The devil, of course, is in the details. And this is where the discussion of “exchange regime” becomes very important: the CSP only depends on the amount of bound protein for systems that are in “fast exchange”, i.e. the difference in the chemical shifts between the free and bound state (delta\_omega), that is the starting and end points, are much smaller than the rate of interconversion between free and bound forms (k\_ex). And only in this scenario does the equation that we used in the paper to fit Kd from CSP apply. Fast exchange is usually written as |delta\_omega| << k\_ex, whereas slow exchange is the opposite |delta\_omega| >> k\_ex and intermediate exchange is in the middle with |delta\_omega| ~ k\_ex.

If we assume that a protein:ligand binding interaction has a single k\_ex, that is a single binding event where k\_ex = k\_off + k\_on[L], then we realize that there can be multiple exchange regimes for different residues throughout the protein, as delta\_omega is a residue-specific parameter that depends on the effect of binding. For example, to put some numbers on this, one first must convert delta\_omega from ppm -> radians per second to enable comparison to k\_ex. So, if the kex is 10,000 s-1 and we observe a |delta\_omega| in the 1H dimension of 0.1 ppm at 600 MHz (which corresponds to 2\*pi\*(600 MHz \* 0.0000001) = 377 rad s-1), we see that |delta\_omega| << k\_ex, so this particular residue will be in fast exchange. However, if the delta\_omega for a different residue is 1.5 ppm (= 5655 rad s-1), we’ve now reached the intermediate exchange regime where delta\_omega ~ k\_ex – and now the CSP for this residue depends on % of the bound state in a very non-linear manner.

The link between CSP and exchange regimes is confounded by the fact that the CSP is a summation of chemical shift changes in two dimensions, and so we have to consider the “exchange regime” in both dimensions.

>> Would you mind explaining the rational of adding more chaperone to the mix?

When ppl show bar graphs of CSPs vs residue number, they generally want to highlight the binding sites, as those should have the largest CSPs relative to the rest of the protein. They may pick a particular stoichiometry where this is most obviously seen. In cases where the binding is very weak and the observed CSPs are very small, then it’s hard to identify the binding site. So you would then add a lot more ligand to see if your CSPs in the binding site become stronger and can be identified more easily. It’s usually best practice to show a titration, either with the raw spectra or graphs of CSPs vs. added ligand, so one can see how the titration progresses. That way, you know what is the starting point and what is the “end state”, i.e. the fully bound form, and from that you can identify the maximum CSP.

>> Do you simply expect higher signal if you add more of your protein of interest, to the tube? Because then you could in theory add as much as needed, to obtain high enough signals, no?

In general, yes, but you will reach a limit of maximum CSP when you’ve reached 100% of the bound form. And this can easily be achieved for systems with nanomolar-low micromolar Kd values. However, for systems that cannot be saturated (very weak binders) where there are solubility/sample limitations, then you cannot (easily) obtain the chemical shifts of the fully bound state. You can, of course, lower the amount of protein and add the maximum amount of ligand to skew the protein:ligand ratio toward ligand, but even then this might not be enough. On modern NMR spectrometers equipped with the latest technology, you can actually record a 2D spectrum on a 15N-labeled protein at a concentration as low as 1 uM. That way, you could easily obtain a 1:1000 ratio of protein:ligand if you can get your ligand up to a 1 mM concentration (and even higher if you can go higher).

\*\* The one caveat to my answer above is that, under certain circumstances, one can exploit chemical exchange to measure the chemical shifts of the bound state. The caveats are the timescale of exchange (k\_ex) and the differences in chemical shifts between free and bound (delta\_omega); assuming those fall in the correct windows, then one can measure this quite easily. In this case, you don’t have to perform a full titration to get the chemical shift of the end state. This is the beauty of experiments like CPMG relaxation dispersion or the more recent CEST experiment 😊 for example, in our paper we are able to use CPMG relaxation dispersion to measure chemical shifts of the bound ACD when it’s only ~1.5% bound to the peptide – we didn’t have to prepare 100% bound to get the chemical shifts. Other groups have used a combination of CPMG and CEST to measure chemical shifts of a disordered protein that folds upon binding to its cognate partner – the beauty here was that, when they titrated the cognate partner into a solution of the 15N-labeled IDP, the IDP signals slowly disappeared and never returned. This is because the interaction was weak and could not be saturated, and so the end state chemical shifts of the IDP remained unobservable in the HSQC spectrum. But via CPMG and CEST, they measured chemical shifts of the bound form, from which they could calculate a structural model of the complex ( <https://www.ncbi.nlm.nih.gov/pubmed/28780862> ).

>> I actually wonder, how long does it take to perform one NMR experiment?

Good question! A 1 uM sample would take ~10 hours or longer to get decent signal in a 2D spectrum 😉 so it’s a trade off between signal and time here. For comparison, a 1 mM sample would give a great 2D spectrum in ~10 minutes. In my case, the ACD of HSP27 expresses tremendously well, and I had a stock of ~5 mM 15N-labeled protein from which I prepared 200 uM samples for our titration experiments. I also purified a ton of 2H,15N-labeled material for CPMG experiments (2 x 500 uL of 1.5 mM samples). So protein was not a limiting factor for me at all! Rather, it was the peptide that was only soluble to ~5 mM.

Note that for a highly concentrated sample, the experiment duration roughly goes as: 1D experiments can be recorded in ~ 10 seconds -> for 2D, ~10 minutes -> for 3D, ~10 hours -> and for 4D, ~1 week. The latter two can be ‘sped up’ with a method called non-uniform sampling.

>> Like, is it easy to perform a number of experiments shortly after each other once you have purified the proteins or does each experiment take up a lot of time to optimize concentrations, … ?

And for the 2nd part, yes, you are exactly right – there can be a lot of time spent on optimizing sample conditions (buffer, salt, pH, temperature, protein concentration, other additives, Cys oxidation, etc.) and only for the lucky ones is it a quick process! Since a standard NMR tube requires 500 uL of relatively highly concentrated sample (the higher, the better in most cases, but up to a point), we usually screen on unlabeled samples and use a high-throughput/low sample consumption method to look at stability over a range of conditions. For example, I was using differential scanning fluorimetry to look at melting temperatures for the same protein at the same concentration over various buffers/pH values. We know that Tm reflects the stability of the sample, and we assume that the Tm is a proxy for the quality of the NMR spectrum. One problem that persists is for proteins that have exposed Cys residues and oxidize over time – since we can sometimes be recording for days/weeks on the same sample, any reducing agent that we add to the buffer will (inevitably) go off at some point. So people usually take one of two routes: mutate all surface-exposed Cys residues to Ser/Ala (not ideal), or deal with oxygen by (1) bubbling argon through your final NMR buffer and blow it into the tube to expel oxygen or (2) preparing your sample under anerobic conditions and flame-seal the tube.

After you’ve optimized conditions, then things go pretty quickly. If the protein is inherently unstable (as are some of the GPCRs that ppl look at with NMR), then you have to record quickly after purifying/doing the membrane insertion step. But in most cases, we are working with stable proteins. So I frequently flash-freeze my samples and store them at -80 until use. In Lewis’ lab here in Toronto, they have a fridge with ultra-stable proteins that are ~20 years old and still give spectra as if they were freshly purified…! So it really depends on the stability of the protein and the quality of the purification (trace amounts of protease will chew up a sample, given enough time).

>> Also the PRE experiments are a little difficult for me to understand

In essence, the PRE experiment looks at an interaction between an unpaired electron radical and the nucleus of interest. The jargon of “spin label” refers to the moiety that contains the unpaired electron or paramagnet. The PRE interaction can take place over a very large distance (by NMR standards, that is) of up to 25 or 35 Angstroms, depending on the spin label, and it’s very useful to probe long-range interactions. For comparison, the NOE, which is the workhorse of NMR structure calculations, only reaches ~5 Angstroms in most cases (but it can go up to ~10 Angstroms in highly deuterated proteins).

For PREs, you first have to introduce a spin label into your protein. For this purpose, people usually exploit thiol chemistry to “clip on” a nitroxide spin label (e.g. MTSL <https://en.wikipedia.org/wiki/MTSL> ) to a particular Cys residue. It’s important that you only have one spin label at a time so you can identify from where the PRE effects originate – therefore, people usually have to remove all surface-exposed Cys residues and reintroduce one at a time. After the MTSL spin label is conjugated, people record either 1H relaxation rates or a simple 2D spectrum; in the latter, they see that certain residues have become very weak and/or broadened into the noise. This is the PRE effect – the unpaired radical greatly increases the relaxation rate of a nuclear spin, and this leads to very rapid signal decay. This is why PRE is called as such: Paramagnetic Relaxation Enhancement. The extent of relaxation enhancement depends on a few things, most notably the distance between the nuclear spin and the spin label (closer = faster relaxation). The distance dependence goes as the inverse 6th power of the distance separating the spin label and the nucleus, so anything within ~10 Angstroms of the spin label is completely obliterated. Beyond this “epicenter”, the extent of broadening basically refers to the proximity to the spin label – a very useful reporter! The nice thing with MTSL is that you can simply add ascorbic acid quench the radical, then you have the diagmagnetic sample from which you can record a reference spectrum as a control (no PRE effect but MTSL tag still on). And that is how people obtain their I/I0 graph of intensity in the paramagnetic state (I) divided by the intensity in the diamagnetic state (I0).

As an example, let’s assume here that you are interested in inter-domain contacts between a dumb-bell shaped protein with domains A and B that are separated by a flexible linker. And you want to know how two things: do domains A and B contact each other and, if so, what is the binding interface? To test this, you could put a spin label at various sites in domain A and look for PREs on domain B (and vice versa). And if we assume that domain B+A dock at a specific interface, you would very quickly find this interface with such PRE experiments – you would see large PRE effects at the interfacial residues. Of course, one could also do the deletion experiment: collect the NMR spectrum of the full-length protein and compare to the spectra of the isolated domains A or B. In situations where there is a stable interaction formed the majority of the time, this deletion experiment would work and you would see that the spectra of the isolated domains do not match the full-length protein (since the complex is not longer formed).

However, if the domain-domain interaction is transient and only formed some of the time, the deletion experiment might not show any changes – it would appear as if the two domains do not contact each other, i.e. their isolated spectra would superimpose with the full-length protein. This is where PREs are extremely useful – because the PRE effect is so large, it “lights up” regions that may come in close spatial proximity, even when this is 1% of the time. And so, if the minor state is the domain-docked conformation and it exchanges with the 99% undocked form, then one would see this in the PRE measurements. For example, the Clore group at NIH used PREs to show evidence of ‘encounter complexes’ involved in protein-protein interactions (C Tang, GM Clore 2006 Nature) as well as ‘scanning’ by a transcription factor along DNA, even though the population of such states were very low ( see Figure 1 -- <https://www.nature.com/articles/nature04673.pdf> ).

For inter-molecular interactions studied by PREs, people will usually put the spin label on the non-isotope labeled sample, and then mix this with a 15N-labeled protein. They can then ‘read out’ the PREs in the 15N-labeled protein that manifest from the non-isotope/spin-labeled sample. i.e. 15N-protein + 14N-protein-MTSL.

PMID: 30975750

Hi Iva and Reid,

I have again a question about NMR… The figure above is from a recent paper of Lakomek et al (PMID: 30975750). Can you briefly explain what we can see in the four panels? What kind of experiments are these? It’s a bit difficult to understand from the paper…

Hope you are enjoying your holidays!! Have a great time in Croatia. I am planning to book an accommodation on the weekend.

Best, Carla.

From a cursory read of the NMR section in the paper, it seems their construct syb2(1-96) is an IDP with some alpha-helical tendencies. This prompted them to measure nuclear spin relaxation rates to characterize the dynamics of the sample in more detail. If it were a fully disordered random coil, the relaxation rates would more or less be homogeneous, except for the termini (e.g. alpha-synuclein). Deviations from homogeneity would imply interesting dynamics.

The rates they measured are (a) 15N R1 which is the longitudinal relaxation rate (15N magnetization on Z-axis relaxing to equilibrium) which is sensitive to rapid ps-ns motions and also the size of the protein. As globular  proteins increase in size, so do their R1 rates. But for IDPs that can otherwise have very rapid and homogeneous ps-ns motions, a locally increased R1 rate (outside of the termini) can signify partially structured regions -- bc this region would be tumbling slower than the faster motions in the IDP.

(C) is the heteronuclear NOE which is extremely sensitive to ps-ns motions. Lower values mean more rapid motions. Thats about it -- hetNOEs are not so sensitive to the size of the protein. A typical value for stable secondary structure at 600 MHz is 0.8 (N.B. the value of the hetNOE scales with spectrometer frequency). For IDPs the hetNOE values are typically below 0.3 and go negative.

(B) 15N R2 which is the transverse relaxation rate. R2 rates, like R1 rates, are highly sensitive to both ps-ns motions as well as the size of the protein. HOWEVER, unlike R1 rates, R2 rates are extremely sensitive to us-ms motions caused by conformational exchange between multiple states. So when ppl measure R2 rates, the measured value can be "contaminated" with additional factors due to conformational exchange, and therefore the measured R2 rate is higher than expected. So ppl will either use R2 measurements to quantify the us-ms motions (R2 + exchange) or measure the "pure" R2 rate that is free of exchange contributions and is therefore only sensitive to ps-ns motions and size of the protein (R2 only).

The experiment used here is a R1rho experiment, which applies a so-called "spin lock field" while the magnetization is in the transverse plane and while the relaxation occurs. This is important bc the spin-lock is applied here at a field of 2kHz, and this suppresses motions that are slower than 2000 s-1. The R1rho rates can be converted to R2 using a simple equation. So the values in (B) are the "pure" R2 with exchange contributions slower than 2000 s-1 suppressed.

The other R2 rates (R2beta) are a measurement of a particular spin state referred to as the TROSY component. This spin state has more favorable transverse relaxation properties -- bc two components of the relaxation process destructively cancel each other --  and so it relaxes more slowly (lower R2 rates). This is why you see TROSY-HSQC spectra recorded on large proteins, it's necessary to have this slowly relaxing state in order to record data.

The experiment used in (D) DOES NOT suppress any conformational exchange processes, and therefore the measured R2 contains contributions from exchange (R2 + exchange). As the magnetic field strength is increased (here from 600 to 800 MHz), the TROSY effect becomes more pronounced and the measured relaxation rates will become slower. ( I think TROSY on 15N has a minimum R2 rate at 1GHz). But  conformational exchange on the us-ms timescale increases the R2+exchange rates as the magnetic field strength is increased. So there are two competing aspects -- the natural tendency of the TROSY component to have smaller R2 rates at 800 MHz vs 600 MHz, and the effect of exchange that increases R2 rates at 800 MHz vs 600 MHz.

They plot the difference in R2+exchange at 800 vs 600 in panel d. Negative values are expected, and positive values indicate regions with us-ms motions.

Hopefully this makes sense... Let me know whatever is unclear!