**NMR spectroscopy for Structural Biology**
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**Lecture: 51**
**Probing Protein Dynamics by NMR Spectroscopy I**

This week we are going to discuss about protein dynamics as proved by NMR spectroscopy. So, you know the dynamics is very important thing, because everything that happens in this world is all about time and motion. So, we will be looking how NMR contribute towards understanding the dynamics in a profound manner. So, life actually is marked by change over time. So, if you look at any event that is happening. Two of the figure I am showing you, like a nerve cell firing.

What is happening? Something changes over time or even any development happens that we can see in the *C. elegans*, which is a small organism. You can probe that by putting some dye on some protein or gene and you see how it is changing. The question is that how it is changing with time and why it is changing.

If we understand this how and why that is happening in life, we understand how life evolves, how life moves, how life develops, and that's the importance of the dynamics which is encoded in time and motion. So to understand these changes that happens in life or in organism or at cellular level or at molecular level, we need to probe the dynamics happening over time and the main factors that probe dynamics or in which changes happen are the protein. So, in the last lectures we have seen that how we can get the structure of a protein.

But you remember protein are not static entity. They are quite dynamic. They have a dynamic personality. So, they have various sorts of motion that we are going to discuss this week. They have various sorts of motion and those motions are needed for their function.

Now they move to do catalysis, they move to communicate with each other or signal transduction or attacking to other organism in pathogenesis. So everything is dictated by the protein dynamics and proteins are the main actor of all the cellular process that happens in the life. So you can consider proteins as a soft matter and that has a multi-dimensional

energy landscape where it is shown. So here what I draw is an entropy axis and here is the energy axis. So you see the entropy of the protein changes with the energy.

This is typically called folding funnel or energy landscape. So at the top of the funnel, you see the energy is high and entropy is high. That means protein is quite dynamic. It is sampling various states and as we come down, it gets constricted, its dynamics reduces and energy also reduces that means it get stabilized and this is typically called native structure of a protein. The structure that we saw how we can solve using NMR. But other than that we can sample the other states which are populated at different location in the energy landscape view or the folding funnel.

These are of higher energy states with more entropy. So, if we can probe what is the relative probability of these states which are not at the ground state, slightly excited state, then we can understand the thermodynamics happening between the two states. What is the probability that it exists at the bottom? What is the probability that it is slightly up? That is given by the thermodynamics and what are the energy barriers like if you draw energy curve this is one state, this is another state. So, what is this energy barrier between these two states? How it crosses from one state to another state is dictated by the kinetics.

So, by understanding the protein dynamics how it samples various states in the folding funnel or energy landscape one can understand the thermodynamics that is happening in the protein as well as the kinetics parameter. So, although you can see any cell is decorated by various proteins. They are typically shown the bigger protein, which is called monoclonal antibody. These are smaller proteins. So all these proteins at all the time samples various state that means they are dynamic. Now the question is how we can understand this.

So, three dimension is a structure right X-Y-Z dimension, the fourth dimension is the time dimension and this fourth dimension is extremely important to decipher the protein function. As I am saying, protein is not a static entity, it is dynamic, it samples various timescale and that is what actually gives the total function of protein. So, what is the importance of the dynamics? I will show you here in a cartoonistic representation. If you take this bead, like a necklace made up of pearl, this is a beautiful necklace, right? Here, each of these beads are amino acid. When you join and fold in, decorate in a certain manner

that is a three-dimensional structure of a protein or three-dimensional structure of a necklace.

But necklace in itself is not beautiful. It becomes more beautiful when it decorates somebody's neck, right? So that's what actually protein dynamics does. It decipher a function to a protein. It decipher a functional state of a protein. The fourth dimension in protein structure is very much needed and that fourth dimension is a time dimension, which tells about the functional aspects of a protein, the different kind of motion that is present in the protein.

So together this structure and dynamics along with the folding determines the function of a protein. This is called a revived structure function paradigm. So a structure not only tells about the function, structure is not sufficient to tell about the function, you require the dynamics information and also how the protein folds, what kind of folds it has. So, folding and dynamics together with the structure tells about the function of a protein. So that's the energy landscape that we are talking about.

This is three-dimensional energy landscape where in one dimension you are talking about the entropy or conformational excursion. Here is a native state, energy stabilized states of a protein. And there are some intermediate states where some of the parts are open that is a high energy state and that is what basically NMR can tell you that how the conformation from this state to this state, this state to this state may change during the during the folding or during the dynamics. So, how protein is doing conformational excursion. So to give you more emphasis on why it is important, you all know the structure of a hemoglobin, right? Hemoglobin that binds to oxygen.

Now you also know from your biochemistry knowledge that it has a cooperative binding to oxygen, right? Here is a hemoglobin, here you can see in the motion oxygen is binding-releasing binding releasing. So, if you look at it is doing conformational excursion or constantly it is moving and that is what actually dictates whether it is in deoxy state or oxy state.

So, to go from deoxy state to oxy state or a vice versa it is doing lots of motion right. Quite a bit of motion, motion happening at a local time scale here and motion happening at the

global time scale like whole protein is moving. So these different kind of motion that we see in a protein which is quite relevant for their function. So, this how to probe this. This came from very early idea of Kurt wüthrich when he was working on a protein called BPTI, doing a basic experiment. BPTI is a basic pancreatic trypsin inhibitor.

So, what he was looking at the dynamics of an aromatic amino acid, in the globular conformation of a BPTI just using simple proton 1D NMR. So, what he was looking basically that there was an aromatic amino acid like phenylalanine and how it is changing over time, that is what he was looking. Because of this change, what was happening, whether it is twisting or something, you can see the distance between them change and NOE pattern between them change.

So this was happening when he was studying the protein and you can see there are lots of these aromatic amino acids encoded here. So, he was probing essentially aromatic amino acid and to probe the motion what he did, he recorded the temperature dependence 1D NMR and look at the chemical shift of these aromatic amino acids. So, essentially because of temperature change, rotation of this bulky aromatic amino acid within the hydrophobic core was probed. If you look at here, the experiments were done all the way ranging from 4 ℃ to 81 ℃.

And you can look at here, we have a sharp line at the lower temperature. As we go, one thing you can notice that peak seems to be shifting. Another thing you can notice, some of the peaks, the intensity is dropping. That means they are getting broader and broader. This tells that the hydrophobic core with temperature shows lots of motion, lots of relevant motion because now peaks are getting disappeared.

So you can imagine that if disappearance happening, some kind of motion is happening and that gives the information what kind of motion this molecule will adopt with changing temperature. So that was the first evidence that yes protein shows motion and you can probe just by recording 1D NMR, looking at these aromatic amino acids, and looking at their signature the chemical shifts peak pattern how it is appearing or disappearing you can learn about some motion. So, various time scale that are there in the motion I gave you example of like a hemoglobin oxy and deoxy. So, we saw that some kind of local motion is happening some kind of global motion happening. So, local motion we can define as local

flexibility and bigger motion like where large domain movement is happening, we can call them as a collective motion.

So, local motion we can define as kind of bond vibration, the whole methyl is rotating, the loop is moving like this or a side chain rotamers like in methyls moving something like this. So, that is like a fan, wings of a fan. These are called local flexibility. Whereas global flexibility, whole large section is doing motion like this. So if you capture all these motion that may happen in a protein, the time scale that can range from femtosecond to second time scale.

The local motion will be at a faster time scale, femtosecond, picosecond, nanosecond, microsecond and larger domain motion can happen in microsecond, millisecond and second time scale. If you look at the different techniques, in fact, can capture all these motions. So one of the prominent that in this course we are discussing about the NMR technique. So NMR relaxation can actually capture the motion that is coming from picosecond to second time scale.

X-ray can also capture, it reports you indirectly in terms of B-factor. It can capture again femtosecond to second time scale. The slower time scale motion using NMR can be captured using hydrogen deuterium exchange. So, if you couple hydrogen deuterium exchange with the NMR, you can capture the slower time scale motion ranging from millisecond to second time scale. We will be looking exclusively on this towards the end of this week, where we are replacing proton with deuterium and looking at with what rate it goes up.

Now the another important technique, fluorescence can also report, then UV-visible can also capture, Raman gives you about motion, infrared or even with MD simulation some of the faster motion you can capture from femtosecond to microsecond time scale. However many of these techniques lack resolution like a fluorescence, you need to have a tag and you are looking at the local motion of that tag or global motion of whole protein. UV visible encodes but actually resolution lacks, Raman lacks resolution. Infrared again is not that resolved. MD is high resolution but it is a computational technique so you do not know how they behave in solution.

You can mimic it but essentially to prove that you have to do experiment. On the otherhand, NMR is well tuned to give the motion at a various time scale in a residue specific manner or at an atomic resolution. So, that is what one can do by probing some of this NH probes in the protein and that will tell about local flexibility like a bond vibration, methyl rotation, loop movement, side chain rotamer or a collective motion. If we understand how these two loops or two domains are showing correlative motion, a larger domain motion that will come in microsecond to second time scale. So essentially NMR is tuned to provide you local flexibility as well as collective motion ranging from picosecond to second time scale. So, basically various atomic resolution methods that we discussed in the last slide, they essentially can probe it.

So, ideally structures of all subset and their interconversion series can be probed by various techniques high resolution technique like x-ray, but for x-ray you require a homogeneous crystal and you can say that many times this homogeneous crystal could be trapped in one of the substate. What I mean, if you draw the energy landscape here, it could be possible that your x-ray structure is trapped here or here or somewhere here or here. So, they can be trapped, it gives the high resolution structure, but actually interconversion between these states may not be possible. You have to acquire the structure of this state, acquire the structure of this state and then understand how it is possible. But, that is again a chance where you can trap it.

NMR gives you high resolution structure and dynamics, but its limitation that it is not suited for large molecule. For large molecule you lose resolution, you lose sense like lines becomes quite merged. So, for large molecule this is not amenable. Cryo electron microscopy on the other hand very well suited for bigger molecule, it is emerging technique. Now, one can even think of combining few of these techniques to work on a large molecule where you can look at the bigger picture using cryo electron microscopy and on a residue specific manner or localized motion using NMR.

So now, days are coming where you have to integrate multiple technique to understand the motion that is happening in protein. There is another technique called SAXS, small angle X-ray scattering. It's not atomic resolution, but this is also very well suited to understand where we cannot crystallize the protein or we cannot do the cryo-electron microscopy. So again, you can club SAXS and NMR, where SAXS will give you broader picture and NMR can give you local picture. Another technique, which is slowly becoming popular is called free electron laser.

The only problem is that it requires high power and it can destroy a sample. So during the experiment sample will be burned out. Now these are some of the high-resolution technique that can be used to get structure as well as dynamics. And nowadays one need to combine multiple techniques to get a correct picture of a biomolecule. So coming back to NMR relaxation, which probes the protein dynamics as I mentioned it samples or it can probe the motion happening from say picosecond to second time scale.

So if this is a clock, what kind of experiment and what kind of motion that we have in a protein. Just let us look at and what are the events that one can capture. So we can start from here. So, say if we want to understand the secondary structure formation. Secondary structure formation can happen very fast. Say it can happen between nanosecond to millisecond time scale. You can basically combine the HD exchange and you can understand how the secondary structure formation happens in a protein.

Now, if you go little slower motion like loop closure or hairpin closure, that is little bit of slow time scale microsecond to 10 of millisecond. This can be probed by various NMR techniques that is called RDC, residual dipolar coupling or CPMG that probably we are going to look at or $R_1\rho$ experiment these can be used to understand the loop hairpin closure. Now another important phenomena that happens in protein is called catalysis that happens in microsecond to second time scale that also can be studied using probably CPMG and $R_1\rho$ experiment. Now the faster time scale motion that is backbone dynamics can be studied using classical $T_1$-$T_2$-NOE or $R_1$-$R_2$-NOE which happens in picosecond to nanosecond time scale. So how the side chain is moving, the side chain rotation, picosecond time scale motions can be captured here.

Now, even further slower motions like some protein folding happens in millisecond to hour time scale. That again can be clubbed with a hydrogen deuterium exchange to study it. The slowest time scale like a protein aggregation can be studied using diffusion NMR, how the protein is coming or self-association in protein is happening. Slowly, slowly they are coming, forming a nucleus and the nucleation growth happens and then forms an aggregate. That can happen in from second to year time scale and can be studied using diffusion NMR.

So, NMR has a various amenities that can be tuned to understand a particular phenomenon that protein can have a catalysis, a loop closure or hairpin movement or backbone dynamics

or protein folding or aggregation. All of these have one or other experiment that can be used to understand. Now NMR relaxation essentially looks at the nuclear spin relaxation and that provide the information. The fast motion time scale is picosecond to nanosecond time scale. This can be done in laboratory from nuclear spin relaxation.

Whereas the slower time scale motion, which comes in microsecond to millisecond time scale can be probed in a rotating frame nuclear spin relaxation. Magnetization exchange spectroscopy that we will be looking at something called ZZ-exchange which an probe motion in time scale of millisecond to second time scale. So here, fast time scale, like typically we know that $R_1$, $R_2$ are fast time scale. It is a laboratory frame relaxation. $R_1\rho$ rotating frame relaxation can probe us microsecond to millisecond time scale.

And then something called magnetization exchanges spectroscopy from millisecond to second time scale. So these are typical experiments that one use. Now typically we use heteronuclear spin, because heteronuclear is very well suited to give you sharp lines and due to its excellent relaxation property, proton is very abundant and it has a different relaxation property or faster relaxation property than heteronuclei. So typically in protein NMR we do heteronuclear spin relaxation and that basically gives us an idea how to characterize the dynamics process in a protein. So, in this week we are going to look at what are the $T_1$-$T_2$-NOE phenomena, how do we measure this and how they report about the dynamics in the protein.

How we can derive the relaxation parameter from these experimental data that we record the $T_1$ experiment, $T_2$ experiment and heteronuclear-NOE, what are the time scale that they capture and that we can measure by NMR and how this relaxation parameter presented to illustrate the motion that is happening in protein. And essentially, we are going to look at how we can set up this experiment to measure the protein dynamics. So let's talk a little bit more about the motion and then that will help us in designing the experiment. So as I was saying, protein motion affects the NMR parameter.

Various kinds of motion, this local motion is a fast time scale motion - picosecond to nanosecond time scale. So each bond is making some motion either rotation or movement like a conformation excursion all these are happening that is called fast time scale motion. The whole molecule, if you look at this protein of two domains, say beta domain and a helical domain, and connected by loop, the whole protein is tumbling, which has a

rotational correlation time, that is a slow time scale motion, so microsecond to millisecond time scale. So if I want to probe this local motion as well as the collective motion, I need different kind of experiment. In one case, it should only probe here, in another case, it should probe whole protein motion.

And that is what we are saying for a local motion, we just can exploit the NH bond, the excursion that NH bond is doing, the relaxation property of NH bond and that can be probed by the bond vibration or a methyl rotation or loop movement. The collective motion, which is essentially a larger domain motion like from here to here this can also be done. So, another orthogonal technique like a fluorescence, you can add a fluorophore here and do time-resolved FRET. You know how they are coming closer, they are going far and you can plot it. Then you are looking at how the two probes are coming together and going together. Essentially, in NMR you do not need to add anything extrinsic, you can look at this collective motion of cross-correlated motion by understanding collective large domain motion. So, if you now draw this energy landscape here, you can see the motion that is happening. The local motion happening at picosecond time scale or nanosecond time scale happening essentially in this local minima of the protein, local minima in the protein energy landscape.

Whereas the microsecond to millisecond time scale motion, this is happening like when there is another state which it has to cross this barrier to go the state. So, protein existing in state A and protein existing as a state B. So, local motion are these and whole domain motion you can see there is a structural change happening when protein goes from state A to state B and these two states are having some energy difference which is of few $K_BT$ or few kilocalorie-mole. So protein is constantly going from A to B with rate $K_{AB}$ and coming back $K_{BA}$.

Now this kind of motion is slower time scale motion or large amplitude collective motion and that basically samples pure state may be 1 or may be maximum 2. However, the local motion since the energy barrier is very low, it can with a thermal fluctuation, it can sample many of those states because here energy barrier is very low. It can easily cross over or here also it can cross over with a thermal fluctuation. So local motion quite a bit of possible.

However, whole domain motion, it requires certain energy. That energy is given here which should be few kilocalorie per mole. So, slower time scale motion like something which is

important in allosteric or structural rearrangement like a cis-trans isomerization that may happen or here the whole domain is opening and closing up these are slower time scale motion. Cis-trans isomerization during catalysis you see some kind of protein is changing from cis to trans, it is a slower time scale. It requires completely bound orientation or even in signal transduction or protein-protein interaction essentially happens at a slower time scale motion.

Now, to sum up what we discussed till now, NMR is a versatile tool for understanding the atomic structure and various time scale of transition. There are various time scales all the way ranging from picosecond to second time scale motion. The frequency if we want to understand, it is a terahertz to hertz frequency and those are basically can be done with various NMR experiment like a faster time scale motion in laboratory frame can be done using $T_1$-$T_2$-NOE, relaxation in rotating frame can be done $T_1\rho$, $T_2\rho$ or ROE. Then we can understand with a line shape analysis or residual dipolar coupling these can capture various time scale motion. Typically in solution state, we do all these experiments here and solid state it is a relatively slower time scale like a protein folding or aggregation or say tumbling of whole protein in the membrane that comes in second time scale which can be captured using solid state NMR.

Next like we are just focusing on the liquid state, solid state I will be discussing in the last week of this course. To sum up relaxation is a process by which the spins return to their equilibrium population distribution and it is governed by the fluctuation that can happen because of local field and spin orientation. What happens here is a $B_0$ main magnetic field and here are my tiny spins. So, they are doing all random fluctuations and they are experiencing the local field and that is influenced by this main magnetic field. So this reorientation of these spins cause variation in their interactions, which we can define as a CSA, chemical shift anisotropy or dipolar coupling. And as I mentioned that the heteronuclei are very well suited for relaxation mechanism, proton has a complex relaxation.

So, mostly liquid state experiments are done exploiting the heteronuclei and the relaxation parameter that contributes CSA and DD. So, we are going to now look at little more detail in the next class, How the CSA-DD contributes to the relaxation phenomena and how we can exploit this heteronuclear like a 13C or N15 for understanding the relaxation mechanism. So in this class, I just give you the idea about why relaxation and why NMR is suited for relaxation, what information the relaxation brings or the motion brings for understanding the protein function in a holistic manner.

With this I am closing here for today. Thank you very much. See you in the next class. Thank you. Thank you very much.