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Simultaneous measurement of residual dipolar couplings for proteins in complex using the isotopically discriminated NMR approach

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Abstract: One-bond residual dipolar couplings (RDCs) measured for the amide groups of proteins partially aligned in a magnetic field provide valuable information regarding the relative orientation of protein units. In order for RDCs obtained for individual proteins to be useful in the structure determination of heterodimer complexes, they should be measured for exactly the same alignment of the complex. Here, an isotopically discriminated IDIS-RDC-TROSY NMR experiment is proposed, which enables the measurement of HN RDCs for two proteins simultaneously and independently, but in the same sample, while they are part of the same complex. The signals for both proteins, one of which should be labeled with ^{15}N and the other with ^{15}N and ^{13}C, are observed in different subspectra, thus reducing spectral overlap. The approach uniquely ensures that RDCs measured for both proteins relate to exactly the same alignment tensor, allowing accurate measurement of the relative angle between the two proteins. The method is also applicable for complexes containing three or more protein components. The experiment can speed up and lead to automation of protein−protein docking on the basis of angular restraints.

Introduction

Determination of the 3D structure of protein−protein complexes can be greatly facilitated by docking proteins with known structures (as rigid bodies) using orientational restraints.[1] These can be derived from residual dipolar couplings (RDCs) measured for proteins partially oriented relative to the magnetic field.[2,3] Although the concept of the docking approach is well developed and is clearly very promising,[1] in practice the measurement of RDCs for protein−protein complexes still remains a challenge.

To yield the angle between proteins in complex accurately, the RDC values for the individual components should be measured within the same (a priori unknown) orientation frame, under precisely the same alignment conditions, ideally, in the same sample. In practice, RDCs for the two proteins are measured in different samples, where only one protein at a time is labeled, e.g., with ^{15}N, and another protein is unlabeled and hence invisible.[4,5] The drawback of this approach is that any difference in sample preparations (pH, temperature, ionic strength, sample content, behavior of alignment media, etc.) may cause changes in alignment tensors, ultimately leading to a distorted measured angle between proteins. More alarmingly, the differences in the alignment tensors for complexes in various samples may go unnoticed, leading to systematic errors in determining the angle between proteins and, consequently, to wrong structures. Comparison with high-resolution crystal structure of the same complex is unlikely to help verifying the structure in this case, as the differences between solution and crystal structures in principle can be attributed both to crystal packing artifacts and to differences in alignment between different solution samples. To ensure the same alignment, the required control experiments in principle should include multiple sample preparations in different alignment media and repetitive measurements, which is very laborious and rarely done. Controlling the correct ratio of protein components in sample mixture presents a challenge by itself, if one of the proteins is unlabeled and hence invisible in heteronuclear spectra.[6] In the

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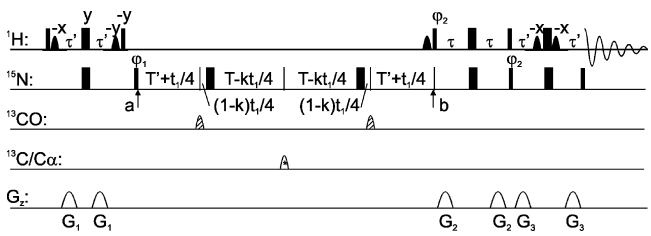

Figure 1. Pulse sequence for the IDIS-RDC-TROSY experiment. Narrow and wide bars represent 90° and 180° pulses, respectively. Solid shapes are selective pulses applied to the water magnetization. The hatched shapes are Q3 pulses applied on resonance to the CO spins. When the hatched pulses are present, the open shape marked with asterisk is a Q3 pulse applied off resonance to the Cα spins to refocus the NCα coupling. In the absence of the hatched pulses it is an adiabatic smoothed chirp pulse centered at 100 ppm, refocusing the NCα coupling as well as removing signal modulation by the NCO coupling in t_1. The delays are $\tau = 2.3$ ms ($1/(4^1J(\mathrm{NH}))$ reduced to partially compensate for relaxation losses), $\tau' = \tau$ reduced by the length of the selective pulse, constant time delay $T = 16.4$ ms ($4T = 1/(^1J(\mathrm{NC}'))$), $T' = T$ reduced by the initial value of $(1 - k)t_1/4$ and additionally reduced to compensate for the contribution of chemical shift evolution during the 90° ^{15}N pulses. The value of k is given by $4*T*sw_1/n$, where n is the number of complex data points in the indirect dimension. For each such data point, four interleaved experiments are recorded and stored separately, with/without the hatched pulses and with φ_2 changed by 180°. Separating experiments 1 and 2 versus 3 and 4 and adding and subtracting odd and even experiments yields the desired subspectra of the differently labeled polypeptide components and the different components of the NH doublet in F2. All pulses are x phase except where otherwise noted. The phase cycles are $\varphi_1 = y, -y, x, -x$ or $y, -y, -x, x$ for consecutive pairs of experiments; $\varphi_2 = -y$; $\varphi_{\mathrm{receiver}} = y, -y, -x, x$. The gradients are sine shaped and have an amplitude of 16, 10, and 24 G/cm, respectively. Phase-sensitive spectra are obtained by using a different setting for φ_1 and inverting φ_2 after pairs of experiments. The complete description of the pulse sequence with more detailed references is included in the Supporting Information. The pulse sequence program for Bruker spectrometers is available from the authors.

presence of exchange between bound and unbound states, component ratio is expected to affect the alignment. Therefore, strictly speaking, the main advantage of RDCs, i.e., providing the *accurate*, *direct and self-consistent* information about relative orientation of protein units *within a common frame*, no longer applies for combined data obtained from different sample preparations. The alternative strategy of using both proteins in ^{15}N-labeled form and measuring RDCs all in one sample can lead to a substantial signal overlap in the spectra and is not practical for larger systems. Here we propose a solution to this problem. A new method is suggested for measuring HN RDCs for both protein complex components *in the same sample* simultaneously, yet separately, avoiding the increased signal overlap. Both proteins are positioned within the common coordinate frame. The direct measurement of relative orientations of complexed proteins thus becomes possible, and the RDC-based approach well-developed for single-chain multidomain proteins[3,7] becomes directly applicable to protein complexes.

Results and Discussion

The 2D IDIS-RDC-TROSY experiment (Figure 1) for RDC measurement is based on the isotopically discriminated (IDIS) NMR principle.[6] Both proteins in the complex should be labeled, one with ^{15}N and another with ^{15}N and ^{13}C isotopes. Similar to other experimental schemes, HN couplings are measured as a distance (in Hz) between signal components of undecoupled multiplets: here TROSY and anti-TROSY components[8−10] are used. In the IDIS-RDC-TROSY, these signal components are additionally separated into different subspectra, according to the presence or absence of NC′ coupling. As such coupling is present only for doubly labeled protein, the scheme allows observation of only one protein per subspectrum, even when two proteins are mixed together. Four experiments in total are recorded for each data point in the indirect dimension. As in the original IDIS-TROSY,[6] two experiments are performed with or without the C′ pulses (shown hatched in Figure 1), and off-resonance C_α pulses or adiabatic carbon pulses, respectively (Figure 1). This leads to the desired isotopical discrimination of the ^{15}N−^{12}C′ and ^{15}N−^{13}C′ moieties. In addition, each pair of experiments is repeated with φ_2 (Figure 1) changed by 180°. Thereby a different, anti-TROSY component of the NH multiplet (shifted in F2) is selected, namely the one that is slower relaxing with respect to ^{15}N, but faster relaxing with respect to ^{1}H.[8] Separation of experiments 1 and 2 versus 3 and 4, as well as linear combination of odd and even experiments, results in four subspectra for the two types of N−C′ moieties and the two components (TROSY and anti-TROSY) of the NH doublet in F2, respectively. This allows the measurement of the HN couplings for two proteins separately (with reduced signal overlap), but simultaneously (in the same sample, for exactly the same orientation of the protein complex). The values of HN RDCs are readily obtained as the difference between the HN couplings in the aligned and isotropic states. It should be noted that for isotropic samples the values of $^1J(\mathrm{NC}')$ coupling constants are uniform (15 Hz) across the protein. For anisotropic samples these values additionally become modulated by the residual dipolar couplings, dependent on the orientation of individual NC′ vectors relative to the magnetic field. In practice, the usage of the same value of $4T$ (Figure 1) for all samples does not give rise to noticeable spectral artifacts. No significant "wrong" signals appear in the subspectra (e.g., see Figure 2). The sensitivity of the IDIS-RDC-TROSY experiment and the level of cross-talk between differently labeled protein components are the same as for the original IDIS-TROSY.[6]

The performance of the experiment was assessed using the 22.6 kDa barnase−barstar complex[11] partially aligned in 5% liquid crystalline (LC) media[12] (Figure 2). The proposed experiment enabled clean separation of signal components into four different subspectra. Despite the relatively high viscosity of LC media and substantial degree of alignment (with 31 Hz quadrupole splitting for ^2H_2O deuterium signal), no significant drop in intensity of anti-TROSY components due to relaxation was observed (Figure 2). The chosen anti-TROSY component largely preserves ^{15}N magnetization during the 66 ms $4T$ period used in the pulse program (Figure 1). (In the alternative, trial version of IDIS-RDC-TROSY experiment, a significant signal loss due to relaxation was observed when another anti-TROSY component was selected, which was shifted upfield along F1). Hence, this experiment will be useful for complexes well in excess of this molecular weight. In comparison with the

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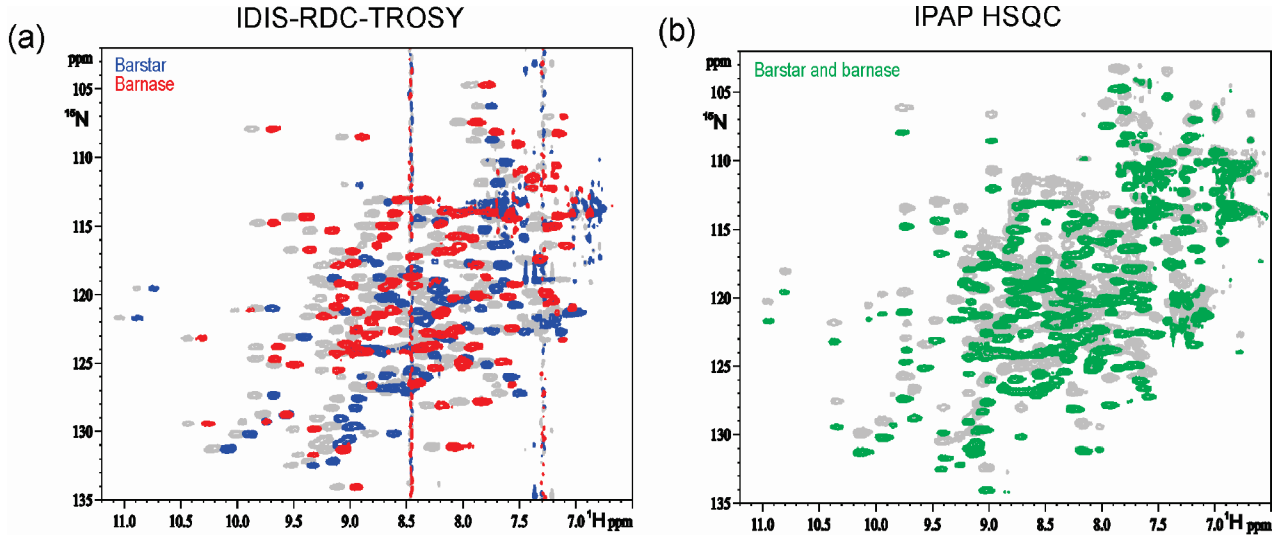

Figure 2. Overall comparison of IDIS-RDC-TROSY and IPAP HSQC[13] spectra of protein complex. The sample is an equimolar mixture of 0.2 mM ^{15}N,^{13}C-labeled barnase and ^{15}N-labeled barstar[11] in 5% C8E5/octanol liquid crystalline media[12] aligned in the magnetic field at 20 °C. (a) IDIS-RDC-TROSY ^{1}H−^{15}N(^{12}C) and ^{1}H−^{15}N(^{13}C) subspectra are overlaid. Red and blue signals, belonging to barnase and barstar, respectively, are observed separately in different subspectra. This enables measurement of one-bond HN couplings for each of the two proteins independently, while they are part of the same complex. The t_1 noise stripes at 7.29 and 8.45 ppm originate from sharp signals from unlabeled low-molecular components of the sample. (b) In the conventional IPAP-HSQC experiment[13] the signals from the two proteins in the complex (green) are indistinguishable from each other and are heavily crowded. The gray shades in (a) and (b) denote the positions of the second component of each amide signal doublet observed in different subspectra. The distance between the two components is used for the measurement of HN couplings, in direct F2 and indirect F1 dimensions for IDIS-RDC-TROSY and IPAP-HSQC, respectively.

conventional IPAP-HSQC,[13] the signal overlap and spectral complexity are dramatically decreased, while the overall experimental sensitivity is very similar (Figure 2; also see Supporting Information for more detailed spectral comparison).

The proposed IDIS-RDC-TROSY measures signal splitting in the directly observed F2 (^{1}H) spectral dimension, yielding RDC(F2). Different to that, the conventional IPAP-HSQC[13] experiment, which is often used as a RDC-measuring benchmark method, obtains the splitting in the indirect F1 (^{15}N) dimension, yielding RDC(F1). Although measurement of RDCs as splittings in the F1 dimension by IPAP-HSQC has well-known advantages (absence of major artifacts and simple line shape with a single peak maximum), it suffers from deterioration of the upfield signal component due to relaxation.[14] This limits the size of the systems that can be studied by this method. Reducing signal-to-noise ratio for this upfield component also decreases the attainable precision of the measured couplings.[14] For the barnase−barstar complex used here, the upfield signal components of IPAP-HSQC were indeed noticeably deteriorated in intensity, whereas both TROSY and anti-TROSY signal components of IDIS-RDC-TROSY subspectra were equally well preserved (for the detailed comparison see Supporting Information). The drawbacks of measuring signal splitting in F2, however, include much larger linewidths and more complex lineshape with multiple maxima, due to unresolved $^{3}J(H^{N}-H^{\alpha})$ couplings and ^{15}N−^{1}H and $^{1}H^{N}-^{1}H^{\alpha}$ cross-correlated dipolar relaxation.[10,14] As a consequence, the separation has to be measured between signal centers rather than between maxima. The expected precision of RDC(F2) determination is therefore inherently lower. Previously it was also demonstrated that the RDCs measured in F2 are systematically smaller than those measured in F1 dimension, which was explained by the effects of cross-correlated dipolar relaxation and influence of unresolved $^{3}J(H^{N}-H^{\alpha})$ couplings.[10] Moreover, signal phase distortions in IDIS-RDC-TROSY due to mismatch between the delay for ^{1}J(NH) evolution τ (see Figure 1) and apparent value of the HN coupling in anisotropic samples lead to noticeable shift in apparent signal positions. In principle, these phase distortions can be corrected when selecting and analyzing the corresponding 1D spectral slices.[15] However, it is worth noting that for the similar linewidths generally observed within a protein, these shifts in signal positions are largely proportional to the value of phase distortions,[14] which would simply result in additional systematic scaling of the values of RDCs measured in F2. We show in the following sections that these artifacts do not present a problem for the application of the proposed method and can be largely ignored. A mismatch of the delay τ' does not result in any phase distortion, but in a cross talk in the selection of the TROSY and anti-TROSY signal components. As the intensities of these "wrong" peaks do not exceed a few percent of the normal signal intensities (3% intensity for 20% mismatch) and the components are reasonably well separated, the analysis of the data is not compromised.

To check if RDC scaling and increased experimental errors have a detrimental effect on the alignment tensor parameters and, more importantly, on the derived angle between proteins, we measured and compared the RDC values using both IDIS-RDC-TROSY and IPAP HSQC. Despite the complex signal line shape in F2, we found that the signal centers can be conveniently identified, e.g., by fitting the F2 signal slices to the symmetrical Gaussian shapes using the commonly available NMR software tools (see Experimental Section). This fitting procedure effectively finds a center of mass of the peak in F2 and feeds the necessary information directly into the peak lists, which can be conveniently analyzed further. Such a fitting routine in principle can be automated. The estimated experimental uncertainty for

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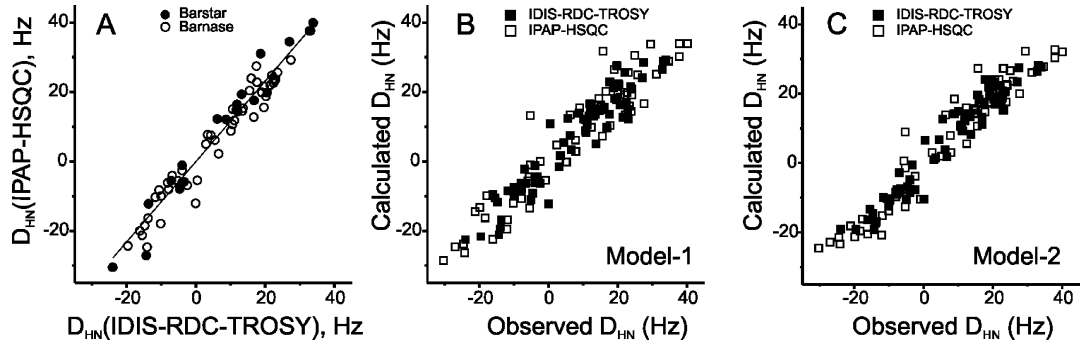

Figure 3. Comparison of RDCs values (D_{HN}) measured by IDIS-RDC-TROSY and IPAP-HSQC for the barnase−barstar complex. (A) Correlation between dipolar couplings measured using two different experiments. The linear regression slope is 1.16 ± 0.03. Data points for barstar and barnase within the same complex are shown separately. (B) Experimental RDCs obtained from IDIS-RDC-TROSY and IPAP-HSQC versus those predicted from the nonoptimized structural Model-1. Pairwise rms deviations are 4.71 and 5.52 Hz, respectively. (C) Experimental RDCs obtained from IDIS-RDC-TROSY and IPAP-HSQC, versus those predicted from the optimized structural Model-2. Pairwise rms deviations are 3.81 and 4.65 Hz, respectively. Model-2 is expected to have reduced structural noise due to additional adjustment of the geometry of attached hydrogen atoms taking into account the RDC-derived angular restraints.

Table 1. Quality of Fit between the Experimental and Back-Calculated RDC Data and Comparison of Alignment Tensor Parameters for Protein Complex Models without and with Optimized Hydrogens

structural segment selected		rms, (Hz)[a]	*Q*-factor[a]	correlation coefficient[a]	D_A^{HN} (Hz)[b]	rhombicity[b]
			IPAP HSQC			
Model-1, without optimization[c]	barnase+barstar	5.52	0.323	0.950	17.767	0.462
	barnase only	5.81	0.363	0.933	16.444	0.501
	barstar only	3.65	0.199	0.985	19.406	0.389
			IDIS-RDC-TROSY			
	barnase+barstar	4.71	0.326	0.950	15.218	0.404
	barnase only	4.88	0.346	0.938	14.812	0.427
	barstar only	3.54	0.241	0.979	15.556	0.385
Model-2, RDC-optimized[d]			IPAP HSQC			
	barnase+barstar	4.65	0.301	0.965	16.468	0.360
	barnase only	4.04	0.283	0.968	14.958	0.428
	barstar only	4.39	0.255	0.976	18.858	0.218
	IDIS-RDC-TROSY					
	barnase+barstar	3.81	0.292	0.968	14.154	0.286
	barnase only	3.56	0.285	0.968	13.388	0.338
	barstar only	3.57	0.259	0.977	15.163	0.211

[a] rms deviation between the observed and calculated RDCs and *Q*-factor are supplied by the DC program.[29] [b] Tensor parameters obtained using SVD[16] fitting using the program DC.[29] D_A^{HN} is the normalized axial component of the alignment tensor. [c] Hydrogen atoms were added to the crystal structure of barnase−barstar complex using standard geometry. The molecular frame of the structure coincides with the frame of alignment tensor derived from IPAP-HSQC experimental data. [d] Same heavy-atom positions and orientation as in Model-1 structure, but with the hydrogen atom positions optimized with RDC-based energy terms included.

RDC(F2) and RDC(F1) values was ±3 and ±2.5 Hz, respectively. It should be noted that the test sample used here contained barnase−barstar complex at a very low concentration (0.2 mM) leading to overall poor signal-to-noise ratio, a situation typical for the "real life" samples. More concentrated samples with better signal-to-noise ratio are expected to yield higher precision in RDC measurements. Experimental RDC(F2) and RDC(F1) have a correlation coefficient of 0.975, and the scattering of experimental points both for barnase and barstar are very similar (Figure 3A). As expected, RDC(F2) appears to be systematically scaled down by a factor of 1.16 relative to RDC(F1), with 4.0 Hz root-mean-square (rms) deviation between the two sets. These experimentally measured RDCs were then compared to the structure-based calculated values. The fitting was done using singular value decomposition (SVD) algorithm[16] which also determines axial and rhombic components of the alignment tensor and the orientation of the principal alignment frame relative to the molecular frame (as Euler angles). Two full-atom structural models of the barnase−barstar complex were used, Model-1 and Model-2. The second model had hydrogen atom positions additionally optimized against RDC(F1) data to reduce the possible effect of local structural imperfections (structural noise), caused by using the idealized covalent geometry. Orientation of both models was the same and for convenience of analysis, corresponded to the alignment tensor also determined from the RDC(F1) data. This enabled the estimation of the change of alignment tensor orientation relative to the chosen reference molecular frame when using different RDC data for the calculations. Both measured RDC(F2) and RDC(F1) correlated comparably well with the predicted values of RDCs (see Table 1). Not surprisingly, the correlation was noticeably improved when Model-2 was used for the prediction (Figure 3B,C). rms deviations from the calculated values were slightly lower for the IDIS-RDC-TROSY data, although the Q-factors were very similar (Table 1). The quality of fit between the calculated values of RDCs and those measured by the IDIS-RDC-TROSY is comparable to that expected for medium-resolution structures.[14] The overall scaling down of RDCs(F2),

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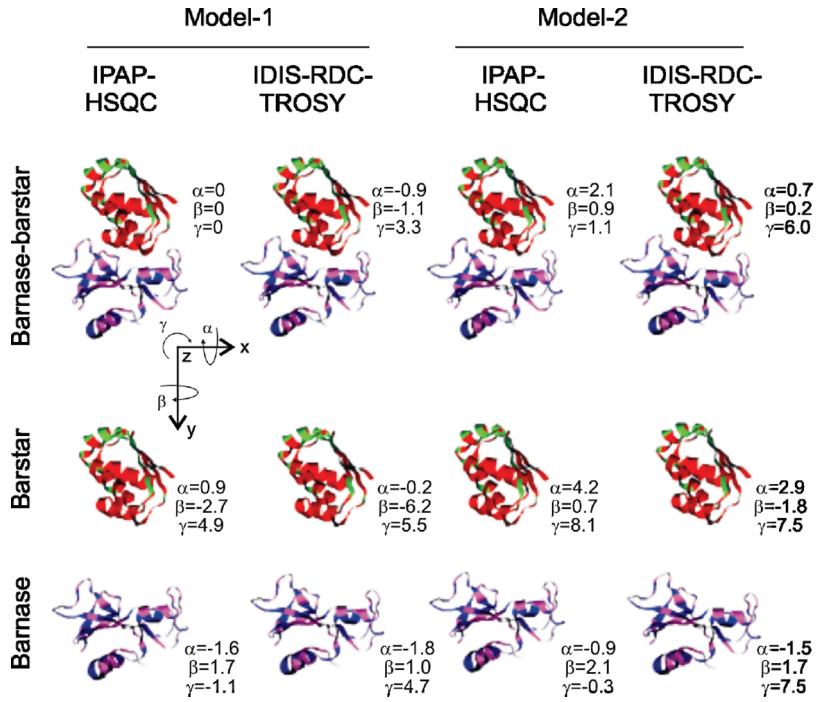

Figure 4. Orientations of structures of the barnase−barstar complex and of separate barnase and barstar units relative to the measured alignment tensor frames. The alignment frames were derived from the RDC data obtained using IPAP-HSQC and IDIS-RDC-TROSY experiments. Barstar and barnase are shown in red and blue, respectively, with the sites for which RDCs were measured marked in green and magenta, respectively. Each individual structure is rotated so that its molecular frame coincides with the principle alignment frame. The values of Euler angles α, β, and γ defining rotations around x, y, and z axes, respectively, are shown. The orientation of the reference Cartesian principal alignment tensor frame is displayed on the inset.

as compared to RDCs(F1), simply leads to scaling down of the values of axial D_A^{HN} and rhombicity components of alignment tensors (Table 1). However, the angular orientations of the tensors (Euler angles relative to the reference molecular frame) are essentially preserved (Figure 4). Moreover, subdividing the complex structure into barnase and barstar segments yielded very similar orientations of their individual tensor frames relative to the chosen molecular frame, to within several degrees (Figure 4 and Table 1). Overall, the effect of using RDCs(F2) (which are systematically scaled down compared to RDCs(F1)) is similar to that of using more dilute LC media for the protein alignment: only the axial component and rhombicity are affected but not the tensor orientation. The correct coordinate frame orientation was similarly well preserved when using either nonoptimized Model-1 or optimized Model-2. Differences in the molecular orientations relative to the alignment frame are difficult to notice upon just a visual inspection of structures (Figure 4). This demonstrates that the errors associated with the individual RDC measurements do not significantly affect the orientation of rigid structural units relative to the external coordinate frame.

The price paid for the convenience of the IDIS-RDC-TROSY experiment is a slightly lower precision of individual RDC measurements. Interestingly, addition of artificial 2−3 Hz rms Gaussian noise (as opposed to usually much lower experimental uncertainty) has been proposed previously[16] while running the SVD calculations to allow for structural model imperfections. This is similar to the 3 Hz experimental uncertainty afforded by the IDIS-RDC-TROSY here. In theory, a minimum of five RDCs are required per each protein to determine its orientation tensor parameters in the absence of experimental and structural noise. In practice, more than 20 RDCs are required to obtain reliable tensor parameters.[17] The errors in determining the angular parameters reduce as the number of measured RDCs increases.[17,18] Due to isotopic discrimination and hence reduced signal overlap (see Figure 2), a much larger number of RDCs can be measured using IDIS-RDC-TROSY for each of the two interacting proteins than by using IPAP-HSQC for the same sample. The effects of local structural imperfections (structural noise) and errors in measurement of individual RDCs are expected to be mostly averaged out in the process of data fitting, yielding the correct relative orientation of the two proteins. It should be noted that the relative orientation is subject to the usual 4-fold degeneracy (defined by the 180° rotations of the Cartesian coordinate frame), which however can be resolved if more than one alignment medium is used.[7]

Consistent complex orientation in different sample preparations has been achieved recently by using lanthanide ions and pseudocontact shifts as a source of angular and dynamic information.[19] Unlike that method, the IDIS-RDC-TROSY does not require the presence of lanthanide binding sites or labels and uses standard isotope labeling schemes readily available for recombinant proteins, making it more universally applicable for obtaining angular restraints for protein rigid-body docking. Although the practical upper molecular size limit for the IDIS-RDC-TROSY experiment remains to be determined, it is expected to be in line with other methods for measuring RDCs.[3,7,20] Protein deuteration is expected to reduce the effect of cross-correlated $^{15}N-^{1}H$ and $^{1}H^{N}-^{1}H^{\alpha}$ dipolar relaxation,

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remove $^3J(H^N-H^\alpha)$ couplings and narrow lines in F2, raising this size limit significantly and increasing precision and accuracy of RDC(F2) measurements. As the IDIS-RDC-TROSY spectral data for both proteins are obtained simultaneously, there is no need to compensate for differences in sample heating which would be otherwise required if separate pulse sequences were employed to record isotopically filtered spectra for each of the two proteins. Intensities of TROSY signal components in IDIS-RDC-TROSY directly reflect the relative amounts of both proteins, thus helping with the sample preparation and establishing stoichiometry.[6] Conveniently, the same signals can be used for chemical shift mapping of the binding surfaces of both proteins.[6] In principle, this makes IDIS-RDC-TROSY a simple and universal experiment for obtaining the information (i.e., relative angular orientation and chemical shift mapping of the molecular interface) potentially required for the fast automated rigid-body docking of protein complexes. It may be anticipated that due to limitations of rigid-body docking, which may underestimate structural changes caused by the complex formation, the docked structural models will contain a strong structural noise. In this situation, the overly precise experimental RDCs may not be necessary, and convenience of IDIS-RDC-TROSY may become a critical factor. Previously uncertainty in alignment tensor determination was assessed dependent on either small experimental errors in RDCs or on structural noise.[17] More simulation studies are however required to assess quantitatively the effect of large experimental uncertainty in RDC determination (i.e., several Hz) combined with the medium-to-large structural noise on the relative orientation of protein units in complex.

Conclusion

The proposed IDIS-RDC-TROSY method for RDC measurement uniquely ensures that two proteins forming a complex are positioned *exactly* within the same orientational frame, relative to the external magnetic field and to each other. Here, we have demonstrated that although the individual errors in RDCs measured by the IDIS-RDC-TROSY for nondeuterated proteins may be as large as several Hz, and the RDC values themselves are systematically scaled down, this does not translate into significant difference in the obtained orientation of proteins. The method also dramatically simplifies spectral analysis. Identifying separate subsets of signals belonging to each protein may assist the RDC- and structure-based signal assignment for individual proteins in the complex.[21,22] In some cases, sequence specific signal assignment and 3D structure may be already available for the free proteins. As complex formation often leaves a significant number (>20) of assigned signals unaffected, the RDCs measured for such assigned signals from both proteins in complex should yield information on their relative orientation. In this situation labor-intensive complete signal assignment of proteins in bound form might not be necessary at all. The proposed experiment thus can conveniently provide experimental data for rigid-body docking or for complete protein complex structure determination.

The described method also uniquely enables direct observation of global or local reorientation of proteins in complex in response to external stimuli, e.g. addition of unlabeled ligand(s) to the sample. Such experiments would be challenging if performed using separate samples comprising different combinations of labeled−unlabeled proteins. The interpretation of the effect of ligand addition in such a situation may be highly ambiguous, and the preservation of complex alignment in different samples during titrations can no longer be assumed. The same considerations are valid for such variable parameters as pH, temperature, or pressure, all of which can change alignment between different samples. The studies of allosteric changes in response to external stimuli can assist in drug design targeted at protein complexes.[23] Measuring RDCs for both proteins simultaneously in the same sample can resolve the possible conflicting data on relative protein orientation in crystal and in solution complexes. The experiment also opens up a way to study complexes consisting of more than two components, by combining angular restraints obtained for several protein pairs within a complex.

Experimental Section

Uniformly ^{15}N,^{13}C-enriched barnase was produced as described[24] except expression here was under the transcription control of the thermoregulated bacteriophage λ P_R promoter. An overnight culture of *Escherichia coli* cells BL21(DE3) harboring the barnase expression vector was grown at 27 °C in a minimal medium M9. Cells were collected by centrifugation and then resuspended at OD_{550} = 0.5 in M9 medium containing $^{15}NH_4Cl$ and ^{13}C-glucose. Expression was induced by increasing the growth temperature to 37 °C, and then the cells were grown overnight. The barnase was secreted mainly into the growth medium, from which it was extracted onto phosphocellulose. The enzyme was purified further by hydrophobic (Phenyl Sepharose FF), cation exchange (Mono-S), and gel filtration (Sephadex G50) chromatography. The yield of barnase exceeded 11 mg/L of medium. Uniformly ^{15}N-enriched C40,82A barstar was produced as described previously.[25]

The experiments were conducted on a 600 MHz spectrometer (Bruker) equipped with a *z*-gradient TXI cryoprobe. The sample contained an equimolar mixture of 0.2 mM ^{15}N,^{13}C-labeled barnase and ^{15}N-labeled barstar[11] in a buffer consisting of solely 25 mM arginine glutamate and 10 mM DTT, 95%/5% $H_2O/^2H_2O$, to obtain the best cryoprobe sensitivity.[26] The anisotropic sample was prepared in 5% C8E5/octanol liquid crystalline media[12] aligned in the magnetic field at 20 °C. The IPAP-HSQC and IDIS-RDC-TROSY data were acquired under the same conditions. The total acquisition time for each experiment was 19 h. The maximum evolution time in the indirect dimension was 36 ms. After data splitting prior to Fourier transformation, each subspectrum contained 76 complex points in the indirect dimension, with 2048 points in the direct dimension. The data were linear predicted forward for another 76 complex points in the indirect dimension.

Sequence-specific signal assignments of complexed barnase and free barstar were taken from BioMagResBank entries 7126 and 6227, respectively. As the assignment of barstar in complex was not available, only the assigned signals preserving their positions upon complex formation were used for the RDC measurements. To allow the direct comparison of RDCs measured using two different techniques, barstar and barnase signals overlapped in IPAP-HSQC spectra were also excluded from the analysis, which constituted around 22% of all assigned signals. In total, 46 barnase and 21 bartar unambiguously assigned RDCs were obtained. The

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splitting between signal centers, rather than between signal maxima, was measured for IDIS-RDC-TROSY by fitting each signal slice in F2 to a Gaussian shape using the standard feature of software Sparky.[27] The uncertainty of RDC measurements was estimated by comparing them with those where signal centers were located manually by visual inspection of cross-peak contours. The structural models of the barnase-barstar complex used for back-calculation of RDCs were based on a 2.0 Å resolution crystal structure (PDB entry 1BRS, chains F and J). Model-1 had hydrogen atoms added using the online server MolProbity (http://molprobity.biochem.duke.edu), without further optimization. The whole structure was then rotated so that its molecular frame coincided with the frame of the alignment tensor calculated on the basis of IPAP-HSQC experimental RDC data, yielding the reference "non-optimized" Model-1 structure. In Model-2, which had the same orientation, the hydrogen atom positions were additionally optimized using CNS[28] with the RDC (measured by the IPAP-HSQC experiment) energy term included. Theoretical values of RDC and alignment tensor parameters, as well as the quality factor Q, were calculated and analyzed using the DC program from the NMRPipe software package,[29] with default parameters for SVD[16] fitting.

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Supporting Information Available: Details regarding pulse program design with a complete list of references; comparison of IDIS-RDC-TROSY with conventional IPAP-HSQC spectra; pictorial representation of alignment tensor orientations; RDC values measured for barnase−barstar complex which were used in the current analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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