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Utilizing NMR to Study the Structure of Growth-Inhibitory Proteins

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1. Introduction

The underlying premise of structural biology is that the fundamental understanding of biologic functions lies in the three-dimensional structures of proteins and other biopolymers. The two well-established experimental methods for determining the high-resolution structures of proteins have both contributed to the wealth of structural information available for the tumor suppressor genes. The tumor suppressor proteins whose structures have been determined by nuclear magnetic resonance (NMR) spectroscopy are listed in Table 1. Although X-ray crystallography plays a central role in high-throughput structure determination in the current structural genomics efforts, several features of NMR spectroscopy make it extremely well suited for three-dimensional structure determination as well as for the structure–function analysis of proteins (1,2).

An important advantage of NMR spectroscopy is that it does not require crystals for structure determination, so that NMR structural studies can be carried out in samples that are similar to physiologic conditions in which the protein is normally functional. Indeed, NMR spectroscopy can be applied to a wide variety of samples, ranging from isotropic solutions to crystalline powders, including those with slowly reorienting or immobile macromolecules, such as membrane proteins in lipid environments (3,4). This is especially significant because many proteins are insoluble and do not provide crystals of suitable quality for crystallographic analysis.

NMR is capable of resolving signals from all atomic sites in proteins, and each site has several well-characterized nuclear spin interactions that can be used as sources of information about molecular structure and dynamics, as well as chemical interactions. The spin interactions can be probed through radiofrequency irradiations and sample manipulations, and provide an immediate characterization of the foldedness of proteins in solution, even prior to complete three-dimensional structure determination. They also provide the basis for a structure-guided approach to the design and optimization of high-affinity ligands and to screen libraries of potential drugs (5,6).

1.1. NMR of Soluble Proteins

Solution NMR methods rely on rapid molecular reorientation for line narrowing, and standard multidimensional solution NMR methods can be successfully applied to proteins in the size range of 25–35 kDa (7). A recent analysis estimates that at least 25% of open reading frames in a genome will be suitable for NMR structure determination, and that 15–20% of new protein structures are determined by NMR methods (1). The recent advances of high-field-magnet technology, cryogenic probes, partial sample deuteration (8), and transverse relaxation optimized spectroscopy (TROSY) (9) all increase the sensitivity of the NMR experiments and extend the size limit of proteins that can have their structures determined by solution NMR to 50 kDa. Although large multidomain proteins are not generally suitable for structure determination by solution NMR spectroscopy, these also tend to exhibit flexibility in the domain linker regions, which can impede crystallization. As a

result, the majority of structural information for these systems, including many tumor suppressor proteins and their complexes, comes from studies of their individual domains using both NMR and X-ray.

1.2. NMR of Membrane Proteins

NMR spectroscopy can also be extended to the study of membrane proteins, which do not easily yield high quality crystals, and thus pose a considerable problem for crystallographic analysis (3,4). Solution NMR methods can be successfully applied to relatively small membrane proteins in micelles, although in this case the size limitation is substantially more severe because the many lipid molecules associated with each protein slow its overall reorientation rate (4). Using currently available instruments and methods, it is difficult to resolve, assign, and measure the long-range Nuclear Overhauser Effects (NOEs) between hydrogens on hydrophobic side chains that are needed to determine tertiary structures based on distance constraints. However, the ability to weakly align membrane proteins in micelles enables the measurement of residual dipolar couplings, and improves the feasibility of determining the structures of membrane proteins using solution NMR methods (10,11).

Nonetheless, it is highly desirable to determine the structures of membrane proteins in the definitive environment of lipid bilayer membranes, where solution NMR methods fail completely. Solid-state NMR spectroscopy is well suited for this task, with both oriented-sample and magic-angle spinning methods providing approaches to measure orientational and distance parameters for structure determination (3,4). Several tumor suppressor genes encode membrane-bound proteins, for example, the deleted in colon cancer (DCC) and the neurofibromatosis type 2 (NF-2) genes, and solid-state NMR provides an important approach toward their structure determination.

2. NMR of Proteins in Solution

The determination of protein structures by multidimensional solution NMR spectroscopy is straightforward in principle, and for globular proteins that are soluble and do not aggregate in aqueous solution, the application of this approach is generally straightforward in practice as well, especially if uniformly ^{15}N - and/or uniformly ^{15}N - and ^{13}C -labeled samples can be prepared by expression in bacteria (12,13). The strategy for protein structure determination by NMR spectroscopy is outlined in Fig. 1 and described below.

2.1. Expression of Isotopically Labeled Proteins

The development of expression systems for the production of isotopically labeled proteins is as important as that of pulse sequences or instrumentation for the success of NMR structural studies. The expression of isotopically labeled proteins can be obtained in several organisms including bacteria, insect, yeast or human cells, and in cell-free expression systems (14), however the most commonly used expression strategy is bacterial expression via an inducible T7 RNA polymerase promoter (4,15,16).

Several *Escherichia coli* expression systems are available, all of which involve the use of fusion proteins. The incorporation of engineered affinity tags, such as poly-His tags for metal affinity chromatography, is often used to simplify protein isolation and purification. This process can be further facilitated by selecting fusion partners that form inclusion bodies. After inclusion body isolation, and fusion protein affinity purification and cleavage, the resulting target protein is purified and then dissolved in the appropriate buffer for NMR studies.

The ability to express proteins in bacteria provides the opportunity to incorporate a variety of isotopic labeling schemes into the overall experimental strategy, since it allows both

selective and uniform labeling. For selective labeling by amino acid type, the bacteria harboring the protein gene are grown on defined media, where only the amino acid of interest is labeled and the others are not. Uniform labeling, where all the nuclei of one or several types (^{15}N , ^{13}C , ^2H) are incorporated in the protein, is accomplished by growing the bacteria on defined media containing ^{15}N -labeled ammonium sulfate, or ^{13}C -labeled glucose, or D_2O , or a combination of these. The availability of uniformly labeled samples is a prerequisite for triple-resonance $^{13}\text{C}/^{15}\text{N}/^1\text{H}$ spectroscopy, which is essential for the structure determination of larger proteins and protein complexes in solution.

2.2. Protein Sample Preparation

The primary goal in NMR sample preparation is to reduce the effective rotational correlation time of the protein as much as possible, so that resonances will have the narrowest achievable line widths. Careful handling of the protein throughout the purification is essential, since subtle changes in the protocol can have a significant impact on the quality of the resulting spectra. It is essential to optimize protein concentrations, counterions, pH, and temperature, in order to obtain well-resolved two-dimensional heteronuclear correlation NMR spectra with narrow ^1H and ^{15}N resonance line width. Narrow line widths in both frequency dimensions, and the presence of one well-defined resonance for each amide site in the protein, reflect a high-quality sample (4,16). As the protein size increases, solubilization generally becomes more difficult and aggregation more likely.

2.3. Protein Structure Determination

2.3.1. Resolution and Assignment of Backbone and Side-Chain Resonances—

The resolution and assignment of backbone and side-chain resonances are based on both through-bond and through-space spin interactions, and are observed in two- and three-dimensional NMR spectra. There are basically two strategies for assigning resolved resonances to specific residues in a protein. One involves short-range homonuclear $^1\text{H}/^1\text{H}$ NOEs (12,13), and the other relies on spin-spin couplings in uniformly ^{15}N - and ^{13}C -labeled proteins (17–19). The procedure starts with heteronuclear edited TOCSY experiments, supplemented with triple-resonance $^{13}\text{C}/^{15}\text{N}/^1\text{H}$ experiments. Selective isotopic labeling may be necessary in order to resolve and assign some of the resonances, especially in cases of limited chemical shift dispersion. Further, the incorporation of ^2H is often needed in studies of larger proteins or protein complexes, in order to limit spin diffusion and line broadening.

2.3.2. Measurement of Structural Constraints—The measurements of as many homonuclear $^1\text{H}/^1\text{H}$ NOEs as possible among the assigned resonances provide the short-range and long-range distance constraints required for structure determination. The cross-peaks between pairs of ^1H nuclei in the protein structure are grouped into three classes of strong, medium and weak intensity, corresponding to interhydrogen distances of 1.9–2.5 Å, 1.9–3.5 Å, and 3.0–5.0 Å, respectively. These are supplemented by other structural constraints, such as spin-spin coupling constants and chemical shifts, in order to assign resonances, obtain torsion angle and H-bond constraints, and to characterize the secondary structure of the protein. The $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ chemical shifts are particularly useful for characterizing secondary structure in the early stages of structure determination (20,21). The amide resonances detected in a two-dimensional $^1\text{H}/^{15}\text{N}$ correlation spectrum at different times after the addition of D_2O to the sample can be used to assign hydrogen bond constraints.

The measurements of residual dipolar couplings from weakly aligned protein samples provide direct long-range angular constraints with respect to a molecule-fixed reference frame, which can be used for structure determination (22,23). Aqueous solutions containing

bicelles (24), purple membrane fragments (25), or rod-shaped viruses (26,27) have all been successfully employed to obtain residual couplings in soluble proteins and other macromolecules, although these media can also complicate studies of large proteins and complexes, since the increased solvent viscosity leads to reorientation rates that are too slow to give adequately resolved spectra. In addition, lanthanide ions can be used to weakly align membrane proteins in lipid micelles (10,11).

2.3.3. Structure Calculation and Refinement—Structure determination involves the interpretation of the distance and angular constraints in terms of secondary and tertiary protein structure. This is achieved through a combination of distance geometry, simulated annealing, molecular dynamics, and other calculations, and yields a family of energy-minimized, three-dimensional protein structures (13). This final stage of the structure determination procedure requires essentially complete assignment of the protein resonances. The lack of a significant number of unambiguously assigned long-range NOEs has limited the ability of solution NMR spectroscopy to determine the tertiary structures of larger proteins, protein complexes, and membrane proteins. Fortunately, the measurement of residual dipolar couplings from weakly aligned protein samples offers an additional set of constraints for structure determination. These couplings can be used to overcome limitations resulting from having few long-range NOE distance restraints. Structures are calculated by inclusion of all available distance and orientational constraints (28,29).

3. NMR Structural Studies of Tumor Suppressor Proteins

3.1. Structure of the p53 Tumor Suppressor

The p53 tumor suppressor protein is a 393-residue transcription factor that activates genes involved in the control of the cell cycle and apoptosis, in response to DNA damage (30). Because over one-half of all human cancers involve mutations or deletions of p53, this molecule has been the subject of several structural studies aimed at understanding the differences between the wild-type and mutant molecule (31). The full-length protein comprises an acidic trans-activation domain (residues 1–70), a DNA-binding domain (residues 90–300), a homo-tetramerization domain (residues 324–355), and basic regulatory domain (residues 355–393). The structures of several domains of p53 have been determined by NMR and/or X-ray crystallography. Recently, the NMR spectrum of a 67-kDa dimer of p53, comprising the DNA-binding and oligomerization domains, has been assigned for structure determination (32). This was possible through the use of triple resonance and TROSY spectroscopy of ^{15}N -, ^{13}C - and ^2H -labeled protein.

Structures of the DNA-binding domain in complex with target DNA and with p53-binding protein 2 (33,34) have been determined by X-ray crystallography. The structure of the trans-activation domain complexed with the MDM2 oncoprotein (35) was determined by X-ray crystallography, and multidimensional NMR spectroscopy was utilized to identify chalcone derivative MDM2 inhibitors that bind to a subsite of the p53 tumor suppressor-binding cleft of human MDM2 (36). Solution NMR spectroscopy was utilized to compare the structure of the p53 DNA-binding domain in wild-type and mutant p53, and monitor the structural changes introduced by hot-spot mutations. By following changes in chemical shifts, the mutation R248Q, which was believed to affect only interactions with DNA, was shown to introduce structural changes that perturb the structure of the p53 DNA-binding domain (37).

The structure of the tetramerization domain has been determined by both NMR spectroscopy (38–40) and crystallography (41,42). The tetramerization domain is required for tumor suppressor activity (43), and since it is only 30 residues long and its function can be easily assayed, it is well suited for structural studies. Its solution structure, shown in Fig. 2, consists of a dimer of two primary dimers, with a well-defined globular hydrophobic core, whose

subunits form a β -strand, followed by a tight turn and an α -helix. NMR studies demonstrate that conservative hydrophobic amino acid mutations influence the helix packing and disrupt tetramerization of the p53 complex (44).

Recently, two new p53 homologs, p63 and p73, have been identified (reviewed in **ref.** 31). The high level of sequence identity in critical functional regions of the p53, p63, and p73 molecules suggests that the three-dimensional structures of their respective domains will be very similar. In addition, the new family members have a conserved C-terminal domain with a predicted regulatory function. The solution structure of this domain has been determined by NMR spectroscopy and is shown in Fig. 3 (31). It forms a 5-helix bundle similar to those of sterile α -motif (SAM) domains from Ephrin tyro-sine kinases, suggesting that it is a protein-protein interaction module, possibly involved in developmental processes.

Finally, the structure of the Ca^{2+} signaling protein S100B in complex with p53 has been determined using NMR spectroscopy (45,46). Upon Ca^{2+} binding to its EF hand, S100B undergoes a large conformational change that is a prerequisite for its interaction with p53 (47,48). This, in turn, inhibits protein kinase C-dependent phosphorylation of p53 at residues Ser376 and Thr377 in its C-terminal regulatory domain, and provides a mechanism for regulating the cellular functions of the tumor suppressor. S100B inhibits p53 tetramerization, and promotes dissociation of the p53 tetramer (49). In addition, it has been shown to protect p53 from thermal denaturation and aggregation in vitro. The solution structure shows that the S100B homo-dimer recognizes two molecules of p53 and inhibits its posttranslational modification.

3.2. Structures of the Tumor Suppressors INK4

The cyclin-dependent kinase (CDK) inhibitors bind to CDKs and inhibit their kinase activity, thus regulating some of the most fundamental decisions in the cell cycle. The INK4 (inhibitor of cyclin-dependent kinase 4) family consists of four tumor suppressor proteins, p15, p16, p18, and p19, ranging in size from 13.7 to 18 kDa (50–53). Among these, mutations in p16 have been tied to the development of cancer, and the tumor suppressor function is well established for p16 and to a lesser extent for p15. Three-dimensional structures of the INK4 proteins have been determined using both X-ray crystallography and NMR spectroscopy, with the following structures reported in recent years: the solution (54) and crystal (55) structures of p19; the solution (56) and crystal (57) structures of p18; the solution structure of p16 (58,59); and the solution structure of p15 (59).

All the INK4 family members are highly homologous in sequences and structures, and fold as ankyrin repeats, arrays of four (p15, p16) or five (p18, p19) 33-residue helix–turn–helix motifs connected by long loops, as shown in Fig. 4. Despite their considerable homology, they also show appreciable differences in conformational flexibility, stability, and aggregation tendency. Because the smaller INK4 proteins, p15 and p16, display the highest degree of conformational flexibility and instability, no crystal structures have been reported for their free forms. However, their NMR structures could be determined in solution, and were refined at high resolution through the use of high-field spectroscopy at 800 MHz (59).

3.3. Structural Studies of the Wilms Tumor Suppressor Protein

NMR spectroscopy has been used to study the structural changes resulting from post-transcriptional modification of the Wilms tumor suppressor protein (WT1) in the 4-zinc finger DNA-binding domain (60). WT1 is a transcription factor that contains a C-terminal DNA-binding domain with four Cys2His2 zinc fingers, a Pro/Glu-rich N-terminus, an activation and a repressor domain, nuclear localization signals, and self-association domains. Its function is modulated by a posttranscriptional modification that adds three amino acids

into one of the linker regions between the DNA-binding zinc fingers. NMR resonance assignments and chemical shift changes were used to characterize the structural differences between two isoforms of the WT1 DNA-binding domain, with a (Lys-Thr-Ser) sequence insertion and without it. These studies were carried out both with WT1 free in solution and in complex with a 14-base DNA duplex corresponding to the WT1 recognition element. In the absence of the DNA, the two isoforms are nearly identical in structure; however, the linker regions become more structured upon DNA binding, and insertion of the Lys-Thr-Ser sequence disrupts important interactions of the linker region with the adjacent zinc fingers, thus lowering the stability of the complex with DNA (60). Using NMR, it was also shown that DNA binding induces a conformational change and helix capping in the conserved zinc finger-linker region of WT1 (61).

3.4. Binding of Elongin C to a von Hippel–Lindau Tumor Suppressor Peptide

NMR spectroscopy was used to study the structural basis for the interaction of Elongin A, an F-box-containing protein, with Elongin C, a SKP1 homolog, and the modulation of this interaction by the tumor suppressor von Hippel-Lindau protein (VHL) (62). Elongin is a hetero-trimeric transcription elongation factor composed of subunits A, B, and C in mammals. Complexes of elongin C with elongin A and with a peptide from the VHL tumor suppressor were analyzed by NMR. Elongin C was shown to oligomerize in solution and to undergo significant structural rearrangements upon binding of its two partner proteins.

4. Conclusions

NMR spectroscopy is extremely well suited to determine the structures and dynamics of tumor suppressor proteins and to study their interactions in complexes with proteins, DNA, or drug molecules. The methods for expression and purification of proteins from bacteria and the preparation of samples are as important as the instrumentation and methods for the NMR experiments. Recent technological advances in NMR spectroscopy enhance the sensitivity of the experiments, and extend the size range of molecules that can have their structures determined by NMR. Thus, the prospects for expanding the current tumor suppressor gene structure database are excellent, as structural studies are extended beyond the single domain, to multiple domains or full-length proteins and their complexes (1,32), and as solid-state NMR spectroscopy is used to determine the structures of membrane-bound tumor suppressor proteins (3,4).

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Fig. 1.
Strategy for protein structure determination by solution NMR spectroscopy.

Fig. 2. Solution NMR structure of the p53 tetramerization domain (PDB ID 3SAK) (40). The residues that switch the domain packing and stoichiometry upon substitution are shown (44). The letters N and C respectively identify the amino and carboxy termini of the protein.

Fig. 3. Solution NMR structure of the p73 SAM domain (PDB ID 1COK) (64). The letters N and C respectively identify the amino and carboxy termini of the protein.

Fig. 4. Superposition of the solution NMR structures of the tumor suppressor INK4 p15, p16 and p18 proteins (PDB IDs 1D9S, 1DC2, 1BU9) (56,59). The helix–turn–helix ankyrin repeats are numbered I through V. The letters N and C respectively identify the amino and carboxy termini of the protein.

Table 1

Tumor Suppressor Proteins Whose Structures Have Been Determined by NMR Spectroscopy in Solution, with Protein Data Bank Identification (PDB ID) Codes Shown for Reference (<http://www.rcsb.org/pdb/>)

Tumor suppressor structures determined by NMR spectroscopy	PDB ID
Refined solution structure of the oligomerization domain of the tumour suppressor p53 (39,40)	1SAE, 1SAF, 1SAG, 1SAH, 1SAI, 1SAJ, 1SAK, 1SAL
Solution structure determination of a p53 mutant dimerization domain (44)	1AU1
NMR solution structure of designed p53 dimer (63)	1HS5
Solution structure of a conserved C-terminal domain of p73 with structural homology to the Sam domain (64)	1COK
Solution structure of P18-Ink4C, 21 structures (56)	1BU9
Tumor suppressor P16Ink4A: determination of solution structure and analyses of its interaction with cyclin-dependent kinase 4 (58)	1A5E, 2A5E
Solution NMR structure of tumor suppressor P16Ink4A (59)	1DC2
Tumor suppressor P15(Ink4B) structure by comparative modeling and NMR data (59)	1D9S
High-resolution solution structure of human pNR-2/pS2:a single trefoil motif protein (65)	1PS2
NMR solution structure of the disulfide-linked homo-dimer of human Tff1 (66)	1HI7