Weak alignment NMR: a hawk-eyed view of biomolecular structure

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Imposing a very slight deviation from the isotropic random distribution of macromolecules in solution in an NMR sample tube permits the measurement of residual internuclear dipolar couplings (RDCs). Such interactions are very sensitive functions of the time-averaged orientation of the corresponding internuclear vectors and thereby offer highly precise structural information. In recent years, advances have been made both in the technology to measure RDCs and in the computational procedures that integrate this information in the structure determination process. The exceptional precision with which RDCs can be measured under weakly aligned conditions is also starting to reveal the mostly, but not universally, subtle effects of internal protein dynamics. Importantly, RDCs potentially can reveal motions taking place on a timescale slower than rotational diffusion and analysis is uniquely sensitive to the direction of motion, not just its amplitude.

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Introduction

Under isotropic solution conditions, large internuclear dipolar couplings and other orientation-dependent magnetic interactions average to exactly zero as a result of Brownian rotational diffusion, which is many orders of magnitude faster than the time it takes to record an NMR signal. The resulting absence of anisotropic interactions is key to the sharpness of resonances typically seen in solution NMR spectra, thereby permitting resonance assignment for proteins as large as 80 kDa [1]. Under such isotropic conditions, the principal source of structural information is the ^{1}H-^{1}H nuclear Overhauser effect (NOE), which corresponds to semi-quantitative distance information for proximate pairs of hydrogens and has been the mainstay of NMR structure determination [2]. The information contained in anisotropic interactions can be recovered by generating a very weak force on the protein that results in a small tunable degree of alignment with respect to the magnetic field. Under such conditions, the instantaneous distribution of protein orientations in the sample is no longer uniform, although in practice the deviations from uniformity are kept very small (typically on the order of 10^{-3}). As a consequence, the orientation-dependent dipolar interaction, averaged over the time it takes to collect the NMR signal (tens of milliseconds), is scaled down to a non-zero value. At the same time, the nearly (~99.9%) complete removal of the large anisotropic dipolar interactions results in spectral simplicity and sensitivity that are comparable to that of conventional solution NMR, while nevertheless permitting measurement of the time-averaged orientation of internuclear vectors. For intrinsically very large interactions, such as the one-bond dipolar coupling between a ^{15}N or ^{13}C nucleus and its directly attached hydrogen, even after being scaled down by orders of magnitude, the residual dipolar couplings (RDCs) can be measured quite accurately. Initially, the feasibility of such measurements was demonstrated for paramagnetic myoglobin [3]. Subsequently, more generally applicable alignment procedures have been developed that rely on introducing anisotropic 'barriers' into the solution, either by means of a suspension of particles ordered in a liquid crystalline manner [4] or by using a very dilute (2–7% w/v) anisotropically compressed acrylamide gel matrix [5]. The most widely used liquid crystalline media include oriented bilayers, filamentous phages and rod-shaped cellulose particles; these have been reviewed extensively in recent years [6–10]. Anisotropic gels tend to be most generally applicable, as they are detergent resistant and can be used over the entire pH and temperature ranges applicable to biological solution NMR. However, they can decrease the rate of rotational diffusion in a manner that depends non-linearly on the volume fraction occupied by the acrylamide gel [11,12] and it can be problematic to diffuse larger molecules into the gel matrix. Various recent advances can alleviate these problems, including the use of electrophoresis and the introduction of charged components into the gel matrix, allowing higher levels of gel hydration [13,14,15•,16••,17•].

This review addresses recent advances in techniques for the measurement of RDCs and anisotropic chemical shifts, and will also highlight new developments in the use of anisotropic interactions for studying biomolecular structure and dynamics.

Measurement of anisotropic interactions

Structural information is contained not only in RDCs, but also in the effect of incomplete averaging of the chemical shift anisotropy (CSA), resulting in a residual effect of chemical shift anisotropy or RCSA. RCSAs are also scaled down by three orders of magnitude relative to the static CSA, yielding changes in chemical shift between isotropic and aligned samples that are on the order of parts-per-billion (ppb). Particular care is needed in the measurement of these effects, as slight changes in solvent conditions between the isotropic and aligned samples can also affect chemical shifts. Nevertheless, RCSA effects, in particular those of the backbone carbonyl carbon, $^{13}C'$, in proteins [18] and ^{31}P in nucleic acids [19], have proven useful in structure determination. A host of other RCSAs, including those of protein backbone ^{15}N and ribose ^{13}C, also may prove useful in this regard [20,21••]. Among the various RCSA measurements, $^{13}C'$ in proteins is particularly useful as it can readily be measured even for large perdeuterated proteins, using the quite sensitive TROSY-HNCO experiment, for which $^{1}D_{NH}$ couplings are often the only easily accessible alternative anisotropic parameters [22••].

For relatively small and well-behaved systems, with rotational correlation times less than about 10 ns (corresponding to about 20 kDa at room temperature), many different types of dipolar interactions often can be measured. Besides the large $^{1}D_{CH}$ and $^{1}D_{NH}$ couplings, these include the much smaller $^{1}D_{CC}$ and $^{1}D_{CN}$ couplings, as well as $^{2}D_{CH}$ [23–25] and longer range D_{HC} and D_{HH} couplings [26]. In favorable cases, interproton interactions over distances exceeding 10 Å can be detected [27].

Structure refinement and cross-validation

Provided that a very complete set of RDCs is available, it has been demonstrated for several model systems that structures can be calculated exclusively based on these anisotropic interactions, without recourse to NOE restraints. However, no new complete protein structures have yet been reported that are based exclusively on RDCs. One problem with using dipolar couplings in structure determination is that a dipolar coupling does not uniquely describe an internuclear vector orientation; it simply limits allowed orientations to the surfaces of two opposing cones [7]. Even selecting which of the two cones applies for a given RDC can be difficult, with a tremendous number of possible combinations (2^{N-1}) for N measured dipolar couplings. However, if an approximate structural model is available, inclusion of a dipolar energy term during the refinement protocol can fine-tune the structure such that the internuclear vector orientations become compatible with the measured RDCs. For relatively simple systems, such as a structure consisting of at most a few α helices, which typically can be recognized on the basis of their chemical shift and 'dipolar wave' pattern [28•], the relative orientation of the helices often can be established from RDCs, not only for water-soluble proteins but also for systems solubilized in detergents or embedded in lipid bilayers [15•,29••].

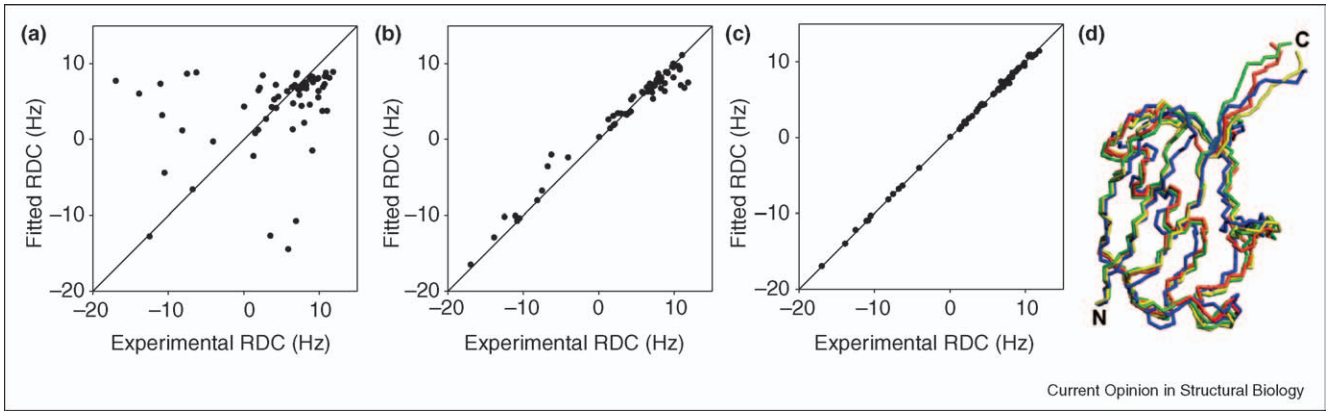
In numerous recent structural studies, RDC restraints have been included during structure refinement as a supplement to the regular NOE and torsion restraints, but using only a relatively small set of RDCs. Although this indeed can improve structural accuracy, the fact that a limited set of RDCs can be satisfied by the resulting structure is by no means proof of its correctness. For example, if only $^{1}D_{NH}$ couplings are measured, they frequently can be satisfied to within experimental error, even if the structure is incorrect. The inverse also applies: if we randomly permute the measured dipolar couplings (i.e. assign the dipolar coupling measured for each backbone amide to another randomly selected backbone amide in the protein), good agreement between these erroneous RDCs and the calculated structure can nevertheless be obtained (Figure 1b). Note that this agreement is better than when comparing the correct RDCs with a structure calculated in the absence of RDCs (Figure 1a), despite the structure having deteriorated as a result of incorrect input restraints. This example serves to show that, for cases in which very few RDCs are available, it may not be easy to tell to what extent the inclusion of RDCs improves the accuracy of a structure. Typically, inclusion of a correct set of RDC restraints will result in structures that exhibit more favorable Ramachandran map distributions than structures based solely on NOE data (see Figure 1).

In cases in which the number of experimental RDCs becomes larger than the number of torsional degrees of freedom (i.e. >2N backbone RDCs for an N-residue protein), the chance of serious errors in a structure becomes increasingly small. However, for validation purposes, it is recommended that randomly selected subsets of dipolar couplings are withheld from the input restraint list and used for cross-validation purposes [30,31]. Pearson's correlation coefficient between the omitted experimental couplings and those predicted for the structure then can be used as a measure of structural accuracy, at both the global and local level. In practice, reasonable-quality structures, comparable to a 2.5 Å X-ray structure, will yield correlation coefficients (R_P) of 90% or higher, whereas values as high as 99% can be obtained for structures that have been solved at very high resolution, such as the 1.1 Å X-ray structure of the GB3 domain [16••]. In practice, instead of R_P, the goodness of the correlation is often expressed as a quality factor (Q), defined as:

$$Q = \mathrm{RMS}(D_i^{obs} - D_i^{pred})/\mathrm{RMS}(D_i^{obs}) \quad (1)$$

where RMS refers to the root mean square function, and D_i^{obs} and D_i^{pred} are the observed and predicted RDCs for interaction i, typically normalized for the types of nuclei

Figure 1



Effect of dipolar couplings on structures calculated for the protein ubiquitin, in the absence and presence of 63 experimentally measured $^1D_{HN}$ RDCs. In each case, an identical subset of the deposited NMR restraints, consisting of 602 backbone amide NOEs, 140 methyl–methyl NOEs and 27 hydrogen bonds, was used. **(a)** Comparison of experimental RDCs and those predicted by the structure calculated without RDCs. **(b)** Randomized experimental RDCs versus those predicted by the structure when the incorrect, randomized RDCs are used as input restraints. **(c)** Same as (b), but with the correctly assigned RDCs used as input restraints. **(d)** Corresponding backbone structures of residues 1–74: yellow, no RDCs; blue, randomized RDCs; green, correct RDCs; red, high-resolution NMR structure (PDB code 1D3Z). The backbone coordinate rmsd relative to 1D3Z is 0.89 Å (no RDCs), 1.27 Å (with their assignments randomized) and 0.79 Å (correct RDCs). The respective percentages of residues in the most favored region of the Ramachandran map are 81, 62 and 87, versus 97% for 1D3Z.

involved when multiple sets of different couplings (e.g. C–H and N–H) are evaluated simultaneously. If the alignment tensor can be accurately estimated from the available data, the denominator in Equation 1 may be replaced by $(D_a^2[4 + 3Rh^2]/5)^{1/2}$, where D_a and Rh are the magnitude and rhombicity of the applicable alignment tensor, respectively [31]. This substitution makes Equation 1 independent of the non-uniformity of the distribution of bond vector orientations. An alternative R_{dip} factor is also in use, which is $\sqrt{2}$ smaller than Q [31]. If bond vector orientations are uniformly distributed, there is a direct relation between the Q (or R_{dip}) factor and Pearson's correlation coefficient between observed and predicted dipolar couplings, with $R_P = 0.9$ corresponding to Q = 42%, $R_P = 0.95$ to Q = 30% and $R_P = 0.99$ to Q = 14% [32]. It should be noted, however, that structure validation based on one-bond RDCs only reports the orientation of bonds and yields no direct information on translation. Therefore, for a multisubunit system, such as a protein–protein complex, low Q or R_{dip} values do not report on the accuracy of the intersubunit spacing of the model.

Even more so than for the analogous R and R_{free} factors in X-ray crystallography, it is important that RDC restraints are not included during structure calculations when reporting Q or R_{dip} values. As mentioned above, when relatively few ($\leq$1) RDCs per residue are available, the model can always be adjusted to fit these few RDCs, regardless of the correctness of the structure or the couplings. This is particularly true when structures are calculated in Cartesian instead of torsion angle space, when minor deviations from ideal bond angles and impropers can 'fudge' a better fit to experimental RDCs. Although validation by means of a Q or R_{dip} factor is only meaningful when the RDCs in question are not used as input restraints, recent literature does not conform to this practice, and it is perhaps useful to add the superscript 'free' in Q^{free} or R_{dip}^{free} to clarify the distinction.

When a given RDC is measured in different media, one could argue that measurement in one medium is independent of that in another medium, but in practice this is rarely the case. Therefore, for Q^{free} calculations, it is recommended that a given bond vector is excluded from all input restraints when RDCs have been measured in multiple alignment media [33]. In principle, a concern might be that multiple RDCs for any given group of atoms in a known substructure, such as $^1D_{NH}$, $^1D_{NC'}$ and $^1D_{C\alpha C'}$ for a peptide plane, are not independent of one another [34]. In practice, however, simulations indicate that, for randomly oriented peptide planes, there is very little correlation between $^1D_{NH}$, $^1D_{NC'}$ and $^1D_{C\alpha C'}$. On the other hand, if both $^1D_{C\alpha C'}$ and $^1D_{NC\alpha}$ are available for a single peptide plane, the nearly parallel orientation of the corresponding vectors requires that both are excluded from structure calculations if either of these couplings was to be used to derive Q^{free}. Similarly, up to seven dipolar couplings have been reported for a single nucleic acid base [23,24]; at most two such interactions can be included as structural restraints if any of the remaining RDCs (not parallel to those included) are to be used to calculate Q^{free}.

Fast answers to specific structural questions

The straightforward use of RDCs to provide direct and unambiguous answers to whether any given structural

model is compatible with a system studied in solution is a compelling aspect of this technology. For example, RDCs measured in a recent study of a C_3-symmetric homotrimeric enzyme involved in phosphoryl transfer elegantly revealed close similarities of the relative orientations of two of the three helices compared to those seen in the X-ray structure of a homologous system, but a distinct difference involving the kinking of a third helix [35•]. Questions that involve the relative orientations of units of known structure indeed are ideally suited to study by weak alignment NMR. In principle, the orientation of a structural subunit, which may be as small as a turn of α helix or as large as an entire domain, can be established (albeit at fourfold degeneracy) from as few as five RDCs per subunit. In practice, the coordinates of the subunit are not known at infinite accuracy and contain so-called 'structural noise', which adversely affects the precision with which the subunit's orientation can be established. However, the more RDCs available, the smaller the effect of structural noise [36]. For α helices, Opella and co-workers [15•] have noted that the pattern of dipolar couplings frequently fits an idealized helical structure better than the corresponding experimental X-ray structure, making such units particularly suitable for study by RDCs and offering a potentially powerful approach to the study of small, helical membrane proteins.

Frequently, questions may concern structural changes, such as the effect of mutations or ligand binding, particularly in relation to allostery. Provided that the structural changes between the two states of a given system are small and the molecular alignment tensor does not change much in its orientation or rhombicity, RDCs can be particularly sensitive reporters of the magnitude of the change. To first order, measurement of the change in the relative orientation of subunits or domains of the structure is then independent of structural noise. This allows accurate determination of the change using smaller numbers of measured RDCs than would be needed to derive their relative orientation *de novo*. As an example of this application, substitution of center dT nucleotides by dT analogs, with a C3′-endo/C1′-exo locked ring pucker, indicated a 6° bend of the B-form DNA helical axis toward the major groove, associated with the naturally occurring C2′-endo to C3′-endo sugar switching [37••].

Docking of intermolecular complexes

With the rapid increase in available genetic information, much attention is focusing on systems biology and biomolecular interactions in particular. Considering the often weak and transient nature of such interactions, the use of X-ray crystallography to address these questions can be problematic. NMR spectroscopy often also has its own problems, related to the typically large size of the complexes involved, and the very large amount of data and labor needed to solve such structures by conventional methods. For this reason, there is much interest in potential short-cuts that combine molecular modeling with the limited amount of experimental data that can be gleaned easily from NMR data [38•]. Both chemical shift perturbation, indicative of the region of a protein's surface most affected by the interaction, and saturation transfer techniques are particularly useful for this purpose [39•]. Other biochemical and/or biophysical data, including mutagenesis results and fluorescence, can be used to identify areas in which contacts occur. RDCs in turn can establish very accurately the relative orientations of interacting components in a complex and therefore provide an ideal complement to these other sources of information.

Incorporating RDC restraints in the process of modeling a biomolecular complex from its known constituents, often referred to as docking, can be carried out in a semi-automated manner [39•,40,41]. In contrast to *de novo* structure determination, full advantage of the alignment technology can be taken, even with the measurement of only a moderate number of RDCs per component of the complex. In principle, five couplings suffice to define the five independent components of the molecular alignment tensor. However, in practice, at least a few dozen $^1D_{NH}$ couplings are needed per subunit of the complex to mitigate the effect of structural noise [42•]. In cases in which the alignment tensor is known *a priori* to be axially symmetric, such as for a C_3-symmetric homotrimeric system [43•], the alignment tensor contains only three independent parameters and fewer RDCs suffice to define it.

If complexes are very weak, it can become impossible to reach the approximation of a pure complex, without free monomers being present in solution. Williams *et al.* [44••] elegantly solved such a case, a complex between HPr and IIAMan, by measuring RDCs in the presence of an excess of HPr, and separately measuring and correcting for HPr RDCs in the unbound state. The same group used RDC technology to solve the structure of a 34 kDa ternary complex composed of a double-stranded DNA oligomer, Hoxb1, and the Oct1 and Sox2 transcription factors [45•]. In other elegant applications, RDC technology was used to rapidly establish the type of complex formed in solution between calmodulin and various target peptides [46,47•].

Evaluation of dynamic processes

A very elegant and unambiguous method for exploring domain dynamics relies on paramagnetic alignment instead of external alignment of the protein. When only the N-terminal domain of calmodulin is chelated to Tb^{3+} or Tm^{3+}, the alignment of the N- and C-terminal domains can be established unambiguously by fitting their RDCs to the known structures of these domains. Chelation of the N-terminal domain yields alignment that is nearly an

order of magnitude higher than that of the C-terminal domain, providing a very direct measure of the flexibility of the linker. When combining such information with paramagnetic relaxation effects, an even more detailed outline of the conformational ensemble becomes feasible [48••].

Perhaps the most intriguing use of RDCs is in the study of dynamic processes. Conventional NMR relaxation studies can quantitatively evaluate the timescale and amplitude of bond vector motions on timescales faster than the rotational correlation time of a system (10^{-8} s). Albeit more qualitatively, NMR can also identify slower conformational exchange processes, on a timescale slower than $\sim 10^{-4}$ s, through their effect on transverse relaxation rates or the appearance of separate resonances. However, in the biologically important 10^{-8}–10^{-4} s range, NMR has a 'blind spot' that potentially can be filled by RDC analysis. RDCs report the average of a bond vector, integrated over the entire timescale of the measurement (i.e. milliseconds). In general, internal motion of a bond vector relative to the molecular alignment frame scales the size of the RDC relative to a static average orientation. This scaling factor is dependent on both the amplitude and the direction of such motion relative to the alignment tensor; scaling factors therefore will differ with the alignment medium used. A thorough theoretical and computational analysis has shown that quantitative evaluation of the underlying motional process is feasible in a model-free fashion if more than five different alignment media are available [49]. However, considering that, for small-amplitude motions ($<\sim\pm 20^{\circ}$), the averaged dipolar coupling falls very close to that of a static vector in the averaged orientation [50•], the RDC approach to studying dynamics is most robust for large-amplitude processes. Inversely, small discrepancies between measured dipolar couplings and those anticipated for a static model result in very large amplitudes for motions extracted from such data if they are entirely attributed to dynamic effects [51]; this has led to heated debate. An alternative strategy, using rapid exchange between two conformers, can reconcile the RDC and structural data to within experimental error using much smaller structural fluctuations, yielding increased cross-validation [52,53••]. In related work, S^2-dynamics-restrained multiple-conformer refinement of the NMR structure of ubiquitin in the absence of dipolar coupling data was shown to predict both the RDCs and sidechain J couplings considerably better than conventional single-conformer refinement [54••].

Although the dust has not yet fully settled on how much motion on a timescale slower than rotational diffusion is required of 'typical' proteins to reconcile RDCs and average structure, a statistically significant improvement is generally observed when invoking the Gaussian axial fluctuation (GAF) model of peptide bond N–H vectors instead of the commonly used model whereby the N–H vector diffuses in an axially symmetric cone [55]. In the GAF model, peptide group motions around the Cα–Cα vector of sequential residues are found to be of larger mean amplitude than fluctuations around the two axes orthogonal to this vector.

Even more debated has been the interpretation of RDCs in describing folding intermediates, pioneered by Shortle and co-workers [56]. In these highly dynamic systems, the approximation of a static average alignment breaks down and different conformers of the ensemble are predicted to align to different degrees [57••], biasing the outcome to favor the stronger-aligning extended conformations of the backbone. Nevertheless, important insight was obtained from RDCs regarding the monomer/trimer equilibrium of the trimerization domain of T4 fibritin and its thermal unfolding [58]. It is also conceivable that the coupling between structure and alignment could be accounted for quantitatively in the analysis, in which case RDCs will become another important set of parameters for addressing this important problem [59].

Conclusions

The introduction of weak alignment in solution NMR recovers the important orientational information lost in conventional solution NMR. In contrast to NOEs and J coupling restraints, the RDC restraints are not relative to nearest neighbors, but define orientations relative to a common frame and therefore have a 'global' character. Their use can sharpen considerably the definition of NMR-derived structures, and generally results both in a considerable improvement in Ramachandran map quality and in better agreement with crystallographically derived structures. The use of RDCs for independent cross-validation of structural accuracy is straightforward, although the correlation between coordinate accuracy and cross-validated Q^{free} factors is not unique: a low Q^{free} essentially guarantees accurate domain structures, although not necessarily a correct relative domain positioning; a high Q^{free} can be the result of moderate local errors, even while the global structure is of reasonable quality [60•].

The access provided by RDCs to the study of motions occurring on the timescale of microseconds is likely to enhance our understanding of dynamic processes involved in biologically relevant structural transitions, which often take place in this time regime. Most importantly, RDCs report not only on the amplitude of such dynamics but also on the direction in which the motions take place. Analysis and interpretation of such data still require further development of a comprehensive theoretical framework. However, considerable progress in this area is already being made [9,48••,49,50•,51,52,53••,54••, 55, 61••].

Update

Recent work by Skrynnikov and colleagues [60•] quantitatively evaluates the relation between dipolar cross-

validation and structural accuracy. Blackledge and co-workers [61••] show a distinct correlation between micro-second backbone dynamics and the degree of solvent exposure of the sidechain. Evidence of correlated motions of amide groups connected by hydrogen bonds is presented.

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