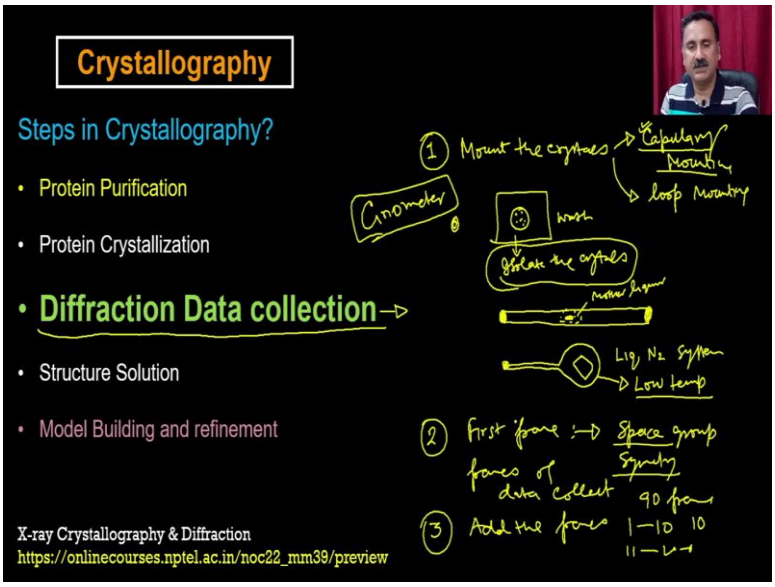
Enzyme Science and Technology
Prof. Vishal Trivedi
Department of Biosciences and Bioengineering
Indian Institute of Technology, Guwahati

Module - II
Enzyme Structure
Lecture - 09
Tertiary Structure of Enzyme (Part II)

Hello everyone. This is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering, IIT Guwahati. And in the course, Enzyme Science and Technology, we are discussing about the different aspects of the enzyme. So, what we were discussing? We were discussing about the crystallizations, right.

(Refer Slide Time: 01:02)



So, the next step is you are actually going to use these crystals for diffraction data collection. So, differential data collection is a multi-step process. In the first step, you are actually going to use; so, within the diffraction data collections first you are going to mount the crystals, right. The mounting of the crystals can be done by two methods. One is called as the capillary mounting, and the second one is called as loop mounting, ok.

So, capillary mounting is a slightly older method where people are you know putting the crystal into a capillary. So, you can imagine that I have sucked the crystals from the

drop. So, what you are going to do in the mounting of the crystal is that you are going to open the crystal the you know cover slips, right.

So, once you open the cover slips, you are going to see a drop and in these drops you have the crystals, right. So, first you are going to do is you are going to wash this drop and then you are actually going to isolate the crystals, right. And then, these crystals can be placed either into the capillary or in the loop.

So, in the capillary, what you have is you have a capillary like glass capillary, right. So, glass capillary you can just place the crystals, right and you can place some amount of the mother, right mother liquor, right. So, you can actually be able to place some amount of mother liquor. And then, it has to be sealed from both the ends or both the sides with the help of the clay. And then, this you can actually be able to use for mounting.

Whereas, in comparison to this when the you are going to do the loop mounting, you are going to have a rod on which you are actually going to have the loop. And this loop you can be able to place the crystal in between, ok. Now, loop mounting is requiring additional infrastructure where you are actually requiring the liquid nitrogen system, right. So, that your crystal is going to be at very high low temperature.

So, that is beneficial because it actually increases the viability of the temperature and viability of the your crystal because when the crystal is going to face you know the X-rays, it is actually also developed some kind of breakdowns. And if you keep it in a very very low temperature like the liquid nitrogen temperature, it is going to reduce the damages.

Now, the step 2, you are actually going to collect the first frame, ok. So, once you collect the first frame that actually will allow you to calculate the space group and it is also going to calculate the symmetry. So, once you calculate the symmetry and the space group, it is actually going to allow or it will actually going to tell you that how many frames of data you are supposed to collect, right.

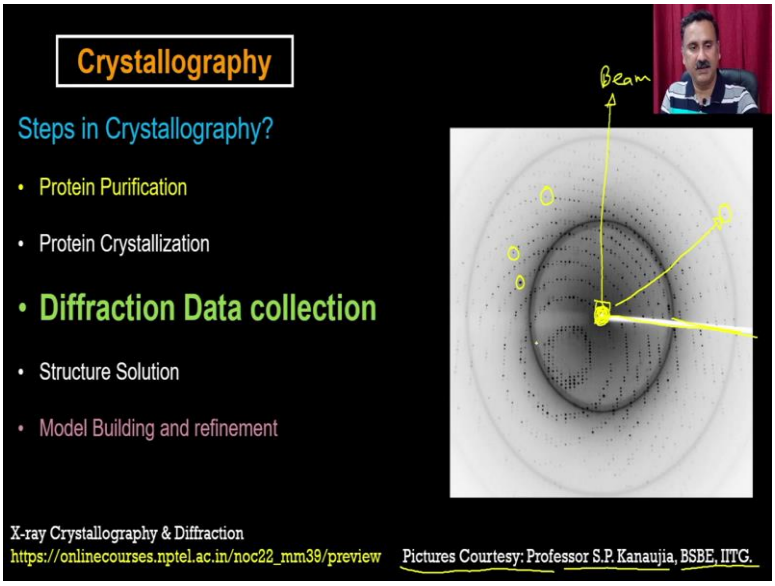
So, you can imagine that if I have the crystal, and if I have the two-fold symmetry or three-fold symmetry and four-fold symmetry and all that, I can be able to collect the data accordingly. So, once you collected the data, the third step is that you are also going to add the frames after frames, right.

So, you can actually for example, if I have collected the 90 frames, right. So, what I will do is I will take the 1 to 10 frames, I will you know add them. So, that I am going to have the data of 1 to 10 frames. Similarly, from 11 to 20, I will add it into another 10, right. So, that is how all the frames data you are supposed to collect.

And I am not showing you the X-ray machine or X-ray place, but all these mounting has to be done on a goniometer, right. And goniometer is a place where which is actually going to be parallel which is going to be perpendicular to the X-ray and that is how X-ray is going to illuminate the X-crystals.

Once you collect the first frame, you are actually going to get the diffraction data. How the diffraction data will look like? Right.

(Refer Slide Time: 05:25)



So, this is the diffraction data what you are going to look like. What you see here is in the center, this is actually called as beam stop, ok. So, in the machine, you are actually having a beam stop which will not allow the undiffracted X-ray beam to hit the detector actually.

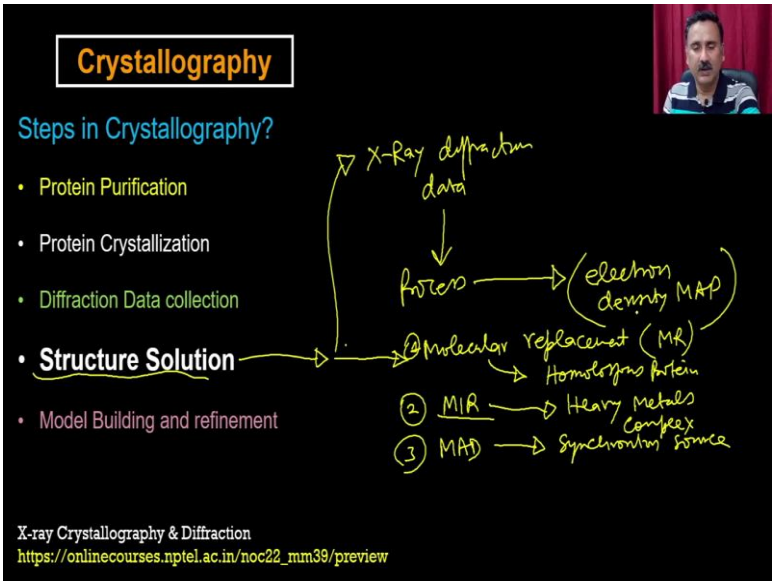
And you see this rod, right, so that is the beam stop actually. So, it has as a rod and then in front of that there is a stop, right. So, that is not going to allow the X-rays to hit the detector, because if that happens it is actually going to affect the brightness and contrast of the detector and also going to detect.

So, what you see is if black color spots, all these are actually the diffracted rays which are falling on to the detectors. And all these this picture is also being provided by the Professor Shankar Prasad Kanaujia from our department only. And the. So, what you see here is that this is the origin of the diffraction pattern, right.

And as you move towards this side, it is actually going to tell you that how good the diffraction you are getting. So, as long as you are getting the diffraction pattern; that means, the resolution is bigger and bigger and higher and this diffraction is good actually. So, you see all the spots are discrete, all the spots are not merged.

In some cases, what you will see is that the one spot and you are getting the second spot. That is actually going to create trouble when you are going to do with the structure solutions, because it is actually going to interfere in terms of preparing the electron density map.

(Refer Slide Time: 07:14)



Now, the second step we are going to go with the structure solutions, ok. So, in the structure solution, what you are going to get, what you are going to do is you are going to have the X-ray diffraction data, right. Now, this diffraction data is going to be process, right and ultimately it is actually going to allow you to generate the electron density map, right. And there are many method or many approaches what you can actually be able to use for structure solutions.

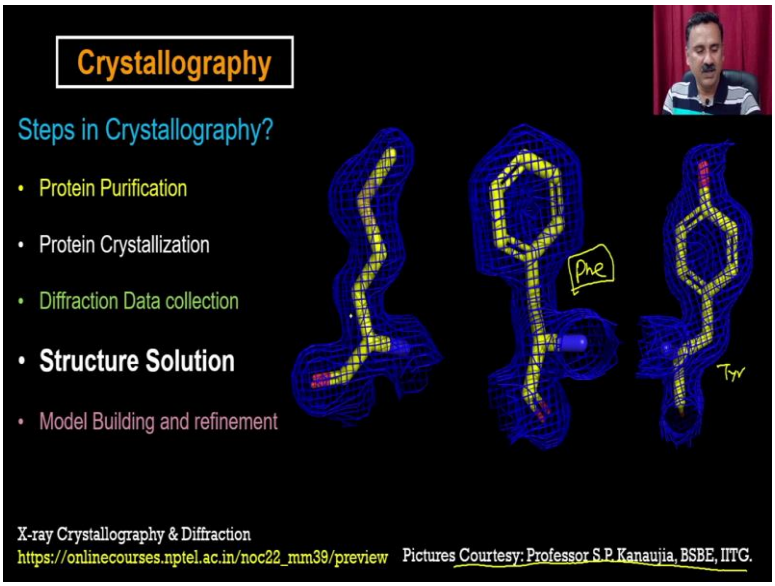
So, you can actually be able to use the molecular replacement or MR, right which is called MR, right. So, these are the molecular replacement approach which is only for the protein if you are; if you are suppose solving a protein which is homologous to the existing protein in the protein structure database. So, this is actually for the homologous proteins, ok. I am not going to deal in detail about any of these methods because they itself are you know very big.

Then, you can also use the MIR and that is actually where you are actually going to prepare the heavy metal complexes, right of the protein and it is actually going to allow you. And then, you also can use the MAD or the multiple anomalous diffraction and that there you are actually going to use the instead of using the X-ray, you are going to use the synchrotron source.

And it is actually going to use for you know varying the X-ray wavelengths, and that is how it is actually going to help you in terms of the solving the phase problem and it also going to help you in terms of developing the electron density map. Now, either of these method depend on the what is the condition, if you are having a homologous protein you can use the molecular replacement.

If you are working with the new protein and the new fold then you have no choice, but to either try out the MIR method or the MAD method. So, after that you are going to have the electron density map after this structure solutions, right. And then how the electron density map will look like, right.

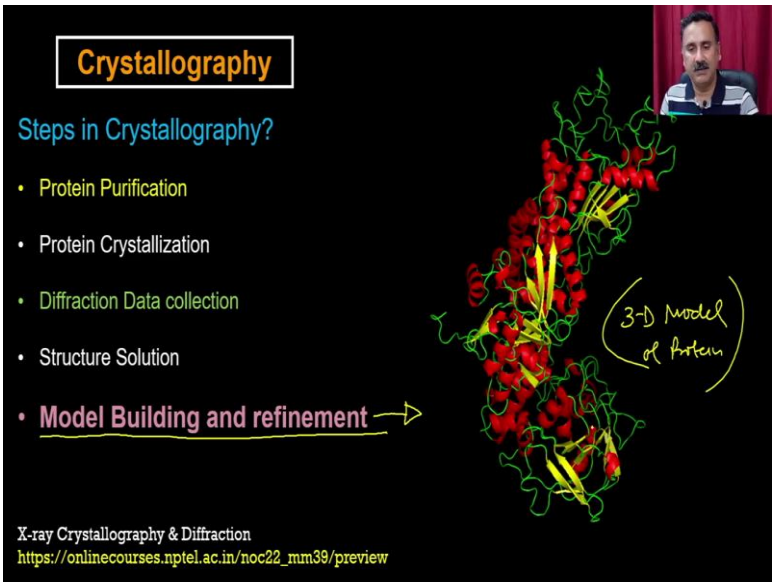
(Refer Slide Time: 09:34)



So, electron density map will look like as a kind of a web like structure which is surrounding the protein. So, this is what you see here is electron density map of the different protein side chains, right, ok. This is the phenylalanine what you see. So, this is the phenylalanine ring and then it is connected to the main chain. So, all these pictures are also being provided by the Professor S. P. Kanaujia and these are the tyrosine what you see and so on, ok.

So, now, in the next step is that where you are actually going to do the model building is that looking at the electron density map you have to place the protein sequence and that is how you are actually going to refine the structure, and based on the using the information from the electron density map.

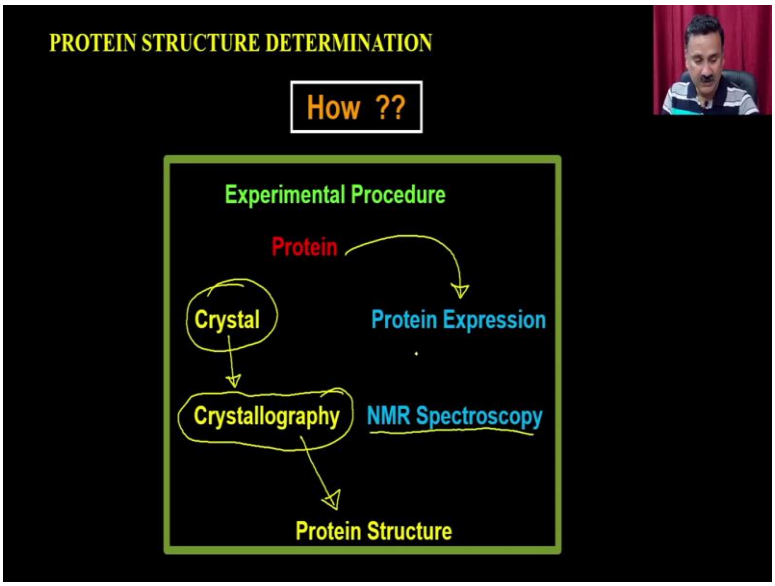
(Refer Slide Time: 10:26)



So, that is what you are going to do when you are going to do the model building and refinement. So, you are actually going to you know fit the protein side chain into the this electron density map, then you are actually going to calculate whether the structure is having the more error or less error. And that would be one of the guiding force to tell you whether you are doing a solution correct or not, right.

So, that is how you are actually going to get the final structure. So, this is actually a final structure, the 3D model of the protein, and what you are going to get after the structure solution using the X-ray crystallography. So, this is all about the X-ray crystallography what we have discussed, right.

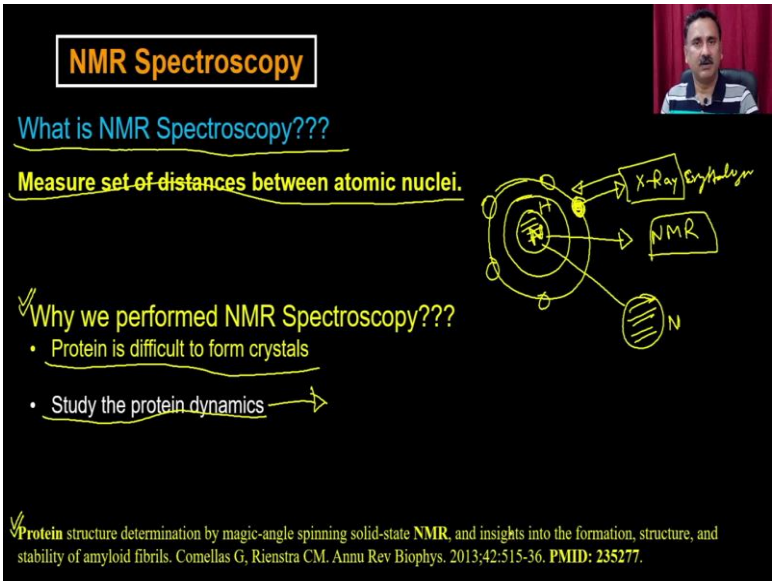
(Refer Slide Time: 11:12)



So, we have discussed about the crystallization process and once you got the crystallization you are actually going to use that for collecting the diffraction data. And then, subsequent to that you are going to do the structure solution and the model building and refinements. And at the end you are going to get the protein structure using this approach.

Now, let us move on to the second approach, and the second approach is the protein structure solution by the NMR spectroscopy.

(Refer Slide Time: 11:48)



So, let us discuss about what is NMR spectroscopy. So, what is NMR spectroscopy? It is its measure the set of distances between the atomic nuclei. So, remember, there is a clear cut difference between the X-ray crystallography and the NMR spectroscopy. NMR is more about; so, see if you see the atom, right, so in the atom you are going to have the electrons, right on the periphery, right. So, it is going to for example, you have the electrons, and then in the center you are going to have the nucleus, right.

So, if you are actually going to study the nucleus then it is actually going to called as NMR spectroscopy. If you are going to study the nuclei electrons then it is actually going to you say X-ray crystallography. Because X-ray is actually going to you know having the wavelength which is actually going to you know activate or going to be hit the electrons.

Whereas, in the NMR you are actually going to study the nucleus. So, you are actually going to measure the distances between the two nuclei. For example, you can have the hydrogen nuclei, you can have the nitrogen nuclei and so on. So, you might have seen the structure different structures of the amino acids, right.

So, if you are mapping with the X-ray crystallography, you will be able to see the arrangement of the electrons, right. That is how you are generating the electron density map whereas, in the case of NMR, you are actually going to see the differences or distances between the nucleis.

Why we perform the NMR spectroscopy? Right. We performed the NMR spectroscopy because the crystallization is very difficult. In some of the cases, the protein structure is dynamically so unstable that it does not form a single conformation. So, it does not get stabilized into a single conformations.

So, in those kind of cases, you are always going to get either the amorphous powder or precipitate or in some cases you are going to get the crystal, but those crystals are actually going to have the very high degree of mosaicity or disorderness or they are actually going to form the crystal, but they may not diffract.

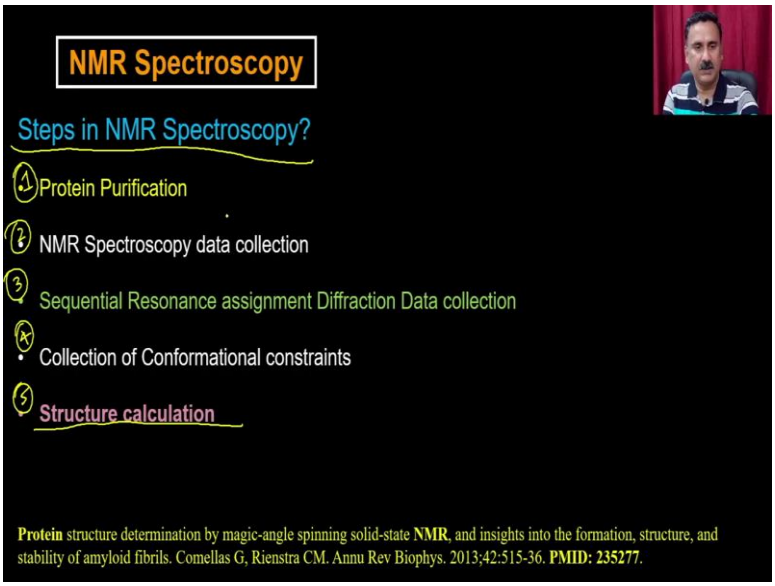
So, either of these method, either of these problems, whether the protein is not forming the crystals or protein is forming the crystal, but they are not giving you a very high

resolution diffractions, you have no choice, but to use the NMR spectroscopy to solve the protein structures.

Then, the second advantage is, that when you solve the protein structure with the help of the NMR, you since the NMR is going to be performed into the buffer or the water you can also be able to study the protein dynamics. Because they are free to move see compared to the X-ray crystallography, where the structures are going to be rigid or they will stick to each other, they are not allowed to move, NMR is going to be done in the solution, right.

And they are, so the proteins are free to move and that is why you are can be able to study the dynamics. For example, if you add the substrate to the protein, right, it is going to change the conformations. So, those conformations can be mapped with the help of the NMR spectroscopy. So, I have given you a reference for the protein NMR spectroscopy as well. And you can be able to study or you can be able to read all of these steps, what are we are going to discuss now onwards from this particular review articles.

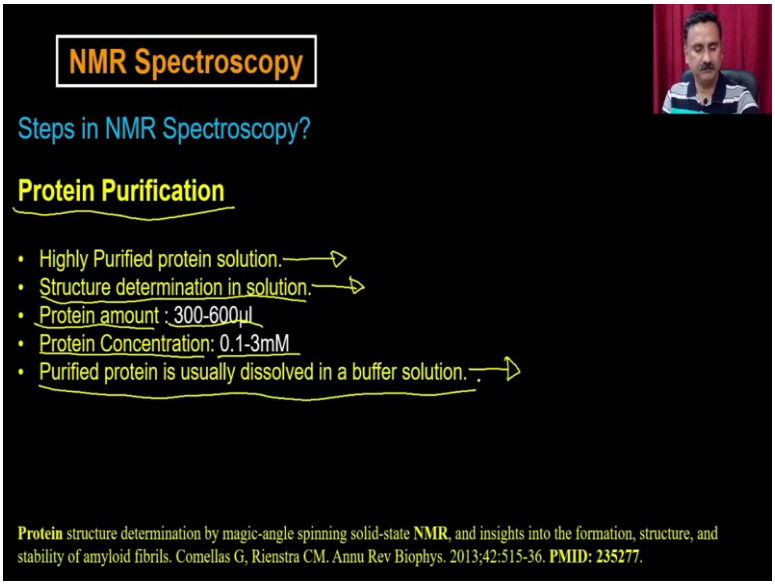
(Refer Slide Time: 15:28)



So, what are the different steps in the NMR spectroscopy? So, the first step is the protein purification the second step is the NMR spectroscopy data collections, the third step is the sequential resonance assignment, and fourth step is the collection of the conformational constraints, and the fifth step is the structure calculations.

Remember that, it is written as structure calculation rather than determinations because in the case of NMR, you always going to calculate the structure, right.

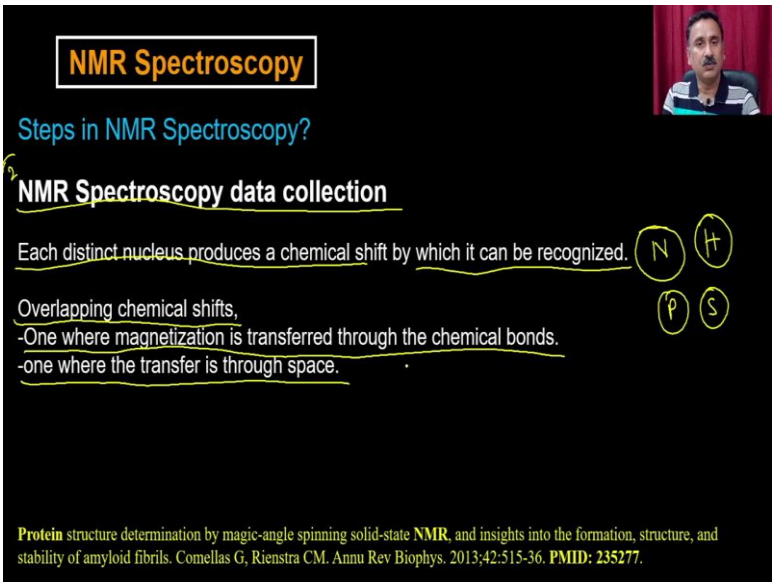
(Refer Slide Time: 16:05)



In first step is the protein purifications. So, when we talk about the protein purifications the requirement for the NMR spectroscopy is also a highly purified protein. It is a highly purified protein you require, so that the other proteins or other kind of molecule should not interfere in producing the NMR data.

Since, the structures are going to be determined in the solution phase, right, you require the protein content that is from 300 to 600 micro liter and the protein concentration should be in the 0.1 to 0.3 millimolar. And the purified protein is usually dissolved in a buffer solutions.

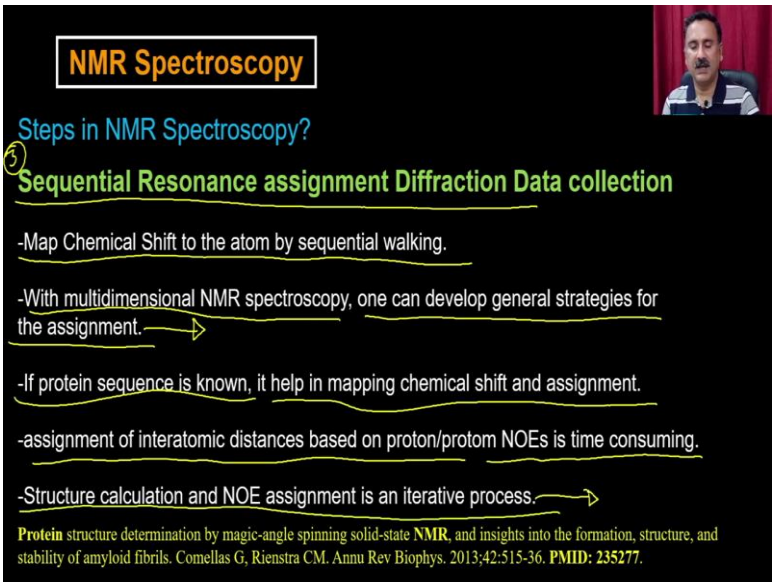
(Refer Slide Time: 16:44)



Then, we the second steps, second step is about the NMR spectroscopy data collection. So, each distinct nuclei produces a chemical shift by which it can be recognized. For example, you can have the speak for nitrogen, you can have for hydrogen, you can have for phosphorus, you can have for sulfur and so on.

So, all these are going to be collected with the help of the different types of NMR probes. For example, nitrogen probes, hydrogen probes, phosphorus probe, sulfur probes and so on. Then, you can also have the overlapping chemical shift. So, one where you have the magnetization is transferred to the chemical bond and one where the transfer is through the space.

(Refer Slide Time: 17:29)

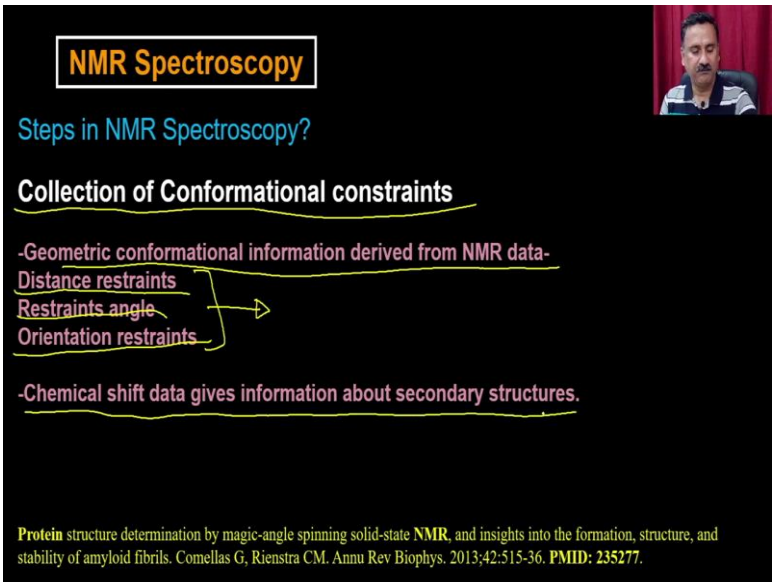


Then, you have the third step, and the third step is the sequential resonance assignments, right. So, you can map the chemical shift to the atom by the sequential walking, right. And with the multidimensional NMR spectroscopy one can develop general strategies for the assignments, right. You can actually be able to use not only the nitrogen probe or you can use phosphorus sulfur and all that.

So, a combination is actually going to give you more complicated multi-dimensional NMR spectra and that actually can be used to assign the different peaks. If the protein sequence is known, it helps in mapping the chemical shift and as well as the assignments. The assignments of the inter atomic distances are based on the proton protom NOEs, and it is time consuming, right.

So, structural calculation and NOE assignment is a iterative process. Just like as we were discussing about the X-ray crystallography, the NMR spectra solution is also like that. If you have to first assign the peaks you have to assign the peaks like nitrogen, ammonia, like that. And if that is correct it will actually going to make the structure more reliable. So, that is how you are going to reduce the R-factors.

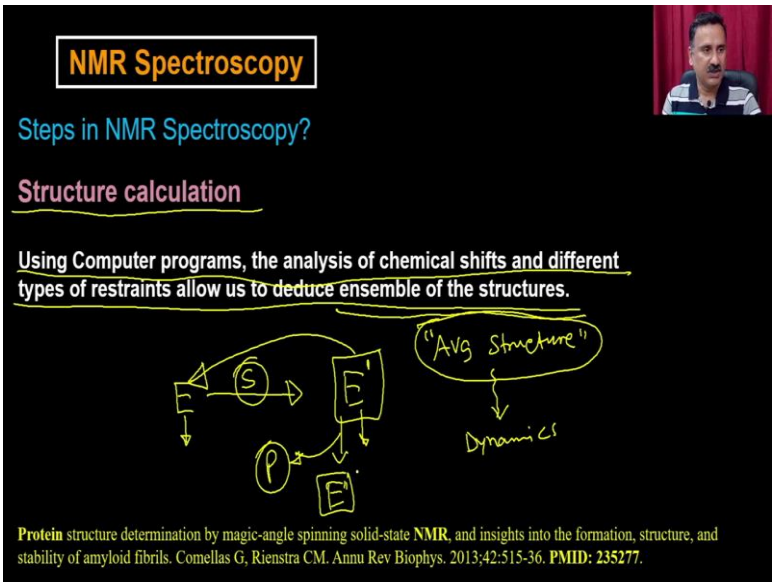
(Refer Slide Time: 18:51)



Then, we have the 4th step, is the collection of conformational constraints. So, geometric conformational informative derived from the NMR data, you can actually have the distance restraints, you can have the restraint angles, and you can have the orientation restraints. So, all these are very important for seeing that the protein you know structurally stable, and it is actually going to have the distance restraints, right restraint angle and orientation restraints.

So, all these are very very important. Then, you can have the chemical shift data given from the secondary structures.

(Refer Slide Time: 19:27)



Then, we can also have the last step that is the structural calculation. So, using the computer programs, the analysis of the chemical shift and the different types of restraints allow us to deduce the ensembles of the structure. Remember that the NMR is going to do the protein structure determination in the solution, right.

So, in the solution, some structure is going to be one conformation, the other structures are going to be second conformation and so on. So, that is why it is actually going to deduce the ensembles of the structures, which means it is going to give you a average structure of that particular protein, at that particular moment because average structure may change if you change the conditions. And that is how it is actually going to use for studying the dynamics, right.

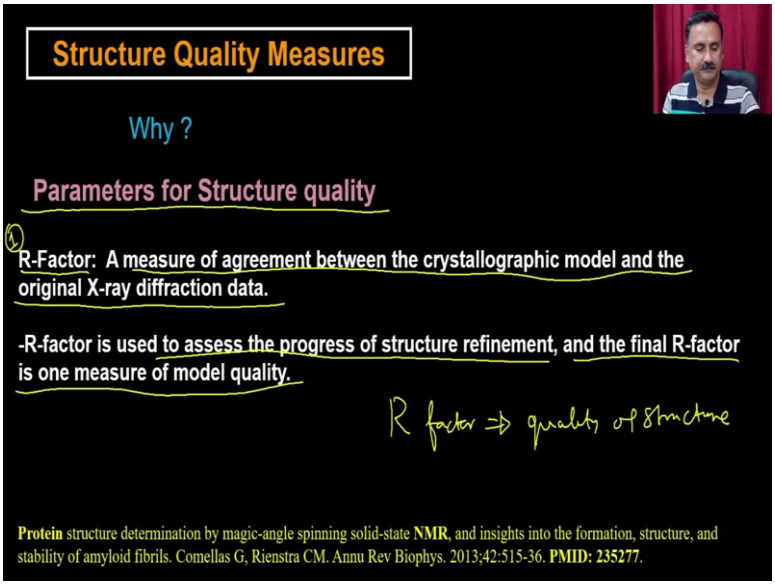
So, if you add for example, if you take the enzyme, right and if you add the substrate, enzyme is going to be enzyme prime because it is actually going to induce the conformational changes. And that is how the average structure for this and average structure is this is going to be change. And that is how you can be able to detect at which portion of the protein is actually responding to the substrate addition, and how that particular portion is determining the structure solution or enzyme catalysis.

Similarly, E prime probably can you know go back to the E or it can actually be go to the E prime, E double prime actually when the product is actually going to be released. So, these kind of NMR is a very very robust technique, right to actually see that you are

actually going to study all these events, like where you can actually be able to see the conformational changes in the enzyme structures when the substrate is binding and you can also be able to see the changes when the product is getting released.

Either it is going to adopt the original confirmation or it can actually be able to adopt the other conformation as well.

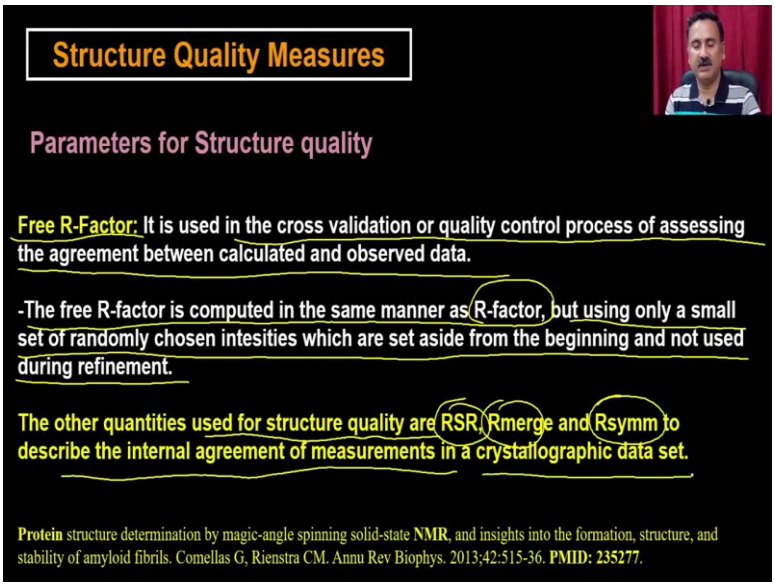
(Refer Slide Time: 21:24)



Now, once you solve the structure either by the X-ray crystallography or the NMR spectroscopy, you have to define or you have to determine whether the structure what I have solved is of good quality or not, right. So, what are the parameters for the structural quality? So, the first parameter is the R-factor.

So, R-factor is a measure of the agreement between the crystallographic model and the original X-ray diffraction data, which means it is R-factor is used to assess the progress of the structure refinement and the final R-factor is on a is one which we measure the model quality, which means R-factor is actually going to decide the quality of the structure.

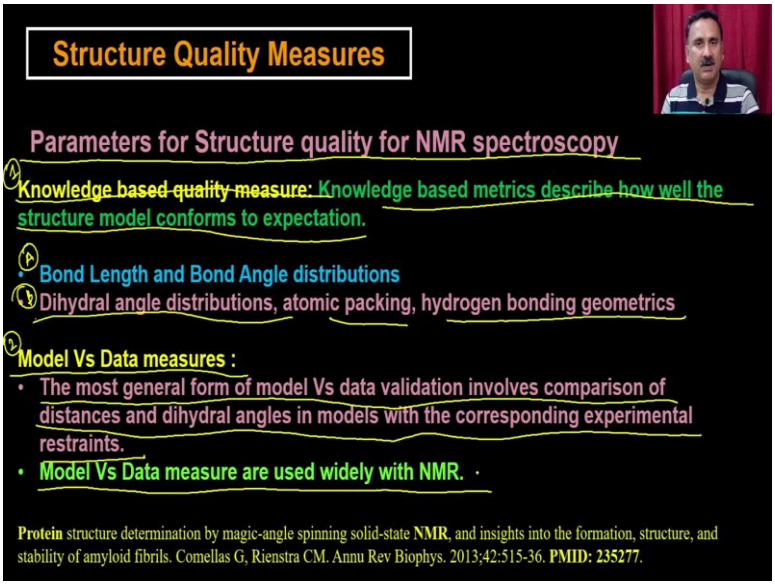
(Refer Slide Time: 22:20)



You can have the free R-factor. So, it is used in the cross validation or the quality control process of assessing the agreement between the calculated and the observed data. The free R-factor is computed in the same manner as the R-factor, but usually only a small set of randomly chosen intensities which are set aside from the beginning and not used during the refinements, ok.

So, other quantity used for the structure qualities are RSR, Rmerge and Rsymm to describe the internal agreement of the measurement in a crystallographic data set.

(Refer Slide Time: 22:58)

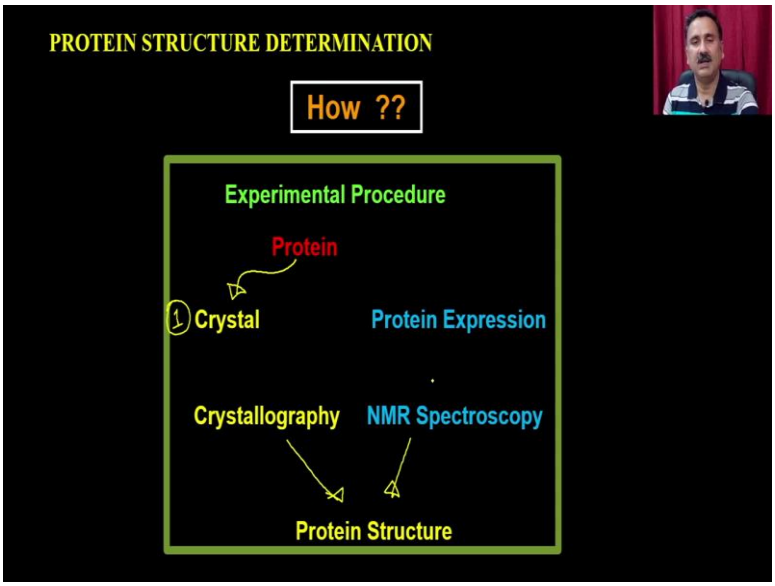


Now, the parameters for structure quality for the NMR spectroscopy. So, you can have the first parameter that is called as the knowledge based quality measures. So, knowledge based matrices describe how well the structure model confers to the expectations. It means you are going to see the bond length and the bond angle. You remember that Ramachandran plot, right. So, that is very important to satisfy the structure what you have solved by the NMR spectroscopy.

Then, you also have the dihedral angle distributions, atomic packing, and the hydrogen bonding geometrics. So, that is also very important. Then, you can also have the second parameter that is the model versus the data measurements, right. So, what model you have prepared and what the data is suggesting in terms of model is also very important.

So, the most general form of model versus data validation involves the comparison of the distances and the dihedral angle in model with the corresponding experimental restraints. The model versus data measurements are used widely with NMR to assess the structure quality.

(Refer Slide Time: 24:06)



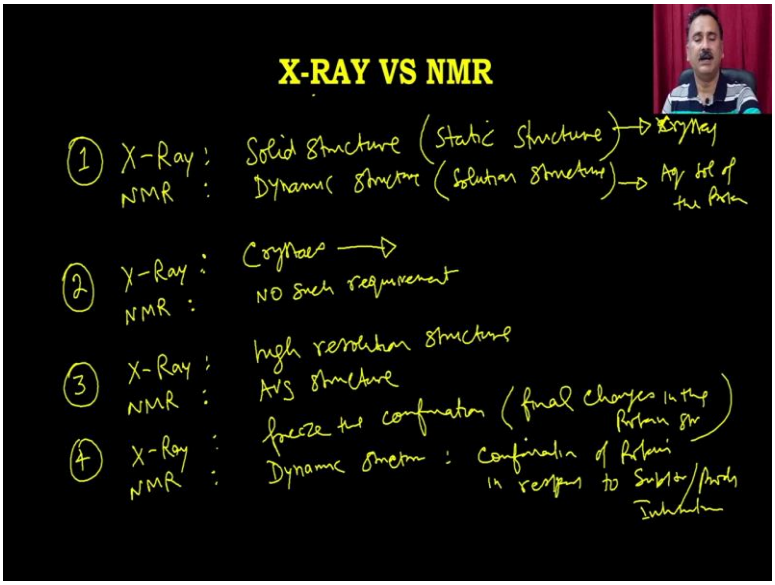
So, this is all about the protein structure determination. So, we have, what we have discussed so far? We have discussed about the experimental procedures and in that discuss, while discussing about the experimental procedure we discussed about the two methods, we have discussed about the two in under two situations. In one situation when

you can be able to produce the crystals, you are actually going to use the X-ray crystallography to solve the protein structures.

And in the second step second process you can be able to use the you can be able to use the NMR spectroscopy. And both of these methods are actually going to give you the protein structures. Now, the question comes what is the difference between the protein structure solution either by the X-ray crystallography or the NMR spectroscopy.

So, what is the advantage and what are the disadvantages when you are going to use the X-ray versus NMR?

(Refer Slide Time: 25:05)



So, X-ray, so what are the difference? In the X-ray, X-ray is going to give you a solid structure or I will say the structure under the static conditions. So, it is actually going to give you the static structure which means that structure is not going to change it is going to be final confirmation.

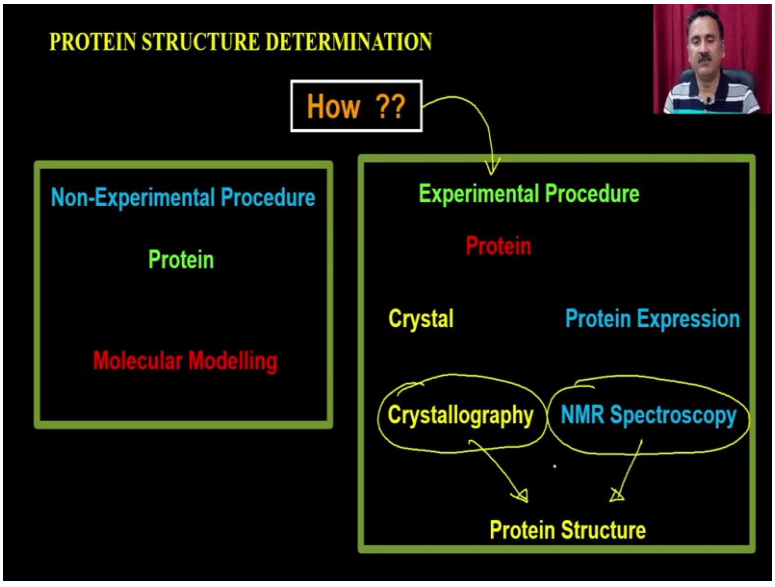
Whereas, in the case of NMR it is actually going to give you a dynamic structure. Now, because it gives the dynamic structure. So, it is actually going to give you a solution structure. So, it is called as solution structures. Why it is so? Because the static structure is coming from the crystal, right. Whereas, the dynamic structure is coming from the aqueous solution of the protein.

Number 2, in the case of X-ray, you require the crystal, right. So, that is a major drawback or I will say disadvantage because you producing the crystal of a protein is not very trivial, right because and whereas, NMR there is no such requirement, ok. Number 3, X-ray is going to give you a high resolution structure. NMR is going to give you average structure, ok.

Number 4, X-ray is going to freeze the conformations, right because it is going to give you the static structure. So, that is good to study the final conformation or final changes in the protein structure. Whereas, in the case of NMR because it is going to give you the dynamic structure, it can be used very extensively in terms of the studying the conformations of the protein in response to substrate or product or inhibitor.

So, or in some cases, it can be also studied to you know see the conformation of the protein structure, even when the you are adding the another protein, so to which the previous protein is interacting, ok. So, what we have discussed in this particular lecture?

(Refer Slide Time: 28:21)



We have discussed about the protein structure and we have discussed we have taken the two approaches, one is called as the experimental approach. So, where we have discussed about the X-ray crystallography, and as well as the NMR spectroscopy. And both of these approaches can be used to study the protein structures.

When we were discussing about the crystallizations, we discussed about the hanging drop method and as well as the sitting drop method. Subsequent to that, we have also discussed about the how you can be able to use the data collections. So, when we are doing the data collections, we can also be able to do the mounting, mounting into the capillary or mounting into the loop.

And then, subsequent to that we have also discussed about the data collection, X-ray diffraction patterns and structure solutions and the model building and requirements. We also discussed about what are the different parameters you can use for you know calculating the errors into the salt structures and how you can be able to assess whether the structure what you have solved is of good quality or not.

And when we were talking about the NMR spectroscopy, we have discussed about the different steps, how we can be able to you know collect the diffraction collect the NMR spectroscopy data, how you can be able to assign the different peaks and so on. And lastly, we have also discussed about the how we can be able to assess the quality of the NMR structure from the NMR.

And at the end, we have also given you a comparison of the two techniques. So, that you can be able to know what are the robustness of one technique and what are the advantage of the other techniques. So, with this I would like to conclude my lecture here. In our subsequent lecture, we are going to discuss more about the non-experimental procedures which means we are going to use, we are going to discuss about the computational approaches and how you can be able to model a protein structure using the molecular modeling approach.

So, with this I would like to conclude my lecture here.

Thank you.