retracted conformation phosphorylation dependent?

2.5 μM PINK1
10mM MgCl₂/ATP

Wt Ub ➔ CR-Ub

Wt pUb ➔ CR-pUb

Rate at 25°C = 1.76 ± 0.09 S⁻¹

Retracted conformation without phosphorylation? CEST!

State A ➔ State B

Population ~ >99%

Population ~ <1%

• Must have unique chemical shifts in the 2 populations
Gladkova et al (2017) EMBO J. 36 p3555

CEST on Q62...

0.69% / 62 S⁻¹
Temperature dependence...

Is this the retracted conformation?
Is this the retracted conformation?

- Mutants TVLN (retracted) /L71Y (WT lock)
- Phosphorylation time courses

Phosphorylation of mutants...
Implications...

Summary

- In-situ phosphorylation
- Exchange between two forms characterized
- Not observable by crystallography
- Invisible conformation detectable and quantifiable
- Retracted conformation required for PINK1 phosphorylation
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Charcot-Marie-Tooth disease (CMT) and LITAF
• Most common inherited neuromuscular disorder (1:2500)
• Muscle weakness, loss of sensation: effects feet, lower legs, hands and forearms

Jean-Martin Charcot
1825-1893

Pierre Marie
1853-1940

Howard Henry Tooth
1856-1925
CMT: axons and myelin sheath

- Various genes associated with CMT, divided into subtypes
- Autosomal dominant & recessive
- Damage to Axon and Myelin sheath

![Diagram of Axons and Myelin Sheath]

CMT Type 1C: LITAF

- Lipopolysaccharide-induced tumour necrosis factor-α factor / small integral membrane protein of lysosome/late endosome (SIMPLE)
- 17kDa – possibly higher order complex
- Endosome associated
- N-terminal proline rich unstructured domain
- C-terminal “LITAF” domain containing hydrophobic helix

- Topology remained controversial in literature
LITAF - Localisation

Ho et al. (2016) BMC Biology. 14:109

LITAF - Localisation

LITAF – membrane insertion

Ho et al. (2016) BMC Biology. 14:109
Assignment and Secondary Structure Analysis

LITAF domain dynamics


Polyproline arm - interactions

Polyproline arm - interactions

Model Building: Δ114-139
Model building: Δ114-139

Wild-type structure modeling
Membrane mimetics...

- Soluble protein; soluble head group

Phosphoglycerol (PG)

Phosphoethanolamine (PE)

Phosphoserine (PS)

Phosphocholine (PC)

Inositolhexakisphosphate (IP6)

Membrane interactions

Membrane interactions

Phosphoglycerol (PG)


Membrane interactions

Phosphoserine (PS)

Membrane interactions

Inositolhexakisphosphate (IP6)


Membrane interactions

LITAF perturbation with PE

LITAF WT model

Patient Mutations
Patient mutation V126/144M

Patient mutation V126/144M

Ho et al. (2016) BMC Biology. 14, p109

Patient mutation V126/144M

Ho et al. (2016) BMC Biology. 14, p109
Patient mutation V126/144M

References


