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THE DEVELOPMENT OF NMR METHODS TO STUDY PROTEIN STRUCTURE AND DYNAMICS

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Abstract

An understanding of the role played by a protein in cellular function requires a detailed picture of its three-dimensional structure as well as an appreciation of how the structure varies as a function of time due to molecular dynamics. Over the past several years multi-dimensional, multi-nuclear solution NMR spectroscopy has become a powerful technology for obtaining both structural and dynamical information on proteins and protein-ligand systems. However, until recently the methods were limited to the study of molecules having molecular weights on the order of 25 kDa or less. Recent developments making use of fractional or complete deuteration have increased the scope of structural studies by NMR and have also improved studies of sidechain protein dynamics.

Introduction

In the past decade NMR spectroscopy has emerged as a powerful technique for the determination of the three-dimensional structures of proteins in solution. In the early 1980s, studies by R. R. Ernst [1] and K. Wüthrich [2] demonstrated that it is possible to obtain atomic resolution structures of small proteins, with molecular weights less than approximately 10 kDa (~ 100 amino acids). The work of Ernst provided the framework for the extension of the NMR technique from one to two and three frequency dimensions. Recent developments in NMR spectroscopy have had a significant impact on solution structural studies of proteins [3,4]. The improvements in the technology have been several fold and include: (i) the increase in the dimensionality of experiments from two to three and four, providing improved resolution for complex spectra; (ii) the uniform incorporation of ^{15}N, ^{13}C and ^{2}H labels

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into the biological system of interest coupled with the development of sophisticated NMR pulse schemes to transfer magnetization between scalar (through bond) and dipolar (through space) coupled spins; and (iii) significantly improved radio frequency (RF) electronics and increased magnetic field strengths as well as the development of commercially available hardware such as pulsed field gradients. Using this technology a large number of structures of proteins or protein complexes in the molecular weight range of ~15-20 kDa have been reported in the past several years [5,6]. Recently backbone assignments for two 269 residue proteins [3] and a symmetric dimer of molecular weight 38 kDa (Copie and Torchia, personal communication) have been reported.

These new methods utilizing uniform ^{15}N and ^{13}C labeling of proteins are called triple resonance (^{1}H, ^{15}N, ^{13}C) techniques, since the naturally-present ^{1}H resonances of the molecule are recorded along with those of incorporated ^{15}N and ^{13}C nuclei. Triple resonance methods require uniform labeling of protein samples with both ^{15}N and ^{13}C and make use of the large couplings that exist between the ^{15}N and ^{13}C nuclei and between these nuclei and their directly attached protons for efficient magnetization transfer. In this approach, assignment of backbone NH, $^{13}C^{\alpha}$, $^{13}C^{\beta}$, $^{13}C'$ (carbonyl), $^{1}H^{\alpha}$ and $^{1}H^{\beta}$ chemical shifts is accomplished using 3D experiments which correlate nuclei three at a time. Because of their excellent resolution and sensitivity and the redundancy of information, backbone assignment is straightforward. Sidechain assignments utilize the 3D HCCH-TOCSY as well as experiments correlating sidechain ^{1}H and ^{13}C shifts with backbone ^{15}N and NH shifts [3]. Structural information is obtained from nuclear Overhauser effect (NOE) experiments which provide distance correlations between protons within 5 Å of each other. Various dihedral angle restraints can be derived from experiments which measure homo- or hetero-nuclear coupling constants, as reviewed by Bax et al. [7].

Development of ^{15}N, ^{13}C, ^{2}H NMR Spectroscopy

As discussed above, ^{1}H, ^{13}C, ^{15}N triple resonance 3D and 4D spectroscopy has increased the size limits of protein structures that can be determined by the NMR technique to approximately 25 kDa. There are two reasons for this limit using the triple resonance approach. First, as the molecular weight increases, the number of cross peaks in spectra also increases. In the case of the triple resonance experiments developed for backbone assignment the number of peaks increases in a linear fashion with molecular weight. However, considering the excellent resolution afforded in such spectra, this does not represent a serious problem. The number of cross peaks in NOE-type spectra increases rapidly with size and poses a more serious problem for studying proteins of increasing size. However, the second factor, the rapid decay of the NMR

signal that occurs during the multitude of transfer steps in a complex NMR experiment, has the most significant effect on the size of molecules that can be studied by NMR. A typical multi-dimensional NMR experiment can be schematized as follows:

$$\mathbf{A} \rightarrow \mathbf{B} \rightarrow \mathbf{C} \rightarrow \mathbf{D} \rightarrow \mathbf{E} \rightarrow \ldots \rightarrow \mathbf{Z}$$

where the transfer of magnetization proceeds from **A** to **Z** via **B**, **C**, **D**, etc. The amount of time required to transfer magnetization along each link in the chain, from say **B** to **C**, is a function of the strength of the coupling between the participating links. The transfer can vary from ~4 ms if **B** = ^{1}H and **C** = ^{13}C to 20-30 ms if **B** = ^{15}N and **C** = ^{13}C, for example. During this transfer, the signal decays via relaxation processes whose efficiency, for macromolecules, increases linearly with molecular weight. The decay time of the signal varies depending on the type of nucleus (*ie*, whether **A** = ^{1}H, ^{13}C, ^{15}N etc); for ^{13}C nuclei coupled to protons the decay time can be as short as ~ 15 ms for proteins in the 20-30 kDa range. If the decay rate is the same order of magnitude as the transfer rate, a significant attenuation of the signal can be expected. In principle, there are three approaches to increasing the amount of signal observed at the end of the transfer. The first is to increase the inherent sensitivity of the experiment. This approach has been discussed in detail in the literature [8]. The second is to increase the strength of the coupling between the spins involved in the transfer. Unfortunately, these couplings are fixed by spin type and can not be manipulated. The third approach is to decrease the efficiency of relaxation loss that occurs during the transfer. This can be manipulated experimentally, through the substitution of deuterium for carbon bound protons in the protein [9,10].

The relaxation of nuclei is caused by fluctuating magnetic fields that are the result of the overall molecular tumbling in solution as well as internal dynamics. For the case of heteronuclei such as ^{15}N or ^{13}C directly coupled to ^{1}H spins, the major source of relaxation is due to fluctuating dipolar fields caused by the ^{1}H spins. The sizes of these dipolar fields are proportional to the gyromagnetic ratio of the spins which give rise to the fields, in this case ^{1}H spins. By substituting ^{13}C bound protons with deuterons, the size of the dipolar fields which cause relaxation of the attached ^{13}C nucleus is reduced by a factor of ~6.5, since the gyromagnetic ratio of deuterons (^{2}H) is ~6.5 times smaller than that of protons. This results in a substantial decrease in the relaxation rates of the ^{13}C spins. In addition, the proton nuclei remaining in the molecule have much slower decay rates as well, since many of the relaxation pathways which would normally involve adjacent protons are significantly attenuated by the incorporation of deuterium.

The idea of using ^{2}H labeling of proteins as a means of spectral editing dates back to the late 1960s with the pioneering experiments of Crespi *et al.* [11] and Markley and coworkers [12]. However, it was really the elegant work of LeMaster in

the late 1980s [13] that demonstrated the utility of random fractional deuteration of proteins to aid in the sequential assignment of what was then considered to be a large protein for NMR studies, *E. Coli* thioredoxin (108 residues). To obtain a balance between reduced line widths and reduced sensitivity, Lemaster prepared a 75% deuterium labeled sample of thioredoxin. He noted that the resolution obtained is significantly better than observed for fully protonated samples and that the sensitivity of the amide-amide region of the NOE spectrum was substantially better. Moreover, the intensities of NOE cross peaks connecting amide protons and carbon bound protons were comparable to intensities of the corresponding cross peaks in unlabeled protein. Finally, cross peak intensities of aliphatic-aliphatic NOEs were only reduced by a factor of three.

We have recently developed a suite of triple resonance experiments for the backbone assignment of ^{15}N, ^{13}C, ^{2}H labeled proteins with high sensitivity and significantly improved resolution [10]. The methods have been applied to study the 37 kDa ternary complex of the trp-repressor, corepressor and trp-operator DNA and more recently the backbone resonances of a 64 kDa trp-repressor complex have been nearly completely assigned [14] (>95%). Studies of the 64 kDa trp-repressor complex were only possible when performed on a >90% deuterated ^{15}N, ^{13}C labeled sample, stressing the requirement for high levels of deuteration for the study of large molecular weight proteins and protein-ligand complexes. While such a perdeuteration scheme is of benefit for the assignment of backbone NH, ^{15}N and ^{13}C chemical shifts the depletion of protons results in molecules with significant levels of protonation only at exchangeable NH sites. Structural studies by NMR depend to a large extent on the generation of inter-proton distance restraints, established via NOE-based experiments. The absence of large numbers of protons, poses therefore, a very serious problem for obtaining three dimensional structures of proteins using current NMR approaches. With these limitations in mind we have currently developed a biosynthetic approach in which overexpression of proteins in D_2O and with protonated, ^{13}C-pyruvate as the sole carbon source results in molecules which are highly deuterated at the majority of positions, with the exception of methyl groups of Ala, Val, Leu and Ile ($\gamma 2$) [15]. Molecules labeled in this way can be assigned in a straightforward manner using recently developed ^{15}N, ^{13}C, ^{2}H NMR experiments. Most important, is that it is possible to obtain NH-NH, NH-methyl and methyl-methyl NOEs in such systems in order to determine overall folds of the protein. Experimental results on the ^{15}N, ^{13}C, ^{2}H, $^{1}H_3C$-labeled C-terminal SH2 domain from phospholipase Cγ1 and calculations performed on a number of proteins ranging in molecular weight from 15-40 kDa indicate that it will be possible to generate global folds of the majority of proteins in this fashion.

Understanding Protein Dynamics

The identification of Src homology 2 (SH2) domains in many proteins involved in signal transduction has led to a rapid increase in our understanding of the molecular basis of oncogenesis. SH2 domains are regions of approximately 100 residues that bind to specific phosphotyrosine (pTyr) containing sequences. Recently the three dimensional structures of a number of SH2 domains have been solved by nuclear magnetic resonance (NMR) and X-ray crystallographic methods [16]. These structural studies have revealed a similar overall topology for the SH2 domains consisting of a large central b-sheet and an associated b-sheet, flanked by two a-helices. The phosphotyrosine binding site involves a network of charge-charge and hydrogen bonding interactions between residues of the SH2 domain, including arginine residues and the phosphate oxygens and aromatic electrons of the pTyr ring from the peptide. In a collaboration between the laboratories of Julie Forman-Kay and L.E.K. at the University of Toronto, the three dimensional (3D) solution structure of the C-terminal SH2 domain of phospholipase C-γ1 (PLCC) in complex with a phosphopeptide derived from the platelet-derived growth factor (PDGF) receptor Tyr-1021 site (pY1021) has been solved by heteronuclear NMR methods [17]. The topology of this SH2-pTyr peptide complex is similar to that reported for the SH2 domains from Src [18] and Lck [19]. However, the binding site for residues C-terminal to the pTyr is an extended groove that contacts the peptide at residues extending from the pTyr to positions six residues C-terminal to the pTyr. A similar extended binding site has been observed for the amino-terminal SH2 domain of the Syp tyrosine phosphatase (NSyp) in complex with a number of high affinity peptides [20]. The PLCC and NSyp SH2 peptide binding sites are different from the sites observed in structures of Src and Lck complexed with a peptide containing the sequence pTyr-Glu-Glu-Ile. For the SH2 domains of Src and Lck the mode of peptide binding has been described as a 'two pronged plug' interaction, with the pTyr inserting into a large pocket and the Ile into a separate and smaller pocket.

Despite the importance of these static 3D structures, it must be recognized that the picture obtained is not complete. Protein molecules are not static in solution and, indeed, the key to a protein's functionality may lie in its dynamic properties. Recently, Shoelson and coworkers (personal communication) have carried out binding studies where the parent high affinity peptides that bind to a particular SH2 domain were either (i) truncated one residue at a time or (ii) substituted with an alternative amino acid. In both cases binding affinities were measured and compared with the parent peptide. In the case of the Syp SH2 domain it was found that hydrophobic residues at the +5 position (i.e., the position five residues C-terminal to the pTyr) and the +3 position are required for high affinity binding. This is expected on the basis of the extended hydrophobic binding groove of the NSyp SH2 domain which contacts residues on the peptide up to the +5 position. However, a tripeptide centered on the

pTyr was found to bind to the PLCC SH2 domain with only a fifteen fold reduction in affinity (K_d ~ 15 μM) relative to the parent peptide (1 μM). Moreover, truncation of residues at the +2 through +6 positions had significantly smaller effects on binding for the PLCC SH2 domain relative to the NSyp SH2 domain. The results for the PLCC SH2 binding are surprising given the fact that both the PLCC SH2 and the NSyp SH2 domains have similar extended binding sites.

In an effort to understand why the extensive contacts between the PLCC SH2 domain and peptide residues at the +2 through +6 positions do not confer significant binding energy or specificity we have undertaken a study of the dynamics of methyl sidechains in both the free and complexed forms of the PLCC and NSyp SH2 domains. In principle, NMR spectroscopy is a powerful technique for probing sidechain dynamics of proteins and a number of studies of methyl dynamics at specific ^{13}C labeled sites in proteins have appeared in the literature [21]. In practice, however, a number of difficulties with such techniques have emerged. First, only a small subset of the sidechains can be examined in this way. Second, ^{13}C spin relaxation methods are hampered by the effects of cross correlation between ^{1}H - ^{13}C dipoles in methylene and methyl groups which can result in systematic errors in extracted motional parameters. To circumvent these problems we have developed a new strategy for studying picosecond-nanosecond sidechain dynamics based on the fractional incorporation of deuterium into uniformly ^{15}N, ^{13}C labeled proteins [22]. Because the deuterium lines in a protein are extremely broad, the experiments record the relaxation properties of the deuterons indirectly, through measurement of a series of high resolution, constant time ^{13}C, ^{1}H correlation maps where the intensity of the correlations relate to the relevant ^{2}H relaxation property, T_1 or $T_{1\rho}$. In principle, dynamics information about any labeled site in the molecule can be obtained. To date, we have restricted our analysis to methyl groups, due to the excellent resolution and sensitivity in this region of the correlation spectrum. The method allows dynamic information to be extracted from all methyl positions in the molecule simultaneously, in a manner which is free from the effects of cross correlation [23].

Application of these experiments to the PLCC SH2 domain establishes that certain of the residues of the SH2 domain which line the binding site contacting the +1 through +6 positions of the peptide are highly flexible [24]. It is very likely that the high degree of flexibility of the binding surface contributes to the relaxed specificity of the domain for target phosphotyrosine containing sequences. In addition the combination of significant amplitude motions and the steep distance dependence of the van der Waals potential may well result in a substantial decrease in the interaction energy which would otherwise manifest in a static site. In contrast, preliminary dynamics studies of the NSyp SH2 domain indicate that the hydrophobic binding interface is more rigid in this system than in the PLCC SH2:peptide complex; this increased rigidity may explain why hydrophobic interactions in the NSyp SH2 domain are more stabilizing than in the PLCC SH2 domain.

NMR relaxation parameters can be interpreted in terms of (i) an order parameter, S, which is related to the amplitude of the motion, and (ii) correlation times describing the time scale of the dynamics [25]. We have recently developed a relation between changes in order parameters and changes in conformational entropy, thereby providing a link between motional parameters and thermodynamics [26]. Experimentally, global thermodynamic values which characterize a net change in the system associated with a transition between two states, have been measured from optical methods such as circular dichroism and fluorescence or by calorimetric approaches such as scanning calorimetry and titration calorimetry [27]. On the theoretical side, molecular dynamics and Monte Carlo simulations allow estimation of thermodynamic parameters in some detail and offer the possibility of localizing changes in such values to particular sites in the molecule [28]. However, extraction of meaningful values from such simulations does require the use of accurate force fields, simulations of sufficient length and care that the system under study is in equilibrium. The NMR methods developed enable the determination of changes in entropy arising from changes in nano-second to pico-second timescale dynamics on a per residue basis.

Summary

A full understanding of protein function requires both structural and dynamics studies. At present there are only two methods that are able to provide a molecular picture at atomic resolution: X-ray crystallography and NMR spectroscopy. Methodology for extending structural studies by NMR has been developed which involves labeling with ^{15}N, ^{13}C and ^{2}H. The methods promise to extend the molecular weight limitations currently imposed on structural studies by NMR. In addition to structural information NMR can also provide insight into molecular dynamics. It is clear that the static structures of a number of related SH2 domains do not explain their different peptide binding properties; an understanding of the dynamics at the binding interface may be important in this regard.

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