Extending the Size of Protein–RNA Complexes Studied by Nuclear Magnetic Resonance Spectroscopy

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1. Introduction

Increasing evidence supports a model of cellular biochemistry in which most proteins exert their biological role through either transient or relatively stable multicomponent macromolecular complexes.[1] The key to understanding the functions of these complexes lies in their structural investigation by a variety of biophysical methods. For very large systems, cryoelectron microscopy can provide images of the overall shape of the biomolecules, and X-ray crystallographic methods can access the molecular details of multiprotein assemblies. However, the crystallization process is restricted to systems that are scarce in unstructured elements or structural heterogeneity, in contrast to the situation frequently found in the components of signal transduction pathways and transcriptional regulation, among others. Nuclear magnetic resonance spectroscopy is ideally suited to study these latter complexes, but only in recent years have techniques been developed to overcome the size limitation. The use of specific isotope-labeling strategies enables both a simplification of the NMR spectra and reduction in the detrimental relaxation effects that lead to signal degradation in large, slowly tumbling molecules. In addition, applications of orientational restraints from residual dipolar couplings (RDCs) and further developments in utilizing structural restraints derived from spin labels provide novel and powerful data for NMR studies of high-molecular-weight proteins and complexes.

Here, we discuss the emerging potential of NMR to probe the structure and dynamics of increasingly large complexes, especially when structural information is available for smaller regions of such complexes. In particular, aspects of sample production and NMR techniques will discussed. A strategy for quaternary-structure determination based on structures of subdomains, RDCs, and distance restraints is outlined.

1.1. Overall approach

Numerous advances in both sample preparation and spectroscopic techniques have increased the accessibility of systems that include both proteins and nucleic acids. The standard NMR approach whereby a suite of assignment experiments is followed by structural-restraint measurement (usually in the form of NOE values) is good for complexes involving a single RNA-binding domain with a short RNA oligonucleotide ($M_W <$ 20 kDa, reviewed in refs. [2, 3]). With larger systems, a significant decrease in the signal-to-noise ratio as well as an increase in spectral crowding dictates a different approach.

Most eukaryotic proteins are composed of a series of modular domains that are usually connected by flexible linker peptides. These domains can be expressed in isolation, and their atomic resolution structures are either already available or are accessible to standard applications of NMR spectroscopy and X-ray crystallography. A growing role for NMR spectroscopy is to combine these separate structural units by using selective labeling of single components. For this purpose, information about the domain orientations can be derived from residual dipolar coupling, while short- and long-range distance restraints are derived from chemical-shift perturbation, NOE measurements and paramagnetic relaxation enhancement (PRE). Combination of such data might provide an efficient way for deriving a high-resolution model of the multicomponent protein–RNA complex. Applications and a general strategy are discussed in the following with the example of 3′-splice-site recognition during spliceosome assembly.

1.2. Components of the 3′-splice-site-recognition complex

One of the early events in the recognition of introns prior to mammalian pre-mRNA splicing is the formation of complex E, an assembly of both protein and RNA components that ensures fidelity of the 5′ and 3′ intron sites.[4] Three proteins within the complex, SF1, U2AF65, and U2AF35, bind to the intron branch-point sequence, polypyrimidine tract, and the 3′-splice-site AG dinucleotide, respectively (Figure 1). Individual structures have already been calculated for the KH-QUA2 domain of SF1 bound to the consensus branch-point sequence,[5] the isolated first and second RRMs (*R*NA *r*ecognition *m*otifs) from U2AF65,[6] the third RRM from U2AF65 bound to an N-terminal peptide of SF1,[7] and the RRM of U2AF35 bound to an N-terminal peptide from U2AF65 (Figure 1).[8] Some evidence indicates a specific quaternary arrangement within complex E,[9] and further elucidation of the domain arrangement in this complex will be important for our understanding of the definition of the 3′-splice site. The results will provide a struc-

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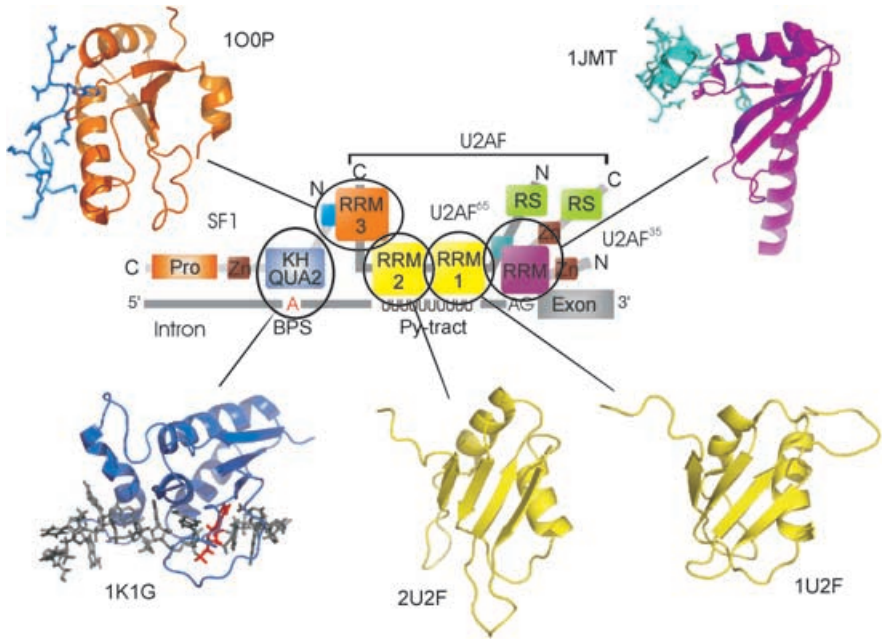

Figure 1. Diagram of the 3′-splice-site-recognition complex detailing the individual domains found within SF1 and the U2AF heterodimer. Also shown are the previously solved structures that are used in the calculation of the ternary complex, accompanied by PDB accession codes. BPS, branch point sequence; Pro, proline-rich; Py-tract, polypyrimidine tract; RRM, RNA recognition motif; RS, arginine/serine-rich; Zn, zinc knuckle.

tural basis for how this modular complex invokes cooperative binding of relatively weak binary protein–RNA and protein–protein interactions to achieve specific and high-affinity 3′-splice recognition, but at the same time allows for disassembly of this intermediate complex in order to proceed with spliceosome assembly. For NMR studies of the assembly of SF1, U2AF and the intron RNA, different subcomplexes are studied initially. Here, we discuss one of these complexes, which involves the relevant regions of SF1, U2AF65, and a cognate intron RNA corresponding to a 74 kDa assembly.

2. Sample Preparation

The first consideration with the study of any large complex by NMR is the production of protein or nucleic acids in the quantity required for a favorable signal-to-noise ratio. Also important is the incorporation of suitable isotopic labels for multidimensional spectroscopic techniques and spectral simplification. The uniform as well as selective incorporation of ^{13}C and ^{15}N atoms into proteins has been utilized for over a decade,[10,11] and many standard procedures are in place for its use with bacterial expression, cell-free protein synthesis,[12] and additional expression hosts.[13]

2.1. Subunit-selective labeling

For NMR studies of multimeric complexes, subunit-selective labeling is advantageous in order to reduce spectral complexity. Note, however, that this requires the possibility of reconstituting the complex in vitro. For example, subunit-selective labeling and the presence of symmetric oligomers were crucial for the NMR study of GroEL–GroES complexes.[14] For our study of the SF1-U2AF65-RNA complex, two types of samples were prepared: one comprising $^{2}H,^{13}C,^{15}N$-labeled U2AF65/unlabeled SF1 and RNA, and another comprising $^{2}H,^{13}C,^{15}N$-labeled SF1 bound to unlabeled U2AF65 and RNA. Different deuteration schemes may be required for the $^{2}H,^{13}C,^{15}N$-labeled protein component. In addition, depending on the size and type of the molecular interfaces, ^{2}H-labeling of the binding partners may be recommended, even though we have not found this to be important in the present study.

2.2. Deuterium-labeling strategies

Significant progress has been made in labeling strategies to aid in the study of large proteins and protein complexes.[15] Improvements in line-width and sensitivity can be achieved through the replacement of side-chain protons with deuterium, with a resulting decrease in transverse relaxation.[16,17] A further improvement in resolution couples deuteration with the use of transverse relaxation-optimized spectroscopy (TROSY)[18] for molecules with decreased molecular tumbling, for example, with systems in excess of 25–30 kDa molecular weight.[19] Since both the U2AF65 construct of three RRM domains (36 kDa) and the N-terminal fragment of SF1 (30 kDa) already exceed this limit, deuteration was employed.

Simple protocols for deuterium incorporation utilize random fractional labeling of protein side chains through the growth of bacterial expression hosts in media made with various amounts of $^{2}H_2O$ (typically from 50% to 100% by volume).[17] Maximal reduction in relaxation requires that the expression host is grown in 100% $^{2}H_2O$ medium with ^{2}H-glucose as the carbon source. Since no aliphatic or aromatic protons remain, however, information derived from side-chain proton resonances is lost. A good compromise between favorable relaxation and retention of enough side-chain protonation for assignment and structural experiments utilizes protein expression in 70% $^{2}H_2O$.[20] This effects an average of 50–60% fractional ^{2}H-labeling, interestingly with a higher than average deuteration level for Cα carbons and a lower level for methyl groups.[17,21] This procedure is significantly cheaper than complete deuteration and does not require stepwise adaptation of the bacteria to the increased $^{2}H_2O$ content, which can sometimes lead to slow cell growth, early stationary phase, or premature death of the culture. In the present study of the U2AF65/SF1/RNA complex, 100% ^{2}H labeling was found to be crucial (Figure 2). For the preparation of such a sample, the growth of cells in com-

mercially available rich ^2H medium, or in minimal recipes[22] containing a 5% supplement of the rich medium, also circumvented the need for adapted growth and produced protein yields comparable to protonated media.

An extremely useful but relatively expensive deuteration scheme involves the use of 100% ^2H_2O medium supplemented with specifically labeled α-ketoisovalerate and α-ketobutyrate biosynthetic precursors.[15,23] This achieves ^1H,^{13}C-methyl labeling in an otherwise ^2H,^{13}C,^{15}N-labeled background. The retention of some key methyl protons is important for helping to define the packing of the hydrophobic core of the protein through methyl NOE-derived distance restraints. The methyl groups can also be used to monitor chemical-shift perturbation upon ligand binding,[24] for example, as observed for methyl groups in ^1H-methyl(I/L/V)-^2H,^{13}C,^{15}N-labeled U2AF65 upon addition of SF1 (Figure 3).

Although not used in our study of the 3′-splice-site-recognition complex, other methods have been used to incorporate deuteration into site-specific regions of proteins. Perhaps the most controllable method for very precise labeling schemes relies on the use of cell-free synthesis,[12] since any amino acid labeling scheme can be incorporated without the risk of isotope scrambling as occurs during bacterial expression. In addition, segmental isotopic labeling holds great promise for the study of large macromolecular systems, since defined regions within a protein can be differentially labeled, for example by

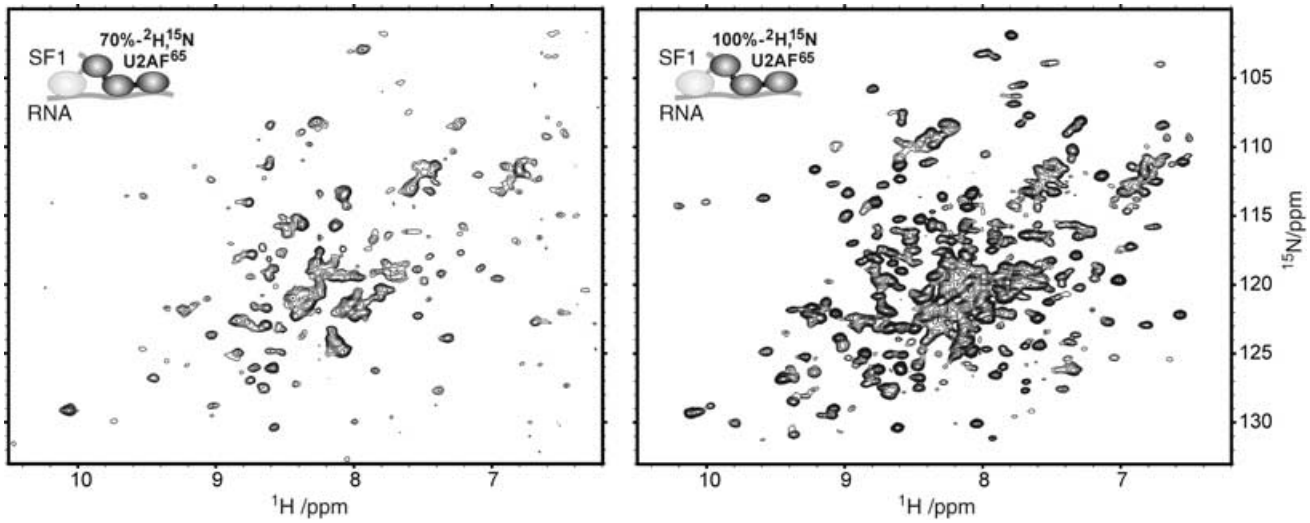

Figure 2. Comparison of ^1H,^{15}N-TROSY-HSQC spectra of 70% (left) versus 100% (right) deuteration of U2AF65(148–475) when bound to unlabeled SF1(1–260) and RNA.

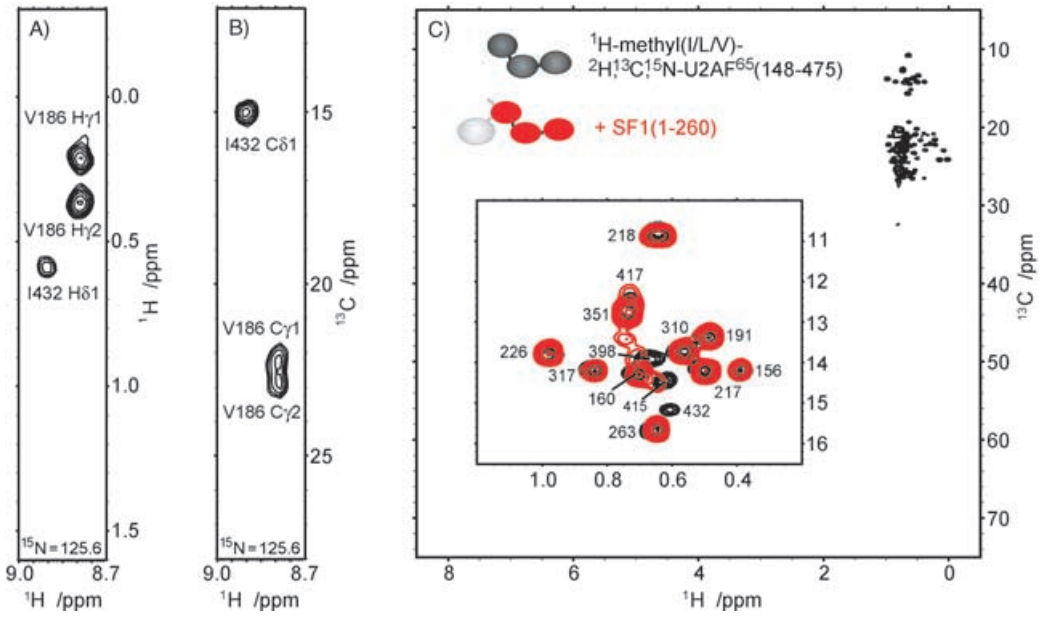

Figure 3. Example A) H(CC)(CO)NH-TOCSY and B) (H)CC(CO)NH-TOCSY assignment spectra for the ^1H-methyl(I/L/V)-^2H,^{13}C,^{15}N-labeled sample of U2AF65(148–475). C) ^1H,^{13}C-CT-HSQC showing that only the Ile, Leu, and Val methyl groups are ^1H-labeled. A close-up view of the Ile $\delta 1$ methyl region includes chemical-shift annotation (black), and the perturbation of I398, I415, I417, and I432 resonances (all found within RRM3) that occur upon binding of unlabeled SF1(1–260) (red).

using ^{2}H, ^{13}C, and ^{15}N nuclei for a single domain within a multidomain protein.[25]

2.3. Preparation of RNA

The production of RNA samples for NMR spectroscopic studies is usually performed by chemical synthesis or by in vitro transcription.[2] For short unlabeled oligoribonucleotides (= 30 nt), phosphoramidite synthesis is feasible and cost-effective. The advantage of a synthetic approach is the ease with which modified nucleotides can be incorporated at any place in the RNA sequence, including the 4-thiouracil required for spin label studies (see Section 4.2.4). In the present example, an unlabeled synthetic RNA has been used. The chemical-shift assignments of the branch-point sequence in this RNA are available from the previous study of the SF1/RNA complex.[5] However, to extend chemical-shift assignments of the RNA in the larger complex, isotope labeling is required. The production of uniformly or nucleotide-selective isotopically labeled RNA relies on in vitro transcription with purified RNA polymerase with either plasmid or single-stranded DNA templates. Incorporation of ^{2}H, ^{13}C, or ^{15}N-labeled nucleotides is straightforward,[26,27] and both the production of these $^{13}C/^{15}N$-NTPs from bacteria grown on labeled media and the recycling of unreacted nucleotides can be used to reduce costs.[26,28] The use of ribozymes in combination with in vitro transcription[29] can help to reduce the current disadvantages in enzymatic preparation of RNA, namely heterogeneity in the size of the final RNA product and difficulty in removing these contaminating oligos, which differ by only a few nucleotides. Finally, segmental labeling is also possible with RNA, and proceeds by preparation of smaller nucleotides that can be modified (for example at the 3′ position) and then joined by using T4 ligase.[30]

3. Chemical shift assignment

For the chemical-shift assignments of the domains or subunits of a complex, standard methods can be employed.[31] For larger proteins, specifically designed NMR experiments are used that take advantage of the TROSY technique and the presence of side-chain perdeuteration.[32] For $^{1}H,^{13}C$-methyl-selectively labeled samples in an otherwise perdeuterated context, a number of experiments are available for the assignment of methyl ^{1}H and ^{13}C chemical shifts through COSY-based, multiple quantum TOCSY and methyl-detected pulse sequences.[32] In the case of ^{1}H-methyl(I/L/V)-$^{2}H,^{13}C,^{15}N$-labeled U2AF65(148–475) HCC(CO)NH-TOCSY experiments were sufficient to assign the free-protein methyl resonances (Figure 3). An alternative way for spectral simplification relies on amino acid-selective labeling, for example, to reduce spectral overlap in $^{1}H,^{15}N$ correlation spectra.[11,33]

The assignment of RNA resonances can be achieved with a $^{13}C,^{15}N$-labeled oligonucleotide and experiments that correlate atoms within the base, sugar, and phosphodiester backbone.[34] When combined with the use of fractional and/or site-specific ^{2}H labeling,[26,27] this procedure is typically applicable to RNA, or protein-bound RNA molecules within moderate-sized complexes (< 25 kDa). For larger complexes, complete assignment of the bound RNA is expected to be hampered by signal overlap in addition to a poor signal-to-noise ratio resulting from large line widths. In this respect, the use of segmental RNA labeling has proven useful in a study of the 100 kDa internal ribosome entry site, in which individual building blocks have been studied by using standard methods, while assignments of the 100 kDa RNA were confirmed by using a ^{15}N-segmentally labeled RNA.[35] An alternative approach was taken for the NMR study of a 101 nucleotide viral RNA, in which nucleotide selective labeling was used to reduce signal overlap.[36]

4. Structure Calculation of Multimeric Protein–RNA Complexes

Traditional NMR structure-calculation methods rely on the measurement of a large number of proton–proton distances, which become more tedious to determine with the increasing size of the molecules.[3] Although it has been shown that these structure-determination methods can be adapted to very large molecular weight systems in favorable cases,[32] such studies require a substantial amount of work that cannot easily be automated. Moreover, in the case of complexes, often only very limited information can be derived from intermolecular NOEs because the interface between the molecules in a complex is not necessarily as rigid and well defined as the core of a protein. It is therefore advisable to employ a strategy that utilizes available structural information for smaller domains and/or subunits of a larger complex. For example, the structure determination of the 38 kDa ternary U1A protein–PIE RNA complex was greatly enhanced by the previous structural analysis of a bimolecular complex of about half the size.[37]

In a general approach, available structures of subdomains should be combined with orientational restraints derived from residual dipolar couplings and with distance restraints to define the molecular interfaces. It is important that these experimental restraints can be obtained for high-molecular-weight assemblies, for which signal-to-noise ratio and spectral crowding have to be considered. The available structures of subdomains serve as building blocks and are used to define the quaternary structure of the higher-order complex based on distance and orientational restraints.

4.1. Structure determination of individual domains and RNA segments

We have chosen to start with the previously defined structures of isolated domains from SF1 and U2AF65 (Figure 1). In general, even if a structure is not yet available for the protein studied, the growing number of available structures from large-scale proteomics initiatives continually raises the probability of finding a close homologue of the desired protein or domain in public depositories such as the Protein Data Bank (http://www.rcsb.org/pdb). Modeling programs such as SWISS-MODEL (http://swissmodel.expasy.org)[38] or MODELLER[39] can then be used to generate adequate starting points for the calculation of the complex, especially when accompanied by NOE or RDC

data to improve the accuracy of the model (see below). When using this approach, it is important to evaluate whether the available subunit structures experience significant changes in conformation upon binding. For the three RRMs in U2AF65, the acquisition of simple $^{1}H,^{15}N$-TROSY spectra confirm that the domains are structurally independent, and that the addition of SF1 affects only the third RRM as previously described (Figure 4).[7]

4.2. Characterization of binding interfaces by NMR

Various methods are available for the characterization of binding interfaces by NMR.[40] These methods are also applicable to identify and characterize interactions between domains or subunits in a larger multimeric complex and can be used to derive distance restraints defining the quaternary arrangement.

4.2.1. Chemical-shift perturbation: The simplest and best-established way for determining molecular interfaces is to monitor chemical-shift perturbation, that is, with $^{1}H,^{15}N$, or $^{1}H,^{13}C$ correlation experiments recorded on the isotopically labeled protein or RNA before and after addition of an unlabeled binding partner.[41] Resonances that display chemical-shift perturbation or residue-specific line broadening[42] indicate contact sites on the protein surface. Although simple to acquire, this approach cannot always distinguish between regions of the protein affected by direct contact with the substrate versus additional changes in conformation in a region distal from the association.

4.2.2. Saturation transfer and solvent accessibility: Saturation transfer from a bound protein[43] or RNA[44] to an isotopically labeled ligand is observed through direct and short-range effects (<6 Å) within the binding interface and thus can complement and validate chemical-shift-perturbation data. However, a distinct subunit-specific isotope labeling scheme is required. NMR spectroscopy can also detect changes in the solvent accessibility of backbone atoms before and after the addition of the substrate, thus providing another method to directly detect the surface in contact with the added ligand(s).[45,46] In a related approach, water-soluble paramagnetic relaxation agents can be added to the protein sample, thereby causing increased relaxation of accessible atoms. Binding of the added ligand will protect the buried association surface from this induced relaxation and thus reveal residues involved in substrate contact.[47,48] An alternative method utilizes changes in the hydrogen/deuterium exchange rate upon ligand binding.[49]

4.2.3. Intermolecular NOEs: If complete chemical shift assignments are available for all binding partners, the collection of NOE-derived distances allows a precise structure determination of the molecular interface. This approach can provide a sufficient amount of structural restraints, but is also the most time-consuming. Edited-filtered experiments can be employed to identify intermolecular NOEs direct-

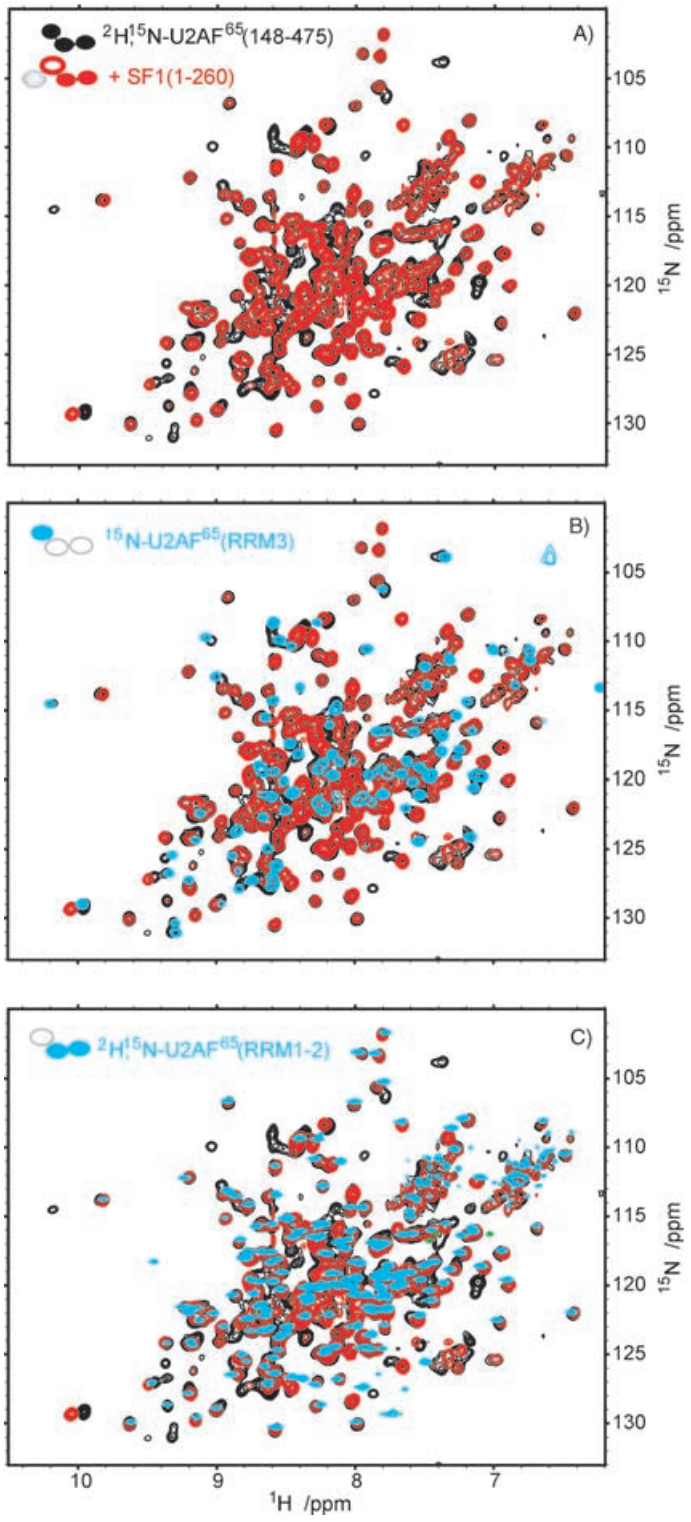

Figure 4. Superposition of $^{1}H,^{15}N$-TROSY spectra from $^{2}H,^{15}N$-U2AF65(148–475) in the absence (black) and presence (red) of unlabeled SF1(1–260). A) Signals corresponding to residues in U2AF65 RRM3 display significant line-broadening within the heterodimer, with peaks often below the level of detection. B) Superimposition of the spectrum of isolated RRM3 (blue) with the spectrum in A) confirms that the line-broadened peaks derive from RRM3. C) In contrast, cross peaks from RRM1 and RRM2 display no significant perturbation, as seen upon superposition of the spectrum from A) with that of an isolated U2AF65 RRM1-RRM2 construct (blue). These results confirm the independent behavior of the RRMs within U2AF65, and validate our approach of reconstituting the structure of the complex from individually characterized components.

ly.[31,50,51] This speeds up data analysis, however, with a concomitant loss in the signal-to-noise ratio. Furthermore, depending on the strength and type of the molecular interaction, only few intermolecular NOEs might be obtainable.[52]

4.2.4. Paramagnetic-relaxation enhancement (PRE) from spin labels: To supplement information about the molecular interface, and especially to obtain long-range distance restraints, the site-specific addition of spin labels can be used. Typically, the spin label is a small paramagnetic molecule with a stabilized electron radical, which is covalently attached to protein cysteinyl groups or ribonucleotide 4-thiouracil bases.[53,54] The presence of a paramagnetic center enhances the relaxation of nuclei within a 25 Å radius. This leads to distance-dependent line-broadening and thus can provide long-range distance restraints.[53] In this regard, spin labeling offers an attractive alternative to the measurement of NOEs, which rapidly decrease with increasing distance and are normally not observable beyond 5–6 Å. Typically, signal intensities in the paramagnetically bleached spectrum are compared to a reference spectrum recorded after reduction of the spin label by using ascorbic acid or sodium hydrosulfite.

For proteins, a paramagnetic nitroxide molecule is coupled to a target protein with only a single defined accessible cysteine. This requires mutation of other cysteine residues in the protein—typically to alanine or serine—and/or incorporation of novel cysteines in the sequence if none are conveniently present. Alternatively, metal chelating tags can be attached and bound to paramagnetic metal ions. In this case, additional structural information can be obtained from pseudocontact shifts and residual dipolar couplings from magnetic alignment.[55–57]

For the study of large protein–RNA complexes, spin-labeling of the RNA component in several locations along the RNA sequence might generate enough distance restraints to sufficiently define the location of the protein and RNA components. A useful spin label for RNA couples 3-(2-iodoacetamidoproxyl) to a 4-thiouridine base,[54] which does not appear to disturb the base-stacking pattern in double-stranded RNA.[58] The location of the spin label and its proximity to the molecular interface is a critical consideration when using this technique, since the incorporation of the bulky covalent paramagnetic ligand should not perturb the binding interface, but still achieve useful PRE.[54] Moreover, the spin label must not be placed in mobile structural elements, since this might cause ambiguous results.[52]

4.3. Structure calculations

Based on the knowledge about binding interfaces between subunits and the structures of these subunits, a general strategy for structure calculation of protein complexes and/or multidomain proteins can be devised. This involves a combination of orientational restraints from residual dipolar couplings (RDCs) to define the relative domain orientation and distance restraints that connect the available substructures. It is advantageous and desirable to rely on previously determined structures of the constituent subdomains or binding partners. RDCs provide efficient orientational restraints for refinement of the initial structures (or homology models) of subdomains and to define their relative orientations.[59,60] The incorporation of orientational restraints from RDCs has been shown to improve the structure determination of protein–RNA complexes.[3,61]

Most previous approaches have employed rigid-body docking of available subdomain structures by using RDC and distance restraints.[62–66] This is applicable when the RDCs measured in the complex can be fitted accurately to the available subdomain structures. However, more generally, a refinement of the initial subdomain structures will be required to consider slight conformational differences and possibly induced-fit binding of the components involved, which is especially important to consider in studies of protein–RNA complexes. The local refinement is crucial in order to obtain an accurate relative orientation of the individual structural elements.[67] We have developed a robust and efficient protocol that provides local refinement of the available input structures against the RDC data prior to determination of the relative domain orientations (Simon et al. unpublished results). Without such a local refinement, the accuracy of the resulting structures and especially of the domain orientation is compromised.

Distance information is required to complement the orientational information contained in RDCs and to resolve the resulting ambiguities. Distance restraints are either obtained from NMR experiments to monitor binding interfaces such as chemical-shift perturbation, saturation transfer, or PRE (see Section 4.2), but can also be derived from biochemical data (i.e. from mutational analysis). In practice, such data are implemented as ambiguous interaction restraints (AIRs).[65,68] More stringent distance restraints can be derived from the measurement of paramagnetic relaxation enhancements.[53] A few well-chosen spin labels can thus provide sufficient distance information to complement RDC-based orientation restraints for the determination of a complex structure.

5. Future Outlook

Recent advances in isotope-labeling strategies, NMR experiments, hardware, and computational methods allow the use of NMR to study larger protein complexes. While the specific methods and experimental strategies employed are still being improved, a number of examples have already demonstrated the utility of NMR for the structural analysis of such high-molecular-weight complexes. With the available methods, NMR can be employed to define the quaternary structure of macromolecular assemblies. Furthermore, conformational dynamics and ligand interactions can be monitored in solution, for example, in the context of rational drug design targeting complexes.

It is foreseeable that a multidisciplinary approach is viable for structural studies of large macromolecules. For example, the utility of combining RDC-based restraints with data derived from small-angle X-ray scattering has been demonstrated.[69] Thus, advances in structural biology methods and their combined and complementary use provide powerful tools for the

structural analysis of large multimeric complexes, which are difficult to tackle by either method alone.

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