Protein complexes studied by NMR spectroscopy

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Recent advances in NMR methods now allow protein complexes to be studied in great detail in a wide range of solution conditions. Isotope-enrichment strategies, resonance-assignment approaches and structural-determination methods have evolved to the point where almost any type of complex involving proteins of reasonable size may be studied in a straightforward way. A variety of isotope editing and filtering strategies underlie these powerful methodologies. Approaches to the characterization of the dynamics of protein complexes have also matured to the point where detailed studies of the effects of complexation on dynamics can be studied over a wide range of timescales.

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Abbreviations

HX	hydrogen exchange
NOE	nuclear Overhauser effect
SH2	Src homology 2
TOCSY	total correlation spectroscopy

Introduction

Advances in the field of NMR since its emergence fifty years ago have been staggering. As the technology has developed, there has been a corresponding explosion in the number of studies of proteins by NMR methods (for recent reviews, see [1–3]). The continuing interest in the interaction of proteins with small ligands and biopolymers has provided a potent driving force for the development of new NMR techniques to probe the structural and dynamic characteristics of protein complexes. This review focuses on the methodologies and strategies that have been developed to study complexes between proteins and various types of ligands, including small organic molecules, peptides and proteins, nucleic acids, carbohydrates, and lipids.

Methodology

One of the basic issues facing the NMR spectroscopist when approaching a noncovalent complex of a protein with a ligand molecule is the question of the lifetime of the complex. Is the complex in slow, intermediate or fast exchange with its dissociated components on the NMR timescale? Significant structural information may be gained in the fast exchange limit by use of the transferred nuclear Overhauser effect (NOE; see, for example, [4•]), although this and related approaches are inherently limited (for a review, see [5,6]). Notable examples of the use of the fast exchange limit to provide detailed structural information about the ligand in the protein-bound state include the characterization of interactions between calmodulin and small peptides [7,8]. However, one must conclude that a complex in slow exchange with its dissociated components on the NMR timescale is the most ideal condition for detailed structural studies. In this situation, the full power of multinuclear, multidimensional NMR methods can be applied to the structural and dynamic characterization of the complex. The use of heteronuclear NMR brings to the forefront the issue of isotopic enrichment of the ligand and/or the protein. Manipulation of the fact that two separable entities are involved is often extremely advantageous. Basically, the resonance assignment and structure determination problems presented by a stable binary complex of proteins is not different from that of a single protein, except for the fact that the former may be isotopically manipulated in a more selective manner. Three distinct isotopic enrichment strategies have been used to simplify the resonance-assignment problem presented by protein–ligand complexes: uniform deuteration of one component; uniform ^{13}C and/or ^{15}N enrichment of one component; and uniform ^{13}C and/or ^{15}N enrichment of both components of the complex.

Ironically, it appears that the uniform isotopic enrichment of proteins by biosynthetic means is often far easier and more cost effective than uniform, or even selective, isotopic enrichment of peptides or other small ligands by chemical synthesis. Enrichment of proteins with ^{13}C, ^{15}N and, to a lesser extent, ^{2}H is now often routinely achieved by bacterial expression of the structural gene during growth on labeled media. Routes to reasonably cost-effective eukaryotic expression of proteins using ^{13}C,^{15}N-containing minimal or rich media are also now available (see, for example, [9,10]).

Uniform deuteration has been used to simplify the ^{1}H spectra of protein–ligand complexes such as that between calmodulin and melittin [11] and between cyclophilin and cyclosporin [12]. In these cases, the subsequent analysis of the structure of the bound peptide relied entirely on ^{1}H–^{1}H interactions. In the context of ^{1}H resonance assignments, which must rely on ^{1}H–^{1}H J-coupling to provide intraresidue side chain resonance correlations and subsequently identification, the size of the complex becomes an issue. Although significant improvements have been made in isotropic mixing sequences employed

in TOCSY (total correlation spectroscopy) experiments (see, for example, [13]), the limitations presented by a long effective correlation time on chemical-shift correlation via direct ^1H–^1H J-coupling places a severe restriction on the size of the complex that can be efficiently studied by this approach. This is especially true when the spectrum of the bound peptide differs greatly from that of the free peptide and therefore requires highly reliable chemical-shift correlation via J-coupling to allow comprehensive resonance assignments to be obtained.

A more flexible strategy is to incorporate ^{15}N and/or ^{13}C into the ligand itself. This serves to provide a heteronuclear chemical shift for purposes of resolution and a means to distinguish the ^1H resonances of the ligand from those of the receptor protein. In cases where the ligand is made by chemical synthesis, the cost effective availability of suitably isotopically enriched precursors may often be limited. This is especially true for peptides and deoxyoligonucleotides. One recent example is the use of $\alpha^{15}N$-labeled tBOC-protected amino acids to prepare a peptide corresponding to the calmodulin-binding domain of the smooth muscle myosin light chain kinase [14].

In some cases, extensively isotopically enriched ligand can be prepared by biosynthetic means. Recent examples include the uniform enrichment of cyclosporin with ^{13}C and its subsequent use to study the conformation of the peptide bound to cyclophilin [15,16]. Surprisingly, fusion protein expression vectors, which can be used to rescue small peptides, have not yet been used extensively for isotopic enrichment of small peptides. Ironically, the availability of reasonably efficient semisynthetic approaches to the preparation of isotopically enriched RNA [17] far exceeds that available for DNA (although see [18•]).

Characterization of the interface of a protein–ligand complex was made much more feasible by the general development of heteronuclear multidimensional NMR. The use of HCCH-TOCSY and related experiments (for reviews, see [1–3]) allows nearly complete resonance assignments for long side chains which are often at the interface between proteins and their bound ligands. Recently, two experiments have been developed that provide a reliable path to the assignment of methionine methyls [19], which are often at the center of hydrophobic interfaces, and to the arginine guanidino group [20], which often participates in ionic interactions. Both of these approaches rely on isotopic enrichment (see, for example, [21]). A number of strategies using both homonuclear and heteronuclear NMR approaches have been developed to study bound water molecules, which are often critical to the structural integrity of protein complexes (for reviews, see [22,23]).

In cases where isotopic enrichment of the bound peptide becomes problematic, a somewhat more difficult spectroscopic approach may be used to isolate ^1H–^1H interactions exclusively involving the unlabeled peptide. This is achieved by uniformly enriching the receptor protein with ^{15}N and/or ^{13}C and editing the spectrum of the complex on the basis of whether or not a given ^1H is J-coupled to a heteronucleus. The standard X-filter selects for those ^1H resonances that are scalar coupled to NMR-active X-nuclei (for a review, see [24]). This same approach applied in the opposite sense is termed reverse X-filtering: only signals due to protons coupled to NMR-inactive X-nuclei survive. However, as pointed out by Fesik and coworkers [25], reverse filtration is a much more demanding task than simply selecting ^1H nuclei that are J-coupled to a heteronucleus. Reverse X-filtered experiments therefore often employ multiple strategies to suppress resonances arising from protons bonded to ^{15}N or ^{13}C. The library of experiments employing X-filtration and reverse X-filtration to simplify the NMR spectra of protein complexes is now relatively stable (for a review, see [26]) with only a few recent additions (see, for example, [27]).

Although the methods available for solving the resonance-assignment problems peculiar to protein complexes are now well established and complete, approaches available to study dynamics at the interface are less evolved. Recent developments in the use of both ^{13}C [28•,29•] and ^2H relaxation [30•] in conjunction with existing ^{15}N relaxation methods (for a review, see [31]) improve the ability of relaxation techniques to probe the effect of complexation on the internal dynamics of both ligand and protein. Deuterium relaxation has recently been employed to probe the effects of complexation by the Src homology 2 (SH2) domain on its phosphotyrosine-containing peptide ligand [32] and ^{15}N relaxation has been used to probe the role of protein dynamics in gating ligand binding to the HIV protease [33•]. It appears that the effects of complexation on the internal dynamics of proteins may be directly related to changes in the fundamental thermodynamic properties of the system [34]. This is a most exciting development.

Use of the exchange of backbone amide hydrogens with solvent is now a well-established approach to the characterization of less frequent motions than those dominating NMR relaxation phenomena. In favorable cases, it has been possible to measure hydrogen exchange (HX) behavior directly in a protein–ligand complex. This approach has been especially illuminating in studies of calmodulin–peptide complexes where HX behavior revealed not only the dynamics of a peptide while bound to calmodulin but also allowed the sequence of steps in the binding and release of the peptide ligand to be inferred [35•]. Access to binding and dynamic behavior within protein complexes that are too large for direct study can be obtained by NMR analysis of HX labeling patterns. A first level application is the delineation of protein interaction surfaces. Here, time-dependent hydrogen–deuterium exchange labeling is performed in the complex. The protein interaction surface, marked by

amide sites that are made slow exchanging in the complex, is analyzed later in one of the separated partners. The method has been applied to protein–monoclonal antibody interactions [36–39], enzyme–inhibitor complexes [40,41] and the interaction of proteins with micellar systems [42]. Similarly, NMR analysis of hydrogen exchange labeling has provided amino acid resolved information on global dynamics in redox pairs [43,44], on local dynamics in the crystalline state [45,46], on long range dynamic effects in DNA–repressor interactions [47], and even on the functional action of massive chaperonin proteins (M_r 800 000) on their bound, partially structured target [48,49].

Protein–small ligand complexes

There have been a number of applications of detailed NMR studies of proteins in complex with small molecules including water. Most of these complexes, studied in the slow exchange limit, provided extremely high resolution information about the binding site and interactions inherent in stabilizing the complex. Notable examples include studies of the complex of phosphatidylinositol-4,5-bisphosphate with the pleckstrin domain [50], the binding of FK506 to its putative target [51], and the dynamic consequences of the binding of folate to dihydrofolate reductase [52•]. Particularly nice examples of the use of NMR spectroscopy to unravel the structural and dynamic consequences of the binding of metals to apoproteins are found in recent studies of calbindin [53•], recoverin [54•,55•], and calmodulin [56•,57•,58]. Water, which has been shown to be integral to the tertiary structure and function of individual proteins (see, for example, [59]) has also been found by NMR to be intimately involved in their complexation of small molecules (see, for example, [51,60•]), other proteins or peptides (see, for example, [51]), and nucleic acids [61,62]. Evidence suggesting the presence of disordered water within a nonpolar cavity of interleukin-1β has also been presented ([63•]; but see also [64,65]).

Protein–protein complexes

Studies of protein–peptide complexes have been commonly used as models of their often much larger parent protein–protein complexes. Early examples include the use of synthetically ^{15}N-enriched peptides to provide the first direct confirmation of the amphiphilic helix model for the structure of calmodulin-binding domains bound to calmodulin [14] and the first characterization of the fast dynamics of a bound domain [66]. The calmodulin–peptide complexes also fueled the development of many of the reverse filtered experiments described above and led to the dramatic characterization of the complex between calmodulin and the calmodulin-binding domain of the myosin light chain kinase [67]. The interaction of calmodulin with peptides corresponding to calmodulin-binding domains of target proteins also serves to illustrate the use of transferred NOE to characterize the structure of a bound peptide [8] and the fast exchange limit to cross-assign spectra [7]. Early work with cyclosporin illustrated a variety of approaches to the protein–peptide complex including deuteration [12] and ^{13}C-enrichment coupled with X- and reverse X-filtering [15,16]. The structural characterization of bound peptide ligands with irregular secondary structure is exemplified by the more recent work on the complex between SH2 domains and phosphotyrosine peptide ligands [68,69] and between thioredoxin and its target peptide from the transcription factor NFκB [70]. Studies of the SH2 domain also serve as useful examples of the use of nitrogen and deuterium relaxation to examine the effects of complexation on the dynamics of the protein [71•,72]. Finally, a particularly difficult complex involving the oligomerization domain of p53 has also recently been solved ([73]; see also [74,75]).

Protein–nucleic acid complexes

Studies of protein–DNA complexes have been somewhat limited by the inability to prepare isotopically enriched DNA. Although there are now a number of highly successful examples of studies characterizing protein–DNA complexes without the benefit of isotopically enriched DNA (see, for example, [76–80,81•,82–84]), recent developments in the preparation of isotopically labeled DNA [18•] will hopefully make the complete structural characterization of protein–DNA complexes as easy as that of protein–protein complexes. Although the methodology for the labeling of RNA is now well established [85•,86] only a limited number of comprehensive NMR-based studies of the structure of protein–RNA complexes have been reported (see, for example, [87•,88]).

Miscellaneous protein complexes

There has been extensive recent development of NMR methods directed at the resonance-assignment problem in carbohydrates (see, for example, [89]), their conformational analysis (see, for example, [90,91]) and the characterization of protein–carbohydrate complexes (for a recent review, see [92]). The transferred NOE approach has been extensively used in studies of protein–carbohydrate complexes (see, for example, [93–96]), and is usually combined with a hard sphere exo-anomeric force field during the refinement of the structure.

The interaction of peptides and proteins within micellar environments has long been the object of study by NMR methods. Detergent micelles have been used to simply solubilize proteins in water (see, for example, [97•,98]). Recent examination of highly asymmetric micelles, termed bicelles, suggests a potential path to the characterization of membrane proteins by high resolution NMR [99•,100•]. This is a most promising area and one which we anticipate will bear significant fruit in the coming years.

Conclusions

The last few years have seen a significant maturation of the techniques available to characterize the structure and dynamics of not only proteins but also nucleic acids, carbohydrates and lipids. The interaction of proteins with

other small molecules and biopolymers can now be studied by NMR in great detail under a range of experimental conditions. The characterization of protein complexes by NMR can be expected to be one of the crowning achievements of the NMR technique.

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