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.

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 \(^1H\)-\(^1H\) 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 $^{1}$DNH 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 $^{1}$DCH and $^{1}$DNH couplings, these include the much smaller $^{1}$DCC and $^{1}$DCN couplings, as well as $^{2}$DCH [23–25] and longer range $^{3}$DHC and $^{3}$DHI 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 $\alpha$ 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 $^{1}$DNH 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. $\geq$2N backbone RDCs for an N-residue protein), the chance of serious errors in a structure becomes increasingly small. However, for validation purposes, it is recommended that randomly selected subsets of dipolar couplings are withheld from the input restraint list and used for cross-validation purposes [30,31]. Pearson's correlation coefficient between the omitted experimental couplings and those predicted for the structure then can be used as a measure of structural accuracy, at both the global and local level. In practice, reasonable-quality structures, comparable to a 2.5 Å X-ray structure, will yield correlation coefficients ($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:

$$Q = \frac{\text{RMS}(D_{i}^{\text{obs}} - D_{i}^{\text{pred}})}{\text{RMS}(D_{i}^{\text{obs}})}$$

where RMS refers to the root mean square function, and $D_{i}^{\text{obs}}$ and $D_{i}^{\text{pred}}$ are the observed and predicted RDCs for interaction $i$, typically normalized for the types of nuclei
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
\[
\left( D_D a \frac{4}{5} + 3 \frac{R_h}{2} \right) / C_{138} = \frac{5}{2},
\]
where \( D_D a \) and \( R_h \) 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 improper 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 \( ^1D_{CaC} \) 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 \( ^1D_{CaC} \). On the other hand, if both \( ^1D_{CaC} \) and \( ^1D_{NCs} \) 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 C3-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 \( \alpha \) 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 \( \alpha \) 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 \( 6^\circ \) 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 fluoroscence, 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\*\*\*]. 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 \( 1D_{N\alpha} \) 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 C3-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 HPt and IIAMan, by measuring RDCs in the presence of an excess of HPt, and separately measuring and correcting for HPt 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). Although more qualitatively, NMR can also identify signals from conformational exchange processes on a timescale slower than ~10⁻⁴ s, through their effect on transverse relaxation rates or the appearance of separate resonances. However, in the biologically important 10⁻²–10⁻⁴ 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 (≪±20°), 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, δ⁰-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 debate 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 fibrin 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 Qfree factors is not unique: a low Qfree essentially guarantees accurate domain structures, although not necessarily a correct relative domain positioning; a high Qfree 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 coworkers [61**] show a distinct correlation between microsecond 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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References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


15. Ulmer TS, Ramirez BE, Delaglio F, Bax A: Evaluation of backbone protein positions and dynamics in a small protein by liquid crystal NMR spectroscopy. J Am Chem Soc 2003, 125:9179-9191. This study uses the 1.1 Å resolution X-ray structure of an Ig-binding domain of protein G as a starting point for its solution structure. Small deviations from peptide bond planarity are needed to satisfy the dipolar couplings, and N-H and Cα-Hα bond vectors are found very close to their idealized positions.


work also indicates that α helices tend to be more regular than observed in X-ray structures.


The alignment tensor is modulated by using two slightly different constraints of the core domain of MerF, rather than different alignment media. Structure calculation is based on both angular restraints from helical regions identified by their dipolar wave pattern and RDC refinement.


The reported structure of a 34 kDa trimer is based primarily on CH3-CH3 NOEs and RDCs.


This work shows that small changes in a structure as a result of a ‘mutation’ or other external factor can be identified with much higher accuracy than the structure itself, even when using only a very limited set of RDCs.


This work discusses and illustrates various sources of data that can be used to map interactions, and their combination with docking methods to generate structural models of biomolecular complexes.


This review discusses the use of cross-saturation experiments to identify the interfaces involved in intermolecular complexes. The technology is suited to the study of very large systems, provided that the moderately sized, labeled protein component exchanges between free and bound states on a timescale of seconds or faster.


The structure of a phosphoryl transfer complex was solved by taking advantage of the high-resolution structures already available of its components and the measurement of dipolar couplings in the complex.


A model is generated of a trimeric complex on the basis of RDCs, exploiting the C2-symmetric properties of the complex.


Arguably the largest structure solved by NMR in the absence of a prior detailed X-ray-based model. Due to the weak affinity of the complex, an excess of HPr was required and the RDCs of unligated HPr were subtracted.


The advanced application of RDC technology to solve the structure of a ternary protein-DNA complex.


The relative orientation of calmodulin’s two domains in a complex, identified on the basis of RDCs, defines the mode of peptide binding.


When alignment of a multidomain system is induced by the paramagnetism of a chelating ion, flexibility in the system has no effect on the alignment of the chelated domain, but results in decreased alignment of other regions of the system. This provides a very unambiguous view of internal flexibility on a timescale faster than milliseconds.


RDC analysis points to amplitudes of N-H motions orthogonal to the peptide chain that are about 10 larger than motions parallel to the chain direction.


The authors used RDCs previously reported for GB3 as input restraints to generate a two-conformer ensemble, and compared the two derived conformers as a model of correlated motion. This yields order parameters that exhibit remarkable correlation with those derived from NMR relaxation studies.

The authors calculated the ubiquitin structure to satisfy an ensemble rather than an individual structure, using 15N-derived $S^2$ restraints and a modified simulated annealing engine. This yields improved prediction of RDCs not used during structure calculation compared to a static structure calculated without RDCs, albeit not quite as good as static structures calculated with RDCs, for which a fraction is omitted for cross-validation purposes.


The interpretation of RDCs for a highly dynamic structure is shown to require particular care when the system is aligned by an external medium, such as a liquid crystal or a compressed gel; uneven weight factors for the different conformers bias the RDC-probed molecular dynamics. Analysis of a paramagnetically aligned system is found to be more straightforward.


Using dipolar couplings simulated for previously deposited bundles of NMR structures for which a crystal structure is also available, the authors evaluate the correlation between dipolar cross-validation and structural accuracy.


The authors identify standing waves of correlated motions across the $\beta$ sheet of the small protein G domain on the basis of a very large set of high-precision RDCs.