

# Bigger is better: megadalton protein NMR in solution

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**NMR spectra recorded on the 900 kDa GroEL–GroES complex substantially raise the bar for the size of macromolecules that can be studied by NMR techniques.**

In recent years a number of techniques have been developed that have dramatically increased the size of macromolecular systems that can be studied using NMR. In a recent issue of *Nature*, Fiaux *et al.*<sup>1</sup> describe a collaboration between the Horwich and Wüthrich laboratories that has raised this molecular weight limit to a dizzying new height. They report high resolution spectra of the backbone amide resonances of the co-chaperonin GroES bound to the chaperonin GroEL in solution. This complex has a molecular mass of ~900,000 Daltons!

The problem one faces when using NMR to look at large molecules in solution is that NMR peaks become broader as molecular size increases. This occurs because the rotational motion of large molecules is slow and less able to completely average the range of energies a given nucleus experiences as the molecules rotate by diffusion through different orientations with respect to the applied magnetic field. The breakdown of the averaging process has two consequences: (i) Nuclear spins corresponding to the same moiety (for example, the amide proton of residue 11) in different molecules experience a range of average energies; as a result, the linewidth of each peak increases. (ii) The heterogeneity of the different averages provides efficient magnetization relaxation pathways; thus the rate of transverse relaxation ( $T_2$ ) also increases. This broadening of the peaks decreases resolution and the corresponding increase in  $T_2$  relaxation rates eventually destroys the desired signal in conventional multi-dimensional NMR experiments.

One approach to reducing the effect of slow rotational motion on linewidth is to narrow the range of energies that a nuclear spin experiences as it reorients with respect to the applied magnetic field. For spin 1/2 nuclei (such as  $^1\text{H}$ ,  $^{15}\text{N}$  or  $^{13}\text{C}$ ) in diamagnetic systems, the orientation-dependent energies that a nuclear spin experiences are dominated by chemical shift anisotropy (CSA) and dipolar cou-

pling. CSA arises from the interaction of the bonding electrons near the nuclear spin with the applied magnetic field. For example, the shift of an amide proton is different if the N-H bond is pointed in the direction of the applied field or perpendicular to it. Similarly, the interaction of two magnetic dipoles is different if the vector joining the dipoles is oriented parallel or perpendicular to the applied field. A simple way to reduce the range of energies is to replace most of the  $^1\text{H}$  atoms in the system by deuterium atoms. Since the dipolar coupling between a  $^1\text{H}$  and a deuteron is much weaker than the coupling between two  $^1\text{H}$  spins, deuteration reduces the dipolar coupling component and therefore results in considerable sharpening of the remaining proton resonances. Perdeuteration can be accomplished by expressing the protein of interest on fully deuterated precursors and then to back exchange in  $^1\text{H}$ 's at the exchangeable amide positions. Similar effects can also be generated using partial deuteration so that individual  $^1\text{H}$  spins are likely surrounded by deuterium spins<sup>2</sup>. Deuteration alone has increased the size of molecules that can be studied using conventional techniques to ~50 kDa.

Even when nearby  $^1\text{H}$  spins have been removed, there are still strong interactions between the desired  $^1\text{H}$  spins and their directly bonded  $^{15}\text{N}$  or  $^{13}\text{C}$  spin. Consider an isolated spin pair such as the  $^{15}\text{N}$  and  $^1\text{H}$  in a peptide amide in proteins. The  $^1\text{H}$  and  $^{15}\text{N}$  resonance will each be split into two components by the scalar (orientation independent) spin–spin coupling between the nuclei. The energies of each component of the  $^{15}\text{N}$  and  $^1\text{H}$  doublets will vary as the molecule randomly rotates due to the CSA and the dipolar coupling between the  $^{15}\text{N}$  and  $^1\text{H}$ . For one member of each doublet the dipolar coupling adds to the CSA contribution, while for the other member of the doublet it subtracts from the CSA contribution. Thus, one component of each doublet experiences a smaller range of orientation-dependent

energies than the other component. After averaging by rotational diffusion, one component of the doublet has a much narrower range of energies to average as the molecule rotates, resulting in a much sharper averaged resonance and longer  $T_2$  relaxation time. As a result, each  $^{15}\text{N}$ – $^1\text{H}$  pair generates four resonances in a two-dimensional  $^{15}\text{N}$ – $^1\text{H}$  spectrum in the absence of spin–spin decoupling, one of which has narrow linewidths in both the  $^1\text{H}$  and  $^{15}\text{N}$  dimensions; another component is broad in both dimensions; and two have mixtures of broad and sharp components. In the traditional methods for observing  $^{15}\text{N}$ – $^1\text{H}$  two-dimensional spectra, decoupling schemes cause these four components to be averaged together. For smaller proteins (<30 kDa) the collapse of this fine structure to a single observable resonance is desirable because the broader components are still useful, but combining the sharp and broad components becomes a very serious problem for larger systems.

Since energies produced by the CSA are dependent on the applied magnetic field, whereas the dipolar coupling is field independent, the range of energies experienced by the narrower component can be tuned to a minimum at the optimal applied field, corresponding to 800–1000 MHz resonance frequency for protons. With the advent of high field instruments, the Wüthrich laboratory and others began developing techniques that exploit the fine structure for application to large macromolecular systems. The Wüthrich laboratory published the TROSY (transverse relaxation optimized spectroscopy) pulse sequence<sup>3</sup> in 1997 that selectively detects the sharp components without averaging them with the broad components. Since then, the combination of deuteration and TROSY detection has become a mainstay for the examination of larger proteins. They were able to get high quality spectra and resonance assignments for a 110 kDa protein — the 7,8-dihydroneopterin aldolase from *Staphylococcus aureus*,

which contains eight identical subunits — using TROSY-optimized versions of the triple resonance methods commonly used for resonance assignment in smaller systems<sup>4</sup>. However, the original TROSY approach needed modification to be applicable to even larger systems. For example, in the GroES–GroEL complex, only relatively disordered residues with fast internal motions could be detected by conventional TROSY<sup>1</sup>.

For <sup>1</sup>H–<sup>15</sup>N (or <sup>13</sup>C) correlation experiments to function there must be coherence transfer between the <sup>1</sup>H and <sup>15</sup>N (<sup>13</sup>C) spins. The original TROSY experiments used the same method to transfer coherence (INEPT)<sup>5</sup> between <sup>1</sup>H spins and <sup>15</sup>N as the conventional NMR approaches. During INEPT, the narrow and broad components are averaged together. In addition INEPT process takes a relatively long time (~5 milliseconds), and substantial signal may be lost to relaxation during this period. To overcome this problem, they incorporated a method for coherence transfer (CRIPT)<sup>6</sup> that exploits cross-correlated cross relaxation between the <sup>1</sup>H and <sup>15</sup>N spins. This process is most efficient for large proteins (>200 kDa) and relies on the rapid decay of one member of the <sup>1</sup>H doublet in an <sup>1</sup>H–<sup>15</sup>N pair. The resulting CRIPT-TROSY<sup>7</sup> version does not directly select for the sharpest of the four components, but in very large systems usually only the sharpest component is observed because the others are lost to relaxation. If internal motions are present, some or all of the four components may be detected. The Wüthrich group also developed CRINEPT-TROSY<sup>8</sup>, an experiment that detects both rigid and mobile components in GroES–GroEL. The different experiments have different sensitivities to internal motions in large systems so by using them all, it is possible to get a qualitative picture of the dynamics that characterize the system.

In their work on the GroES–GroEL interaction, Fiaux *et al.*<sup>1</sup> used the now conventional triple resonance TROSY methods to assign the <sup>1</sup>H–<sup>15</sup>N spectrum of free

GroES, a 72 kDa heptamer of identical subunits. They then examined the effects on GroES upon binding to either the 800 kDa (14 identical subunits) wild type GroEL or a 400 kDa (7 identical subunits) single ring version of GroEL (SR1). In the presence of ADP, one GroES heptamer binds to either the intact GroEL or to SR1. In both complexes they were able to observe essentially all of the GroES amide protons even when GroEL was not deuterium labeled. The majority of GroES resonances do not appear to undergo chemical shift changes upon binding to GroEL; these peaks most likely reflect residues that are not part of the binding interface. Fiaux *et al.*<sup>1</sup> observe a smaller number of resonances that change chemical shift, corresponding to residues 17–32, or the so-called ‘mobile loop’<sup>9,10</sup>. These residues have random coil chemical shifts in free GroES and high temperature factors in the crystal structure of GroES bound to GroEL<sup>11</sup>. These observations are consistent with previous arguments from the crystal structure of the complex, that the mobile loop provides most or all of the binding interactions with GroEL<sup>11</sup>. Some of the shifted residues are detected in <sup>15</sup>N–<sup>1</sup>H TROSY spectra so they appear to retain some mobility in the complex while other shifted residues seem rigidly bound in the complex. The implication being that while the entire ‘mobile loop’ gains structure as evidenced by chemical shift dispersion, one region is rigidly held in the GroEL–GroES complex while another still exhibits substantial mobility with respect to the complex. While we have been provided with a tantalizing glimpse of the dynamic behavior of this loop, a comprehensive structural and dynamic analysis awaits assignment of the shifted residues.

What are the prospects for ‘routine’ NMR analysis of large systems? Because of their large molecular mass, large proteins have low molar solubility and correspondingly poor signal intensity. The seven-fold symmetry of GroES is critical to overcoming this problem and provides an essential seven-fold increase in signal

intensity. The symmetry also reduces spectral overlap, therefore simplifying analysis. There is reason to be optimistic about improving sensitivity so that systems with lower symmetry can be approached. The GroES–GroEL experiments were performed at 750 MHz and it is likely that sensitivity will increase at higher fields. In addition, the cooled pre-amplifier electronics that are available at lower fields will dramatically improve sensitivity when they become available for high field instruments. To date, the 723-amino acid malate synthase G is the largest single chain protein that has been assigned using 3D and 4D TROSY related methods<sup>12</sup>. This size polypeptide chain is larger than the subunits of many of the most interesting and important large protein complexes. Thus the prospects are excellent for detailed analysis of many large systems in the next few years.

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