One-Sample Approach to Determining the Relative Orientations of Proteins in Ternary and Binary Complexes from Residual Dipolar Coupling Measurements

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NMR spectroscopy is a powerful method for analyzing changes at the atomic level in the structure and dynamics of macromolecular complexes.1–5 NMR distance restraints used to define the conformational space of protein–protein assemblies are commonly derived from intermolecular nuclear Overhauser effects,6–7 paramagnetic relaxation enhancements,8,9 cross-saturation,10 and chemical shift perturbations.11 However, because full analysis requires spectral information from each partner in the complex, multiple samples are used for such measurements. Thus the full atomic details of protein assemblies, which only NMR can offer in solution, can be time and cost restrictive. Other strategies have exploited amino acid selective12 or asymmetric labeling patterns13,14 to facilitate measurements of intermolecular distances. Unfortunately, only the interface is characterized and the full backbone conformation and relative orientation of protein–protein complexes is not defined.

In some cases, the approach requires sequences which are insensitive for large assemblies with inherently short transverse relaxation times (T2).

The conformational space of macromolecular complexes also can be determined from orientational restraints derived from residual dipolar couplings (RDCs) under weak alignment.15,16 Again, a common strategy for avoiding spectral overlap and for associating NMR signals with a particular subunit is to prepare multiple samples with differential labeling patterns. Particular care is necessary in the case of RDC measurements because slight changes in the experimental conditions can alter the alignment tensor, such that information from different samples cannot be correlated directly.

The implementation of a spin–echo difference during a constant time period of an HSQC was originally introduced by Bax and co-workers17 for the measurement of side chain dihedral angles. We recently applied a similar constant time spin–echo filter element to replace a two-dimensional version of the triple resonance HNCO pulse sequence to identify sequential pairs of amino acids in large proteins and enzymes18 and extended this idea to enable chemical shift perturbation mapping of samples containing three isotopically labeled species in solution.19

Here we report an approach that, combined with an asymmetric isotopic labeling scheme, enables simultaneous measurement of RDCs from subunits of binary and ternary complexes with high sensitivity. Unlike recently reported schemes,20,21 the pulse sequence used in this approach is shorter and provides higher sensitivity, particularly for large systems. Also, the approach supports the unambiguous identification of spectral information from each subunit of a binary or ternary complex.

Figure 1. Schematic for the gradient-selected TROSY-based pulse sequences for binary (A) or ternary (B) protein mixtures. A reference spectrum is obtained by applying the 180° 13C pulses (open pulses) at position a, while 13C or 15N suppression is obtained with pulses position b or c, respectively. Delay durations: Δ = 2.4 ms; δ = 0.11 ms; τNC = 16.5 ms, τSC = 23.5 ms. Further details, including spectral editing to obtain each subunit, are provided in the Supporting Information.

The complete details for the detection and deconvolution of a spectrum containing three isotopically labeled species were provided in a previous publication,20 and only a brief description is provided here. The subunits of the ternary complex are labeled differentially as follows: uniformly 15N( U

15N) labeled (species A), uniformly 13C (U

13C) labeled (species B), and U

15N and 13C (U

15N,13C) labeled (species C). Selective 15N or 13C labeling can be achieved for recombinant proteins expressed in Escherichia coli by utilizing 1-13C or 2-13C glucose, respectively, as the sole carbon source.22 Signals from either or both 13C labeled species (B or C) can be suppressed in a selective manner by using a [1H/15N]-HSQC sequence containing a modified constant-time period which leads to JSC or JSC modulation (Figure 1). Thus resonances from either species A or species B and C can be produced in subspectra. Linear combinations of these subspectra led to the observation of the three individual subunits.23

To measure RDCs, we adapted these sequences to utilize sensitivity-enhanced TROSY or anti-TROSY spin-state selection and allowed JHN coupling to become active during chemical shift evolution so that 1H–15N splittings could be measured accurately (Figure 1). Alternatively, RDCs can be extracted by measuring the 1H–13C half-splitting between a pair of TROSY and decoupled HSQC spectra.23 Because the introduction of JSC or JSC modulation is used only for the purpose of suppressing the detection of resonances, this modulation has no effect on the accuracy of the 1H–15N splittings measured. As shown in Figure 1, the sequence is simplified to increase sensitivity in cases of a binary complex (Figure 1A), or the full pulse sequence (Figure 1B) is used in the case of a ternary complex. The total constant time period is ~33 ms (1/2 JSC) or ~49 ms (1/2 JSC), respectively.19

As a proof of concept, we applied this approach to a ternary mixture of noninteracting proteins: maltose binding protein (MBP), Glucose-6-phosphate isomerase (GPI), and the carboxy-terminal domain of the Escherichia coli maltose binding protein (MBP-CTD).

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The 15N-labeled species is isotropic couplings for the ternary mixture shown in Figure 2. All isotropic J-coupling values were obtained from spectral editing of TROSY and anti-TROSY data sets as shown for MBP (B), ubiquitin (C), and Kemptide (D). 44 kDa), ubiquitin (8 kDa), and Kemptide (0.7 kDa). Proteins were either expressed in Escherichia coli BL21(DE3) to obtain uniform labeling ([U-15N]-MBP, [U-15N,13C]-ubiquitin, and [15N-Ser5,13C-Ala]-Kemptide). TROSY-detection was used to obtain all 15N resonances in the sample (A). The pulse sequence from Figure 1B was used to obtain the isotropic couplings for the ternary mixture shown in Figure 2. All 1H–15N correlations were observed in the reference spectrum (Figure 2A), and the subspectra for each component were obtained by using spectral editing of the suppressed spectra (Figure 2B–D). Weak alignment was then introduced using pf1 phage (12 mg/mL). The pulse sequence from this single sample were sufficient to define the backbone conformational space of these structured proteins, along with their relative alignment tensors. This new approach to measuring backbone RDC values in a ternary complex eliminates the need for multiple samples, removes errors from sample inconsistencies, and ultimately reduces costs related to the preparation of multiple samples. Although shown only for a tertiary mixture of proteins, this approach will work for soluble and membrane proteins, so long as the reference HSQC experiment can be obtained. In the case of membrane proteins, lanthanides or polycrylamide gels may be used to introduce weak alignment so that the detergent or bicine are not perturbed.

When a binary mixture of proteins is studied, the signal-to-noise ratio (S/N) resulting from the pulse sequence (Figure 1A) used to detect the 15N-labeled species is $\sqrt{2}$ higher than that of approaches that rely on a HNCO-based sequence, as has been proposal recently. In addition, the time period in which magnetization is transverse in HNCO-based experiments is ~68 ms, whereas the sequences presented here only require half this time period (~33 ms) for a binary mixture or ~49 ms for a ternary mixture. Our laboratories have shown that, for large proteins, the constant-time spin–echo filter used in this approach has superior S/N and works well when HNCO-based sequences provide insufficient S/N, particularly in cases in which $T_2$ values are less than ~50 ms.

In summary, we present a new approach for the acquisition of backbone amide RDCs for binary or ternary complexes using a single sample. Together with the applicability of these sequences to monitor chemical shift perturbations in titration experiments, this method provides useful orientational restraints for high-resolution studies of protein complexes. Since numerous biological processes rely on protein assemblies or transient interactions, this approach should be useful for a wide range of applications.