Study of dynamic processes by NMR

• So far we have talked about techniques and experiments used to study ‘frozen’ molecules by NMR. We have made no mention whatsoever about the time frame of the NMR measurement.

• What if we have something in the tube that is suffering some sort of dynamic process? This could be a chemical reaction, conformational equilibrium, exchange between the bound and free states of a ligand/protein complex, etc., etc:

![Diagram of conformational and chemical equilibria]

• We need to analyze a bit how the rate of the process is when compared to the speed of what we are using to measure it. Believe it or not, it is all a matter of the uncertainty principle.

• We will try to explain this by presenting a simple process. Things valid for it will be more or less valid for all other dynamic processes studied by NMR, including ligand binding of drugs to proteins.
Measurement of rate constants

• Say that the process we are looking at is the inversion of NN-dimethylformamide:

\[
\begin{align*}
\text{N} & \text{H} \\
\text{O} & \text{N} \\
\text{C} & \text{H} \\
\end{align*}
\]

• We know that we have an exchange of the red and blue methyls due to the double bond character of the amide bond. Both methyls are chemically and magnetically different, so an NMR spectrum of DMF shows two different methyl signals:

This means that the exchange rate between the two sites is long enough if we compare it with the relative frequency difference between the resonances of the two species (red and blue):

\[
\text{Rate (s) } \gg \frac{1}{\delta_r - \delta_b} \quad \text{or} \quad \frac{1}{\Delta \delta}
\]
Measurement of rate constants (continued)

• Lets now start increasing the 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. What we see in the NMR looks like this:

At a certain temperature, called the **coalescence** temperature, the rate of the exchange between the two species becomes comparable to the difference in chemical shifts of the sites:

\[ \text{Rate (s)} \leq \frac{1}{\delta_r - \delta_b} \text{ or } \frac{1}{\Delta \delta} \]

• Past this point, the NMR measurement cannot distinguish between things in either site, because things are exchanging faster than the difference in relative frequencies.
Measurement of rate constants (...)

- We see that there are two regions as we increase go higher in temperature, called slow exchange and fast exchange:

  \[
  \begin{align*}
  \Delta \delta \times \text{Rate} > 1 & \quad \text{Slow exchange} \\
  \Delta \delta \times \text{Rate} = 1 & \quad \text{Transition (T_c)} \\
  \Delta \delta \times \text{Rate} < 1 & \quad \text{Fast exchange}
  \end{align*}
  \]

- Now, since we can estimate the temperature at which we have the transition taking place, we can get thermodynamic and kinetic data for the exchange process taking place.

- If we did a very detail study, we see that we have to take into account the populations of both sites (one site may be slightly favored over the other energetically), as well as the peak shape.

- As we've been doing with other mathematical derivations, we will use approximate results, which for the time being serve out purposes.

- We will focus on the case of equally populated sites (equal energies), which means that the energy difference will be only from the exchange process being studied.
Measurement of rate constants (...)

• From the $\Delta \delta$ value (in Hz) at the limit of *slow* exchange we estimate the rate constant at the coalescence temperature:

$$K_{ex} = \pi \times \Delta \nu / \sqrt{2} = 2.22 \times \Delta \nu$$

• Here we are using frequencies in radians, and that’s why we need the $\pi$ factor. This equation has many simplifications (we will never now if the lowest temperature is truly *slow* exchange, and we don’t consider linewidths).

• However it works pretty OK. Since we have the coalescence temperature, we can calculate the $\Delta G^\ddagger$ of the process using a similar fudged relationship:

$$\Delta G^\ddagger = R \times T_C \times [ 22.96 + \ln ( T_C / \Delta \nu ) ]$$

• If we do not take into account any entropic contributions to the $\Delta G^\ddagger$, we in principle calculate the rate of the reaction at any other temperature from this data alone.

• With NMR we can measure rates from $10^{-2}$ to $10^8$ s$^{-1}$. 
An example of conformational equilibrium

• As part of my taxol stuff I tried to make a constrained side chain analog, to evaluate if imposing rigidity on the molecule improved or deteriorated activity.

• I decided to make a biphenyl system, which proved to be a really bad choice, because if I had read, I would have known that these things behave funny.

• I made it (it took me a loooooooong time), and when I finally took the $^1$H, I saw that the thing I made had two possible conformations due to the restricted rotation of the biphenyl:

![Chemical structure](image)

• I could see all the signals doubled, (but I had one thing by TLC), so there was some funky business going on. We came to the conclusion that we had a slow equilibrium (slower than NMR) occurring for this sample in DMSO…
• Since I had worked like a mule for 4 months, I refused to leave it at that. Also, we were concerned about having two things. If this was an equilibrium, temperature should affect the rate, so we did a temperature study:

• We found that \textbf{a}) there was coalescence of the two sets of signals at \(\sim 80^\circ\text{C}\), and \textbf{b}) that the process was reversible.
An example (…)

• In this case, the ring inversion is not alone, and we have other conformational changes upon inversion. There may also be H-bond making and breaking, so it’s hard to pick a pair of protons to calculate the barrier for rotation.

• I never did it in Texas, so I’m doing it here. If we pick a pair of aromatic protons (after all, the aromatic rings are flipping), we get a δν of 0.04 ppm, or 20 Hz (at 500 MHz):

![Chemical Spectrum]

• If we apply the approximate formula for the ΔG⁺ (considering that coalescence occurs at 85°C (358 K), we get:

\[
K_{ex} = 44.4 \text{ s}^{-1} \quad \Delta G^\ddagger \sim 18.5 \text{ Kcal/mol}
\]

• Not that off the mark…
Ligand conformation and TRNOE

• One of the most important things when designing a new drug is to find out how it will bind to its receptor, usually a protein.

• If we have this information we can design new drugs that not only have the chemical requirements for activity that we may know from SAR studies, but which also meet conformational requirements of the binding site.

• One way is to find the structure of the isolated molecule by either X-ray or NMR, and then assume that this is the same conformation we’ll see when bound.

• In flexible ligands (99.9% of the interesting stuff…), the change environment (polarity, presence of apolar groups, etc) when going from water to the binding site will most likely change its conformation.

• So, in order to find out the conformation of the ligand when bound, we somehow have to study it either bound to the receptor, or with the receptor present there in some form…
Ligand conformation (continued)

• Depending on the size of the receptor, we can in principle resolve the 3D structure of it plus the ligand.

• There are two problems. First, this is time consuming. After all, we just need the ligand, but if we do it this way we will have to assign the whole protein and compute the structure.

• Second, most receptors are huge, not 10 or 20 KDa, but 100 to 200 KDa, meaning we cannot see anything by NMR. Not only we will have a lot of overlap (even in 3D spectra), but the correlation times are so large that broadening will kill us.

• What in some cases bail us out in this situations are the relative rates of the rise of NOE (cross relaxation) and the binding of the ligand to the receptor.

• Say that we have the following ligand/receptor complex:

![Diagram of ligand/receptor complex](image)

• When bound, the protons in the marked carbons will have an NOE interaction. It will be very hard to see it with the protein also having tons of other NOE correlations.
Ligand conformation (…)

• Now, say that the ligand dissociates from the complex and goes back to solution. It will adopt its solution conformation in a jiffy:

• Usually, $k_{\text{off}}$ (or dissociation constant) is slower than $k_{\text{unf}}$ (the ‘rate’ of unfolding), so we only worry about $k_{\text{off}}$. We define all the constants as follows:

$$K = \frac{k_{\text{on}}}{k_{\text{off}}} = \frac{[\text{protein-ligand}]}{[\text{protein}] [\text{ligand}]}$$

• Irrespective of the $k_{\text{off}}$ rate, the NOE interaction between the two protons that appeared in when the ligand was bound will stay for a time that depends on the spin-lattice relaxation rate.
Ligand conformation (…)

• This means that if the binding/dissociation process is fast compared to the $T_1$ relaxation, the enhancement on the intensity of the two protons that appeared when bound will remain after the ligand is unbound and unfolded. Why?

• We have to consider the whole process:

- Dipolar coupling between $I$ and $S$ occurs for bound ($I_B$, $S_B$) and free ($I_F$, $S_F$) ligand. However, since when the ligand is bound its correlation time is huge (we have the protein), the $\sigma_{IS}^B$ cross-relaxation rate for $I - S$ will dominate.

• This is why we see the NOE enhancements that appeared in the bound state. Furthermore, since when bound the ligand has an apparent $\omega \cdot t_c >> 1$, we will always most certainly be in the spin diffusion limit, meaning negative enhancements and cross-peaks (negative NOE’s).
Ligand conformation (…)

- Additionally, if we have good turnover compared to the spin-lattice relaxation rate, we will have several ligand molecules binding to the same receptor before the NOE enhancement of the first one decayed:

- This means that we can do the experiment with an excess of ligand (10 fold or more), and the signals of the ligand will be in larger ratio than 1:1 with those of the receptor (which will be broad and overlapped).

- Another good thing of measuring the NOEs of bound ligands by TRNOE is that since we will be looking at them in the free molecule, the peaks will be sharp and well resolved:

  - Sharp signals are taller (they have the same area), they stand out from all the protein background, and they give far better resolved cross-peaks in a 2D experiment.
Ligand conformation (...)

• If it looks too good to be true, it is too good to be true. We need to meet several criteria to use \textit{TRNOE}:

  • The ligand cannot bind tightly to the receptor (we need constant exchange between bound and free ligand).

  • The $K_{\text{off}}$ rate has to be much smaller than the spin-lattice relaxation rate, otherwise the NOE dies before we can detect it.

Summary

• With NMR we can study dynamic processes that happen at rates slower than the NMR timescale. We can obtain rate constants and $\Delta G^\ddagger$ values for dynamic processes.

• TRNOE is a variation of the NOE experiment in which we can look at the NOE enhancements of molecules bound to a large receptor.

• Although there are several conditions that need to be met, we don’t have to worry about the size of the receptor.
Magnetic field gradients and diffusion

• So far all our discussions have dealt with ‘perfect’ magnetic fields (i.e., homogeneous $B_o$). We obviously want this for good resolution and sensitivity. However, creating a gradient of known characteristics on $B_o$ can be extremely useful.

• A gradient in the magnetic field results in different $B$s. If we just consider a linear variation along the $z$ axis (i.e., a $z$-gradient, $G_z$) and a sample of water, what we’ll see is that water molecules at different positions along $z$ will have different $\delta$s (because $\delta \propto \gamma (B_o + G_z)$):

• Notice that the signal is proportional to ‘sample mass’…
Magnetic field gradients (continued)

• So, how is this useful? For starters, we can get an image of the sample if we apply the gradient during acquisition. Since spins at different positions along the tube have different $\delta$s, we get a ‘continuous’ spectrum that parallels the shape of the container (MRI).

• In other words, with a linear gradient the spins end up spatially encoded. This means that we ‘know’ to what part of the tube (or “arm”) the spin belongs based on the gradients that we applied. Add contrast based on different relaxation times for different tissue, and you get MRI (sort’a).

• In addition, by combining gradients of different signs we can spatially encode the nuclei, allow them to ‘evolve’ (times, pulses, etc.), and then decode them.

• Spins that did (or did not) behave as expected during the evolution period will (will not) show in the spectrum…
Gradients and diffusion

• Which brings us to diffusion measurements. Measuring self-diffusion coefficients ($D_s$) is extremely important in chemistry and biology. It tells us about interactions between molecules, their motions, etc., etc.

• Pulse-field gradient NMR is ideal to measure the diffusion of particles bearing NMR-active nuclei. The most basic technique involves combining a spin-echo with two gradients of opposite sign and length $\delta$ separated by a delay $\Delta$:

![Diagram of pulse-field gradient NMR with spin-echo and gradients]

• We have to analyze how the spin-echo will look like under the effect of the encoding and decoding gradients for nuclei that diffuse at different rates. Keep in mind that the gradient will make things move faster/slower in the rotating frame (i.e., change their ‘chemical shift').
Gradients and diffusion (continued)

• For a spin that does not diffuse much (after the 90):

• Here the blue/red dots represent the same type of nuclei in different regions of the ‘tube’…

• Basically, since the spin is not moving the gradients have little effect. That is, the spin did not move from the area it was, so the decoding gradient had the same and opposite effect as the encoding gradient. In other words, the signal intensity for this nuclei did not change…

• Things will change quite a bit if we have a fast-moving spin…
In this case, since the nuclei moves away from where it was, the decoding gradient has a completely different effect (i.e., things will dephase further). Therefore, the signal intensity will be greatly attenuated.

The end results is that the the faster the nuclei diffuse, the smaller their signals will be in the spectrum. By selecting the values of \( G \), \( \delta \), and \( \Delta \) we can ‘fine-tune’ the experiment for species with self-diffusivities going down to \( 10^{-12} \text{ m}^2\text{s}^{-1} \) (this is for our Bruker).
Gradients and diffusion (continued)

- In order to calculate the value of $D$, we repeat the experiment for several values of $G$ while maintaining $\delta$ and $\Delta$ constant.

- The resulting intensity versus gradient plot follows the following relationship, and a fit gives $D$.

\[ I(G) = I_0 \times e^{-\gamma^2 G^2 \delta^2 D \times (\Delta - \delta / 3)} \]

- Some real data for [Emim][OAc] at 40 °C: