NMR Spectroscopy

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Approaching the Megadalton: NMR Spectroscopy of Protein Complexes

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One decade ago the upper size limit for biomolecular NMR spectroscopy was thought to be on the order of 30 kDa, but this limit has been extended step by step since then by a number of innovations. Recently, Sprangers and Kay reported quantitative NMR-relaxation studies of the 670-kDa 20S proteasome core particle and identified its binding site in the 1.1-MDa 11S activator–proteasome complex.[1] The results not only represent the largest molecular complex ever studied by high-resolution NMR spectroscopy, but also give insight into the function of the central molecular machine for the removal of damaged and misfolded proteins from the cell: a cluster of flexible residues with fast motion can be found outside the barrel between individual α subunits, and residues undergoing slow concerted motion can be found inside the α_7 ring, which most likely facilitates the transport of proteins into the center of the complex (Figure 1).[1] This work was made possible by the application of a number of recently developed techniques, which are described briefly herein.

Besides the steady improvement of NMR spectrometers, four major achievements build the basis for residue-specific measurements on very large complexes: 1) the use of methyl-TROSY pulse-sequence techniques, 2) the corresponding isotope-labeling schemes, 3) a stepwise strategy for methyl-group assignment, and 4) reliable methods for measuring relaxation for the identification of motion.

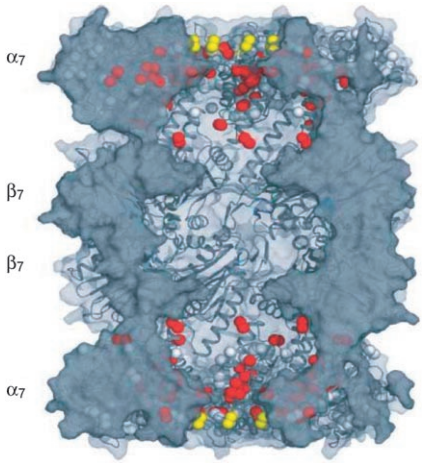

Figure 1. Structure of the 20S proteasome core particle. Methyl groups undergoing slow motions are represented in red, highly flexible gate-keeping N-terminal methyl groups are colored in yellow.

The central technique that enabled the study of larger molecular systems was developed by Pervushin et al. and is called transverse relaxation optimized spectroscopy (TROSY).[2] TROSY is based on the effect of cross-correlated relaxation, which, for certain spin arrangements, leads to partial cancellation of relaxation and therefore to sharpened signals. In this way not only the crucial signal-intensity problem for very large molecules can be overcome, but also the problem of signal overlap, which usually prevents the interpretation of spectra. TROSY was originally developed for the detection of amide groups in proteins[2] and has already led to the identification of conformational changes of the free chaperone GroES upon binding in the 870-kDa complex with GroEL.[3] Several pulse sequences based on the TROSY principle have been developed in the meantime with even further enhanced sensitivity, for example, CRIPT,[4] CRINEPT,[5] CROP,[6] and PC-SPI.[7]

The Kay group further extended the technique to the so-called methyl-TROSY, in which the favorable relaxation properties of methyl groups are used in HMQC-type experiments.[8] The signal intensities of the three degenerate methyl protons are, of course, three times the intensities of amide groups, and their fast rotation in conjunction with cross-correlated relaxation effects of their directly attached carbon spins yielded sufficiently narrow lines for decent spectra of the 670-kDa proteasome complex to be obtained within 90 minutes (Figure 2a).[1]

The success of methyl-TROSY, however, is directly related to highly specialized isotope-labeling schemes.[9] In general, with the exception of selected non-deuterated and ^{13}C-labeled methyl groups, all samples of the proteasome complex were fully deuterated. Depending on the purpose of a specific experiment, the side chains of leucine, isoleucine, and valine were either additionally ^{13}C-labeled or left in their natural isotopic abundance (Figure 2b). Also, for distance measurements with NOE, either the two methyl groups of the three hydrophobic amino acids or only single methyl groups were ^{13}C-labeled and non-deuterated. For deuterium-relaxation studies, residues with single $^{13}CHD_2$

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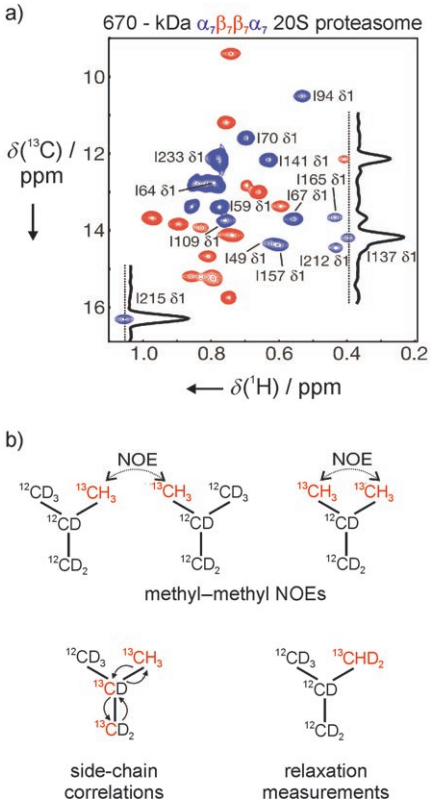

Figure 2. a) HMQC-based methyl-TROSY experiments result in spectra of good quality for the 20S proteasome core particle (cross peaks for the α subunit in blue and for the β subunit in red). b) Highly specializsed isotope-labeling schemes for methyl-containing hydrophobic residues such as leucine for ^{13}C–^{13}C correlation experiments, intra- and interresidue NOESY, and relaxation measurements.

groups were incorporated. In all cases, the correspondingly labeled α-ketobutyrate (for Ile) and α-ketoisovalerate (for Leu, Val) were used as precursors for feeding *E. coli* in deuterated growth media. The resulting high degree of deuteration strongly reduces the dipolar relaxation pathways, which is essential for NMR measurements in very large molecules. It not only provides narrow line widths, but, as spin diffusion between methyl groups as the only proton source is avoided, NOE-derived methyl–methyl distances up to 8 Å can also be measured. Such optimized labeling schemes with a very high degree of deuteration are not limited to hydrophobic amino acids, as has been shown recently by Kainosho et al. with the so-called stereoarray isotope labeling (SAIL) strategy.[10]

But even with measurable methyl signals and the ability to derive distances between them, the assignment of cross peaks to specific residues is a challenging problem. Three prerequisites laid the basis of the assignment process for the 670-kDa proteasome complex: the high symmetry of the complex, which allowed the breakdown into significantly smaller subunits, the availability of a crystal structure of the very same complex,[11] and the use of single-site mutations to identify specific residues.

The 20S proteasome core particle contains 14 identical α and β subunits with a heptameric symmetry and is referred to as $\alpha_7\beta_7\beta_7\alpha_7$. This complex can be dissected into the 360-kDa $\alpha_7\alpha_7$ double ring, which forms spontaneously in the absence of β subunits. By introducing mutations, monomeric α subunits with a molecular weight of 21 kDa were obtained, for which a high-resolution structure could be determined from standard NMR triple-resonance experiments. Having the assignment of the methyl groups in the monomeric α subunit and the expected distances in the complex derived from the crystal structure, Sprangers and Kay were able to transfer the assignment first to the $\alpha_7\alpha_7$ complex and then to the $\alpha_7\beta_7\beta_7\alpha_7$ complex. Isolated methyl groups with no measurable NOE contacts were assigned in a final step by point mutations of the specific residues. In this way, 89 % of the nearly 100 methyl groups could be unambiguously identified.

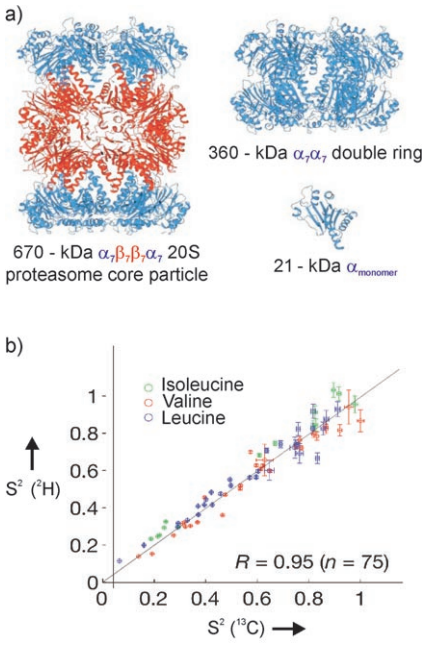

Figure 3. a) The assignment of the core particle could only be obtained by breaking down the 670-kDa complex into subunits that allowed the unambiguous identification of methyl groups. b) The high correlation of order parameters S^2 obtained from ^{13}C- and deuterium-relaxation measurements demonstrates the reliability of the acquired dynamic data.

The assignment of the methyl groups enables determination of the very fast (in the picosecond range) and relatively slow (in the millisecond range) dynamic nature of the complex through relaxation measurements. ^{13}C- and deuterium-relaxation rates, which are sources for identifying characteristic fast motions, can be derived from experiments developed by the Kay group.[12] The good correlation between the two independently derived order parameters S^2 remarkably demonstrates how accurately such motions can be determined (Figure 3). Interestingly, although residues with high mobility show also increased temperature factors in crystallographic data, there is very little correlation between the B factors and corresponding S^2 values.[1] Relaxation-dispersion experiments for the measurement of ^1H,^{13}C multiple quantum dispersion profiles[13] finally allowed slow motions to be identified; these data were fitted to a single two-site exchange model, which suggests a single global process (Figure 1).

With the NMR techniques developed over the past decade and the availability of corresponding crystal structures, molecular complexes of nearly unlimited size seem to be amenable to liquid-state dynamics measurements. These results are an important step in understanding the modes of operation of complicated molecular machines in biological systems.

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