NMR spectroscopy for Structural Biology
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Lecture: 52
Probing Protein Dynamics by NMR Spectroscopy II

This week we are discussing protein dynamics as probed by NMR spectroscopy. So, in last lecture, I mentioned why the dynamics is important for understanding the biological phenomena, biological function, protein function. What are the techniques that can be used for understanding the protein dynamics and then we went ahead and looked at how NMR can be used for understanding the protein dynamics. So, it is all about time and motion and we will continue from there. So, NMR appears to be a versatile tool for the studying transitions of atomic structure at various time scales ranging from say picosecond to second time scale motion or in frequency term from terahertz to hertz, right.

So, there are some motions which can be called as internal dynamics or it is sometimes called molecular diffusion. There are local time scale motion or global time scale motion and those can be probed by various NMR experiments that we mentioned. So, relaxation in laboratory time frame, if we do something like a T1, T2 or NOE that can probe basically the fast time scale motion ranging from picosecond to submicrosecond time scale motion.

Then, we can do the relaxation in rotating frame like T1ρ, T2ρ or ROE and that actually captures the microsecond to millisecond time scale motion and then even slower motions can be probed by exchange NMR like one of them is hydrogen deuterium exchange. So, the faster time scale motions comes from average anisotropic interaction and slower time scale motion can be also inferred from line shape analysis. So, essentially all these can be probed by utilizing some of the NMR experiment. Now, for solid, since molecule tumble slowly, so in solid state generally you have a slower time scale motion. So, what actually relaxation means? So, relaxation is a process by which any spin returns to its equilibrium position, equilibrium population.

So, that means generally in a B_0 magnetic field you have a spins aligned to this. You create some perturbation and the time that takes to come to the equilibrium position is essentially relaxation. So, it is governed by the fluctuation that happens in local field and that local field fluctuation is experienced by these nuclear spins. So, generally what is happening,

our spins are oriented in a stronger magnetic field and some fluctuation happening in this magnetic field and that basically causes relaxation process. So, because of the fluctuation that is happening, these spins will be reoriented and that cause the variation in their interactions between two spins or their chemical shift anisotropy.

So, basically these are contributing towards the relaxation phenomena: chemical shift anisotropy and dipolar coupling, that we are going to look in little more detail. So, typically we also looked at these heteronuclei are well suited for understanding the relaxation mechanism, heteronuclei such as 13C, 15N. Proton has slightly complex relaxation behavior, but yes it can be used. In one case, you can spin dilute it, change with deuterium many of the proton can be changed with deuterons and then few protons can be actually probed in an elegant way to understand the relaxation mechanism.

But typically for all simplistic calculation in protein 13C or N15 or both are exploited to understand the relaxation mechanism in protein. So, relaxation mechanism actually influenced by two of the major interactions, one is called dipolar coupling, another is called chemical shift anisotropy. There are some other which influence the relaxation mechanism is called spin-spin coupling or J-coupling or it can be even quadrupolar coupling or the exchange happening between the spins. So, major population and minor population how they are exchanging. So, all these essentially contributes towards the relaxation mechanism.

Dipolar coupling are between two spins. Chemical shift anisotropy, how these spins are oriented in the magnetic field, what is the anisotropic interactions in them. J-coupling is a scalar coupling between two spins, and quadrupolar like one dipole and one quadrupole interacts, and chemical exchange as I said if it is exchanging between two states, those all contributes to the relaxation mechanism. So some basic theory of spin relaxation in protein. So one of the major contributor is this dipolar interaction.

Another one is chemical shift anisotropy. So suppose in the magnetic field, these are two spins, spin one and spin two, and they are separated by some distance called r_{12}. So, this is distance and there is some angle with the main magnetic field which is θ. So, there will be dipolar interaction that depends upon this distance and also on the orientational angle and that basically contributes towards the relaxation phenomena.

So, in liquid what happens? The tumbling most of the time averaged out this dipolar interaction. In solid, the tumbling does not happen, therefore dipolar interactions are there and that is how lines in solids are broader which we are going to look at in the next weeks. But to understand, even if averaging happen this is one of the cause for dipolar coupling. So, if dipolar coupling is present, you can see there is a line splitting and line becomes broader. Another important phenomena that contributes to the relaxation phenomena is called chemical shift anisotropy.

So, if spins are not tumbling and they are in the magnetic field and they are actually oriented in various direction. So each of this direction will have one resonance frequency, which is shown here. If you take envelope of all these resonance frequency because of this different orientation, you get a really broad line something like this. This is essentially chemical shift anisotropy. So, chemical shift anisotropy is essentially orientation dependence of the chemical shift.

So, when we start tumbling of these spins these anisotropic interactions essentially gets averaged out and you have an isotropic chemical shift. Generally, we see the sharp peaks in liquid state NMR spectrum. So, now in solid, basically you spin very fast to make these anisotropic interaction look like isotropic which again we are going to look at in the next slides, but this anisotropy is present. All over in liquid it is quite averaged out because of the Brownian motion that spins can take, but this is one of the major contributor to the relaxation phenomena. So, these two DD and CSA are dominant source of relaxation. So this is the essentially nuclear spin relaxation depends upon two phenomena.

These are the two dominant contributing phenomena, the dipolar relaxation and chemical shift anisotropy. So, suppose these two spins are in the main magnetic field which is B_0, they oriented along the magnetic field, spin 1 and spin 2, and there is a distance between them which is r_{12} and the angle of orientation is θ. So, the dipolar interaction depends upon this angle which is $(3\cos^2\theta - 1)$ and also the distance between them which is say r_{IS} or r_{12}. So, it is r^3 and this γ_1 and γ_2 are the gyromagnetic ratio of these two spins. This is permittivity (μ_0), π and Planck's constant ($\hbar$).

So, these are the phenomena that contributes to dipolar coupling. Now, one thing you notice this $(3\cos^2\theta - 1)$. So typically, this is the main contributor of the spins. So if the spins

are quite close, that means if this distance is short, dipolar interaction is large, if it is long then dipolar interaction is weaker.

So, when we do spin dilution that means we make spins talk to each other in a less dominant way and therefore, we reduce this dipolar interaction. So, we can spin dilute it by putting deuterons, proton-proton dipolar coupling can be reduced. In solid this is a trick that we are going use. In solid state we always try or tends to make this term 0 by setting or spinning the our sample at certain angle which is called magic angle. So, this $(3\cos^2\theta - 1)$ becomes 0 and that is how we try to reduce the dipolar interaction between those. Another dominant interaction that we talked is a chemical shift anisotropy. This is again an orientation dependent interaction.

So, in magnetic field spins can be oriented in various fashion and since they tumble most of the time this interactions is 0 or it tends towards 0 because of their tumbling or Brownian motion. But suppose, there is some orientation is still there, so they will cause the chemical shift anisotropic. That anisotropic interaction will arise because of these spins that are oriented in different dimension. So, like suppose they are isotropic, we see one peak and that is what we see in liquid state. But when there is a restriction in motion, it is not tumbling very fast, you will see some kind of anisotropic interaction emerges out and you see many lines are there and if you take an envelope of all those lines you see a really broad peaks. So, this is chemical shift anisotropy. Even in solution, quite a bit of these are not there averaged out, but these causes relaxation.

DD and CSA are major source of relaxation phenomena. Therefore, typically an isolated XH spin system is chosen for relaxation rate constant, where X spin like a 13C and N15 are chosen, and the dipolar interaction between that X spin and proton is considered and also the CSA originating from X spin is contributed. So, relaxation rates that can arise because of these anisotropic interactions CSA or DD can be expressed in something called spectral density functions. So, we are going to look at what is essentially the spectral density function.

So, essentially all the relaxation rate that we are talking can be expressed in this term spectral density function. So, I am going to explain you soon what is spectral density function, but before I go to a spectral density function, let me define something called correlation function. So, the correlation function can be given

$$G(t) = \frac{1}{5} exp\left(-\frac{t}{\tau_c}\right)$$

Now, this τ_c essentially is the correlation time.

So, correlation function for an isotropic diffusion of a rigid rotor, let us explain this spin as a rigid rotor, can be given in this term

$$G(t) = \frac{1}{5} exp\left(-\frac{t}{\tau_c}\right)$$

This correlation time which is τ_c is a time constraint for an exponential decay of the function. τ_c is approximate amount that molecule take to make rotation by one radian. So how much time it takes to make rotation by one radian that is a τ_c correlation time. So short correlation time essentially causes the correlation functions to decay rapidly whereas long correlation time makes function to decay slowly. The correlation time essentially depends upon the molecular weight of a molecule, what is the shape, what is the solvent viscosity, what is the temperature.

So let me simplify this. If a molecule is bigger, that means in solution it will tumble slowly. If the molecule is smaller, in solution it will tumble fast. If the solvent is viscous, that means the molecular tumbling will be slow. You raise the temperature the same molecule can now make rotation fast. So, it depends upon the shape and size of a molecule, molecular weight of a molecule, the solvent viscosity whether it is more viscous or less viscous solvent and what is the temperature.

So, suppose a molecule has the correlation time of 50 nanosecond, it's a correlation function will decay slowly and if it has a correlation time of 1 nanosecond, you can say it decays very fast. So, that is what here we are saying, it is time exponential decay of a function, it is approximately the amount of time molecule takes to make one radian. If the correlation function is decaying rapidly here, it takes long time.

So short correlation time, function decay rapidly. Long correlation time, function decay slowly. So, that is a correlation time. Another one we were talking the spectral density function. So, it is essentially the power. So, power is connected with the correlation time.

So, suppose here the correlation time is 100 nanosecond that means the molecule is slowly tumbling. So, you can see the spectral density function which is *J(ω)*. So, ωis a frequency, *J* is a spectral density function. With the frequency, it dies or dies very rapidly. If the correlation time is shorter, like 1 nanosecond, this is very, very slowly decaying.

So spectral density function decays very slowly. So *J(ω)* is given by this function, where τ_c is the correlation time, ω is the frequency. So it stores the power of a molecule, how rapidly or how quickly or how slowly it decays, how it dissipates power that is what the spectral density function is saying. So, for a longer correlation time, it can dissipates power very fast, for shorter correlation time it dissipates power very slowly with the frequency. That is the spectral density function tells about and we say that we can express our relaxation parameters in terms of spectral density function.

So, spectral density function *J(ω)* is a Fourier transform of correlation function just as rapidly accessing domain signal give rise to a broader line. If something is rapidly decaying, it gives the broader line. If something is slowly decaying, it gives the sharper line. So, give rise to broader line, short correlation time like have a broader spectral density.

So, this makes sense that molecule tumbles very rapidly can sample wide range of frequency and molecule that tumbles slowly have a very long correlation time and only samples fewer frequency. So, let me explain again. So, a small molecule, a small protein, a small peptide and all those tumbles very fast. A bigger molecule tumbles slowly. So, if the molecule is tumbling rapidly, that means it can sample a wide range of frequency.

Say, here in this correlation function the molecule, which has a shorter correlation time, can essentially samples the all frequency. So, a smaller molecule can basically samples the wide range of frequency or bigger molecule a protein of 2 kDa can sample many frequency, molecule of 100 kDa which has correlation time is about 100 nanosecond samples only few frequency. So, the molecule tumbles very rapidly can sample a wide range of frequency and molecule that tumbles slowly like a bigger protein have a very long correlation time and only can sample few frequency. That is essentially a spectral density function tells about itself. So now, if we know this, what is correlation function? Now we can express our relaxation parameters in terms of this spectral density function.

So R_1, which is longitudinal relaxation rate, can come because of D dipolar coupling, because of CSA, and you can explain this R_1 in terms of this formula, where the spectral density function of the H spin and X spin is given.

$$R_1 = \frac{d^2}{4}[6J(\omega_H + \omega_X) - J(\omega_H - \omega_X) + 3J(\omega_X) + c^2 J(\omega_X)]$$

So, $J(\omega_H + \omega_X)$. So, this is the joint frequency that H and X are evolving that is a spectral density function for proton and carbon 13. $J(\omega_H - \omega_X)$, this is the difference in the spectral density function or then individual spectral density function, $J(\omega_X)$, of X as well as H spin. So, that all contributes towards the relaxation of these spins in R_1 longitudinal relaxation rate. Similarly, R_2 can be given by these formulae,

$$R_2 = R_2^D + R_2^{CSA}$$

it again depends upon the spectral density function of proton and carbon given by these formulae

$$R_2 = \frac{d^2}{8}[6J(\omega_H + \omega_X) + 6J(\omega_H) + +J(\omega_H - \omega_X) + 3J(\omega_X) + 4J(0)] + \frac{c^2}{6}[3J(\omega_X) + 4J(0)]$$

So, that is what R_2 and then this is cross relaxation. So, these are individual relaxation and this is cross relaxation this again will be given by

$$\sigma_{XH} = \frac{d^2}{4}[6J(\omega_H + \omega_X) - J(\omega_H - \omega_X)]$$

So, R_1, R_2, and σ_{XH} are the rate constant for spin-lattice relaxation as well as spin-spipn relaxation and this one is cross relaxation. So, we can see that these simple relaxation parameter the individual R_1 and R_2, spin-spin and spin-lattice relaxation can be given by the spectral density function of the individual spins X and H, their joint frequency, their difference frequency and the zero frequency. So, in terms of these spectral density function we can explain our R_1 and R_2.

So, dependent of a spectral density function can be evaluated on these five frequencies. What are those five frequencies? The joint frequency $(\omega_H + \omega_X)$, (ω_H), $(\omega_H - \omega_X)$, (ω_X), and 0. So, these are 5 different frequency which can contribute towards this spectral density function. Some of the parameters that I had given in the previous slides like d essentially

these parameter which also depends upon the distance between the X and H and rest are the gyromagnetic ratio, Planck constant and permeability of the vacuum.

XH is essentially the bond length and γ_X, γ_H are gyromagnetic ratio and C is a constant. So, now $\Delta\sigma$ is the CSA of the X spin. So, you consider that the chemical shift tensor is axially symmetric. Now CSA for different nuclei which are typically given, for N15 this chemical shift anisotropy is about 170 ppm, reference is from here, and for carbonyl $\Delta\sigma$ is about 35 ppm, for Cα it is about 30 ppm. So, these are various constant that can be plugged in here to find out their contribution coming from different relaxation rate.

The R_1 and R_2 rate constant are determined experimentally, we are going to look at how we can determine on proteins. And the cross relaxation rate, σ_{XH} is determined from the steady state NOE, that also we are going to look at how we can design an experiment to do this heteronuclear NOE. As we mentioned, this cross relaxation rate depends upon d and the value of d is given here. This spectral density function can be given as,

$$\sigma_{XH} = \frac{\gamma_X}{\gamma_H} R_1 (NOE - 1)$$

So,

$$NOE = 1 + \frac{\sigma_{XH}}{R_1} \frac{\gamma_X}{\gamma_H}$$

So, you can find it out the NOE that we calculate experimentally can basically come from these spectral density function. So, NOE is this cross correlation rate. So, if we do the three basic experiment R_1, R_2 and heteronuclear NOE, we can essentially determine the all spectral density function and τ_c and that is what typically is done in the protein NMR. So, spectral density function at the five frequency cannot be determined from 3 exponentially determined relaxation rate constant by just measuring T_1, T_2 and NOE. So, assumption must be made so that only 3 unknown need to be determined from this 3 values.

So, we are want to determine the 5 frequency, $(\omega_H + \omega_X)$, (ω_H), $(\omega_H - \omega_X)$, (ω_X), and 0. from 3 rates which is not possible. So, we need to make some assumptions. So, that three equation and three unknowns are there. So, there are various mathematical models that maps the spectral density function and one of them is model free analysis widely known as

Lipari-Szabo model free analysis and that basically gives the site specific internal motion of protein.

Towards the end of this week, we were going to briefly touch upon what is the model free analysis, but essentially it can be determined from this relaxation rate by making some assumptions and we will be looking at that. So, today I am going to give you a glimpse and we can continue over it what experiments are done for measurement of relaxation parameter. So, typically whatever we have learned, HSQC or HMQC based experiment can be utilized for understanding the relaxation rate like T_1 and T_2 or even NOE. Concept is that we plugged in these parameters where we can we can determine the T_1 or T_2 or NOE from these HSQC or HMQC based experiment, doing the heteronuclear correlation experiment for determining the heteronuclear relaxation. So, essentially we start with a preparation of desired coherence and then we invoke this T1 delay for like autocorrelation or cross correlation and then we encode the frequency.

So, here is T_1 period for encoding the frequency and then we transfer the magnetization to proton nuclei and then you acquire on proton and then we give some delay like a D1 delay between the scans. So, typically this is the pulse sequence design. We are going to start a preparation phase with invoking the delay, so that we can encode the T_1 relaxation time or T_2 relaxation time. Then we encode the frequency indirect dimension, then we transfer back magnetization to proton, acquire proton, and encode the T_2 frequency, so that we can record delay time-dependent HSQC and finally, towards end of pulse sequence we give the relaxation delay, so that magnetization returns to the equilibrium states.

So, these are typical design of an HSQC, HMQC based T_1 and T_2 relaxation. Next class I am going to discuss how we can basically design a pulse sequence to measure the T_1 and T_2 and how data comes and how we can interpret this data for understanding the relaxation mechanism in protein. So, with this let me close it today and looking forward to have you in the next class. Thank you very much.