Chemical Exchange and Ligand Binding

- NMR time scale
- Slow exchange for tight binding
- Fast exchange for binding constants
- Single vs. multiple binding modes
- Diffusion NMR and its application for ligand binding
Effects of Chemical Exchange on NMR Spectra

• Chemical exchange refers to any process in which a nucleus exchanges between two or more environments in which its NMR parameters (e.g. chemical shift, scalar coupling, or relaxation) differ.

• DNMR deals with the effects in a broad sense of chemical exchange processes on NMR spectra; and conversely with the information about the changes in the environment of magnetic nuclei that can be derived from observation of NMR spectra.
Types of Chemical Exchange

**Intramolecular exchange**
- Motions of sidechains in proteins
- Helix-coil transitions of nucleic acids
- Unfolding of proteins
- Conformational equilibria

**Intermolecular exchange**
- Binding of small molecules to macromolecules
- Protonation/deprotonation equilibria
- Isotope exchange processes
- Enzyme catalyzed reactions

Because NMR detects the molecular motion itself, rather the numbers of molecules in different states, NMR is able to detect chemical exchange even when the system is in equilibrium.
2-state First Order Exchange

\[
A \xleftrightarrow[k_{-1}, k_1]{\text{Lif}} B
\]

Lifetime of state A:
\[
\tau_A = \frac{1}{k_{+1}}
\]

Lifetime of state B:
\[
\tau_B = \frac{1}{k_{-1}}
\]

Use a single lifetime
\[
\frac{1}{\tau} = \frac{1}{\tau_A} + \frac{1}{\tau_B} = k_{+1} + k_{-1}
\]
Rationale for Chemical Exchange

For slow exchange

\[ \frac{dT}{dt} = -\left(\Delta \omega_A\right)M_{AY} - \frac{M_{AX}}{\tau_A} + \frac{M_{BX}}{\tau_B} \]

FT

For fast exchange

\[ \frac{dT}{dt} = -\left(\Delta \omega_B\right)M_{BY} - \frac{M_{BX}}{\tau_B} + \frac{M_{AX}}{\tau_A} \]

FT

Bloch equation approach:

\[
\begin{align*}
\frac{dM_{AX}}{dt} &= -(\Delta \omega_A)M_{AY} - \frac{M_{AX}}{\tau_A} + \frac{M_{BX}}{\tau_B} \\
\frac{dM_{BX}}{dt} &= -(\Delta \omega_B)M_{BY} - \frac{M_{BX}}{\tau_B} + \frac{M_{AX}}{\tau_A} \\
\cdots \\
\cdots 
\end{align*}
\]
2-state 2nd Order Exchange

\[ \text{M+L} \xrightarrow{k_{-1}} \text{ML} \xleftarrow{k_{+1}} \text{M+L} \]

\[ K_d = \frac{[M][L]}{[ML]} = \frac{k_{-1}}{k_{+1}} \]

\[ K_d = 10^{-3} - 10^{-9} \text{ M} \]

\[ k_{on} = k_{+1} \sim 10^8 \text{ M}^{-1} \text{ s}^{-1} \text{ (diffusion-limited)} \]

\[ k_{-1} \sim 10^{-1} - 10^{-5} \text{ s}^{-1} \]

Lifetime \( 1/\tau = 1/\tau_{ML} + 1/\tau_1 \)

\[ = k_{-1} (1+f_{ML}/f_L) \]

\( f_{ML} \) and \( f_L \) are the mole fractions of bound and free ligand, respectively.
Typical Motion Time Scale for Physical Processes

- **SLOW**
  - Very slow: s
  - Slow: ms
  - Fast: μs
  - Very fast: ns
  - Ultrafast: ps, fs

- **MACROSCOPIC DIFFUSION, FLOW**
- **CHEMICAL EXCHANGE**
- **MOLECULAR ROTATIONS**
- **MOLECULAR VIBRATIONS**

- **SLOW**
  - 3: Relaxation timescale
  - 2: Spectral timescale
  - 1: Larmor timescale

- **FAST**
# NMR Time Scale

<table>
<thead>
<tr>
<th>Time Scale</th>
<th>Chem. Shift, $\delta$</th>
<th>Coupling Const., $J$</th>
<th>T2 relaxation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow</td>
<td>$k &lt;&lt; \delta_A - \delta_B$</td>
<td>$k &lt;&lt; J_A - J_B$</td>
<td>$k &lt;&lt; 1/ T_{2,A} - 1/ T_{2,B}$</td>
</tr>
<tr>
<td>Intermediate</td>
<td>$k = \delta_A - \delta_B$</td>
<td>$k = J_A - J_B$</td>
<td>$k = 1/ T_{2,A} - 1/ T_{2,B}$</td>
</tr>
<tr>
<td>Fast</td>
<td>$k &gt;&gt; \delta_A - \delta_B$</td>
<td>$k &gt;&gt; J_A - J_B$</td>
<td>$k &gt;&gt; 1/ T_{2,A} - 1/ T_{2,B}$</td>
</tr>
<tr>
<td>Sec$^{-1}$</td>
<td>0 – 1000</td>
<td>0 – 12</td>
<td>1 - 20</td>
</tr>
</tbody>
</table>

- NMR time-scale refers to the chemical shift timescale.
- The range of the rate can be studied 0.05-5000 s$^{-1}$ for H can be extended to faster rate using $^{19}$F, $^{13}$C and etc.
Slow Exchange $k \ll \delta_A - \delta_B$

- Separate lines are observed for each state.
- The exchange rate can be readily measured from the line widths of the resonances.
- Like the apparent spin-spin relaxation rates, $1/T_{2i, \text{obs}}$
  $1/T_{2A, \text{obs}} = 1/T_{2A} + 1/\tau_A = 1/T_{2A} + 1/k_1$
  $1/T_{2B, \text{obs}} = 1/T_{2B} + 1/\tau_B = 1/T_{2B} + 1/k_{-1}$

  Line width $L_w = 1/\pi T_2 = 1/\pi T_2 + k_1/\pi$

Each resonance is broadened by $\Delta L_w = k/\pi$

Increasing temperature increases $k$, line width increases.
Slow Exchange for \( \text{M+L} \xrightleftharpoons[k_1]{k_-1} \text{ML} \)

- Separate resonances potentially are observable for both the free and bound states \( \text{M}_F, \text{M}_B, \text{L}_F, \) and \( \text{L}_B \)

- The addition of a ligand to a solution of a protein can be used to determine the stoichiometry of the complex.

- Once a stoichiometric mole ratio is achieved, peaks from free ligand appear with increasing intensity as the excess of free ligand increases.

- Obtain spectra over a range of \([\text{L}]/[\text{M}]\) ratios from 1 to 10
Slow Exchange for $\text{M}+\text{L} \rightleftharpoons \text{ML}

- For free form
  
  $\frac{1}{T_{2\text{L},\text{obs}}} = \frac{1}{T_{2\text{L}}} + \frac{1}{\tau_\text{L}} = \frac{1}{T_{2\text{L}}} + \frac{k_{-1} f_{\text{ML}}}{f_\text{L}}$

  $\frac{1}{T_{2\text{M},\text{obs}}} = \frac{1}{T_{2\text{M}}} + \frac{1}{\tau_\text{M}} = \frac{1}{T_{2\text{M}}} + \frac{k_{-1} f_{\text{ML}}}{f_\text{M}}$

For complex form

$\frac{1}{T_{2\text{ML},\text{obs}}} = \frac{1}{T_{2\text{ML}}} + \frac{1}{\tau_{\text{ML}}} = \frac{1}{T_{2\text{ML}}} + k_{-1}$

Measurements of line width during a titration can be used to derive $k_{-1}$ (k_{off}).
$^{19}$F spectra of the enzyme-inhibitor complex at various mole ratio of carbonic anhydrase:inhibitor

- At -6 ppm the broadened peak for the bound ligand is in slow exchange with the peak from free ligand at 0 ppm.

- The stoichiometry of the complex is 2:1. No signal from the free ligand is visible until more than 2 moles of inhibitor are present.
Coalescence Rate

• For $A \Leftrightarrow B$ equal concentrations, there will be a rate of interchange where the separate lines for two species are no longer discernible.

• The coalescence rate

$$k_c = \frac{\pi \Delta \delta}{\sqrt{2}} = 2.22 \, \Delta \delta$$

$\Delta \delta$ is the chemical shift difference between the two signals in the unit of Hz. $\Delta \delta$ depends on the magnetic field.
Coalescence Temperature

- Since the rate depends on the $\Delta G$ of the inversion, and the $\Delta G$ is affected by $T$, higher temperature will make things go faster.
- $T_c$ is the temperature at which fast and slow exchange meet.
- $T>T_c$, fast exchange
- $T<T_c$, slow exchange

\[ \Delta G^\ddagger = R \times T_c \times [22.96 + \ln(T_c/\Delta\delta)] \]
Fast Exchange $k >> \delta_A - \delta_B$

A single resonance is observed, whose chemical shift is the weight average of the chemical shifts of the two individual states

$$\delta_{\text{obs}} = f_A \delta_A + f_B \delta_B, \quad f_A + f_B = 1$$

For very fast limit

$$\frac{1}{T_{2,\text{obs}}} = \frac{f_A}{T_{2A}} + \frac{f_B}{T_{2B}}$$

For moderately fast

$$\frac{1}{T_{2,\text{obs}}} = \frac{f_A}{T_{2A}} + \frac{f_B}{T_{2B}} + f_A f_B^2 \frac{4\pi (\Delta \delta_{AB})^2}{k_{-1}}$$

Maximal line broadening is observed when

$$f_A = f_B = 0.5$$
Fast Exchange $k \gg \delta_A - \delta_B$

For $M$, $\delta_{M,\text{obs}} = f_M \delta_M + f_{ML} \delta_{ML}$

For $L$, $\delta_{L,\text{obs}} = f_L \delta_L + f_{ML} \delta_{ML}$

$1/T_{2,\text{obs}} = f_{ML}/T_{2,\text{ML}} + f_L/T_{2,L} + f_{ML} f_L^2 4\pi (\delta_{ML} - \delta_L)^2 / k_1$

- A maximum in the line broadening of ligand or protein resonances occurs during the titration at a mole ratio of approx. ligand:protein 1:3

- The dissociation constant for the complex can be obtained by measuring the chemical shift of the ligand resonance at a series of $[L]$. 
Measuring Binding Constant \[ \text{P+L} \xrightleftharpoons[k+1]{k-1} \text{PL} \]

\[
\delta_{obs} = X_L \delta_L + X_{PL} \delta_{PL}
\]

\[
\delta_{obs} = X_L \delta_L + (1 - X_L) \delta_{PL}
\]

\[
\delta_L - \delta_{obs} = ([PL]/[L_0])(\delta_L - \delta_{PL})
\]

For the formation of a 1:1 complex,

- \([P] + [PL] = [P]0\)
- \([L] + [PL] = [L]0\)

\[
K_D = \frac{[PL]}{[P][L]} = \frac{[PL]}{([P_0] - [PL])([L_0] - [PL])} \approx \frac{[PL]}{([P_0] - [PL])([L_0])}
\]

*If the ligand concentration is in large excess, \([L] \approx [L_0]\)*

\[
\frac{(\delta_L - \delta_{obs})}{[L_0]} = -\frac{(\delta_L - \delta_{obs})}{K_D} + \frac{(\delta_L - \delta_{PL})}{K_D}
\]
Plot of NMR data

Scatchard Plot

\[
\frac{[L_0]}{\Delta \delta} = \frac{K_D}{\Delta \delta_{\text{max}}} + \frac{[L_0]}{\Delta \delta_{\text{max}}}
\]

Here

\[
\Delta \delta = \delta_I - \delta_{\text{obs}}
\]

\[
\Delta \delta_{\text{max}} = \delta_L - \delta_{PL}
\]

- \frac{1}{K_D}

Plot \( \frac{\delta_I - \delta_{\text{obs}}}{[L_0]} \) against \( \delta_I - \delta_{\text{obs}} \)

Benesi-Hildebrand Plot

Plot of \( \frac{1}{\Delta \delta} \) against \( 1/[L_0] \)
Calcium Titration of Designed Ca2+ binding Protein

Yang et al. (2005) JACS

\[ \Delta S = \frac{\Delta S_1 \times [\text{Ca}]}{K_{d1} + [\text{Ca}]} - \frac{\Delta S_2 \times [\text{Ca}]}{K_{d2} + [\text{Ca}]} \]
Manganese Relaxation of CD2-6D15

Yang et al. (2004) submitted to Cell
NMR Distinction of Single- and Multiple-Mode Binding of Small-Molecule Protein Ligands

1H-13C HSQC spectrum of a 25 μM sample of Bcl-xL that is 13C-labeled only at the methyl groups of Ile, Leu, and Val. 3,4 Black: no ligand, red 25 μM, and blue 250 μM ligand. The ligand is a first-generation compound from the primary screen; KD = 80 μM.

Reibarkh, M... Wagner, G. JACS. 2006
Simulated NMR line shapes in the intermediate exchange regime

Two bound conformations
Kd=80 μM

Single binding mode,
Kd = 20 μM

The line width in the free protein is 20 Hz, Δν is 100 Hz, the effective line broadening, Ra, caused by conformational exchange is 80 Hz. The resonance of the free protein is set to 0 ppm at 500 MHz.
Cross-sections of HSQC spectra of 13C ILV-labeled Bcl-xL

V91

The use of 1H-13C HSQC spectra of methyl-labeled proteins is beneficial compared to 1H-15N labeling: 3-fold higher signal intensity; can be performed in D2O; and methyl resonances appear to be less susceptible to line broadening compared to backbone amides. The effect is probably most easily detectable as a gradual loss of peak intensities.
Diffusion by Pulse Field Gradient

Pulse Sequence

\[ I = I_0 \exp[-(\gamma \delta G)^2(\Delta + 2\tau - 2\delta/3)D] \]

\( \gamma \): gyromagnetic ratio

\( D \): diffusion constant

\( \Delta \): interval between gradient pulse

\( \delta \): PFG duration time

\( G \): gradient strength

\( D = kT/f \)

\( f = 6\pi \eta r \)

\( k \): Boltzman constant

\( f \): friction coefficient

\( \eta \): solvent viscosity

\( r \): hydrodynamic radius
N15 Edited

Calibration of Gradient Strength

500 MHz

\[ y = \exp(-m1 \cdot M0^2) \]

<table>
<thead>
<tr>
<th>Value</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>m1</td>
<td>7.9083e-08</td>
</tr>
<tr>
<td>Chisq</td>
<td>7.6519e-05</td>
</tr>
<tr>
<td>R</td>
<td>0.99999</td>
</tr>
</tbody>
</table>

600 MHz

\[ y = \exp(-m1 \cdot M0^2) \]

<table>
<thead>
<tr>
<th>Value</th>
<th>Error</th>
</tr>
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<tbody>
<tr>
<td>m1</td>
<td>1.465e-07</td>
</tr>
<tr>
<td>Chisq</td>
<td>0.00037522</td>
</tr>
<tr>
<td>R</td>
<td>0.99989</td>
</tr>
</tbody>
</table>

Gradient strength:

\( Gzlv1 = 32768 = 53.2 \text{ G/cm} \)

\( = 64.6 \text{ G/cm} \)
Diffusion Constant is Related to Molecular Weight

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Molecular weight (kDa)</th>
<th>Diffusion constant ($\times 10^7$ cm$^2$/s)</th>
<th>Hydrodynamic radius (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dioxane</td>
<td>88</td>
<td>98.9 ± 0.9</td>
<td>2.12$^a$</td>
</tr>
<tr>
<td>Tris</td>
<td>121</td>
<td>67.6 ± 1.3</td>
<td>3.10 ± 0.06</td>
</tr>
<tr>
<td>Glycine</td>
<td>75</td>
<td>93.3$^b$</td>
<td>2.21 ± 0.02</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>14.1</td>
<td>10.6 ± 0.2</td>
<td>40.2 ± 0.8</td>
</tr>
<tr>
<td>Trypsinogen</td>
<td>24</td>
<td>9.5 ± 0.2</td>
<td>45.4 ± 1.0</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>28</td>
<td>9.1 ± 0.4</td>
<td>47.4 ± 2.1</td>
</tr>
<tr>
<td>CD2</td>
<td>11.3</td>
<td>11.0 ± 0.2</td>
<td>39.2 ± 0.7</td>
</tr>
<tr>
<td>CaM-CD2</td>
<td>12.3</td>
<td>10.9 ± 0.2</td>
<td>39.4 ± 0.7</td>
</tr>
<tr>
<td>CaM-CD2-III-3G</td>
<td>12.4</td>
<td>11.1 ± 0.3</td>
<td>38.8 ± 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.4 ± 0.2$^c$</td>
<td>37.8 ± 0.7$^c$</td>
</tr>
<tr>
<td>CaM-CD2-III-5G</td>
<td>12.5</td>
<td>11.1 ± 0.2</td>
<td>38.9 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.4 ± 0.2$^c$</td>
<td>37.9 ± 0.6$^c$</td>
</tr>
</tbody>
</table>

Lee et al. *Biochimica et Biophysica Acta* (2002) 1598,80–87
Diffusion Dependent on Conformation

ApoCaM
\( r = 22.4 \pm 0.3 \text{ Å} \)
NMR: 22 Å

Ca\(^{2+}\)-CaM
\( r = 22.8 \pm 0.5 \text{ Å} \)
X-ray: 23 Å
Binding Constant Determination

\[ D_{\text{obs}} = X_L D_L + X_{PL} D_{PL} \]

\[ X_{PL} = (D_L - D_{\text{obs}})/(D_L - D_{PL}) \]

\[ K_a = X_{PL}/((1 - X_{PL})([P]_0 - X_{PL}[L]_0)) \]

- 0.5 mM cyclohexylacetic acid in D\textsubscript{2}O; 
  \( D = 6.85 \times 10^6 \text{ cm}^2 \text{ s}^{-1} \)

- 0.22 mM cyclohexylacetic acid plus 0.5 mM \( \beta \)-cyclodextrin in D\textsubscript{2}O; 
  \( D = 5.39 \times 10^6 \text{ cm}^2 \text{ s}^{-1} \)

\[ K_a = 1800 \pm 100 \text{ M}^{-1} \]

DOSY (Diffusion Ordered Spectroscopy)

Limitations

- The transverse relaxation time constant $T_2$ limits the interval where diffusion can be observed. Small diffusion coefficients ($D < 10^{-10} \text{ m}^2 \text{ s}^{-1}$), associated with macromolecules with masses larger than 50 kDa are difficult to measure.
- For weak binding, the change in diffusion constant is too small, and it is difficult to get the binding constant.

Singlet State Diffusion Spectroscopy

- stores the nuclear spin order as a singlet state. This state relaxes with a time constant $T_s$ that can be much longer than both $T_1$ and $T_2$.

Further reading: