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On the Interpretation of Residual Dipolar Couplings as Reporters of Molecular Dynamics

Kai Fredriksson,[†] Martti Louhivuori,[‡] Perttu Permi,[†] and Arto Annila*[,†,‡,§]

Contribution from the Structural Biology and Biophysics Program, Institute of Biotechnology, Department of Physical Sciences, and Department of Biosciences, University of Helsinki, FIN-00014 Helsinki, Finland

Received March 25, 2004; E-mail: arto.annila@helsinki.fi

Abstract: The analysis of residual dipolar couplings from an ensemble of conformations to extract molecular dynamics is intricate. The very mechanism that is necessary to perturb overall molecular tumbling to generate nonvanishing residual dipolar couplings gives rise to convoluted data. The measured values are essentially weighted averages over conformations. However, the weights are not simply the populations of conformations. Consequently, the observed order parameter is not exactly the true measure of motion. In the case of paramagnetic alignment, the apparent order parameter is expected to depend on the number of torsions that separate the locus of interest from the paramagnetic site. In the case of alignment due to steric obstruction, the uneven selection of conformations by their differing Saupe order matrices leads to a bias in the residual dipolar couplings-probed molecular dynamics.

Introduction

A wealth of evidence is accumulating, showing that not only folded proteins but also weakly structured segments and flexible peptides convey biological functions.[1] In many cases, activity is understood to depend on a protein's dynamic and marginally stable nature.[2] These stimulating findings question the classical notion that a function arises from a specific three-dimensional molecular structure.[3]

Recently it has been demonstrated that molecular dynamics could be probed by an NMR parameter known as residual dipolar coupling (RDC).[4−8] In the case of a single conformation, i.e., a folded rigid structure, RDCs relate to internuclear vector directions with respect to the definite molecular coordinate system.[9−11] Small deviations from the average vector directions have been inferred to display molecular dynamics[4−7] using the model-free formalism,[12] but these interpretations have been challenged very recently.[13−15] Nonvanishing RDCs have been recorded also from denatured proteins, i.e., presumably unstructured polypeptides.[16] However, the analysis of RDC data becomes intricate when the frame of reference is time-dependent, i.e., the molecule undergoes substantial internal motion. Although strongly aligned small molecules in ordinary liquid crystals have been investigated earlier,[17] weakly aligned biological macromolecules in dilute liquid crystals display additional phenomena.

In the present work we examine the interpretation of RDCs as reporters of molecular motions with the emphasis on high mobility that generates numerous conformations. In other words, we adopt the ergodic hypothesis and consider the average over the statistical ensemble equivalent to the time average over the motional trajectory.

Theory and Calculations

According to the model-free formalism, the measure of motion in a locus i is the generalized order parameter squared,

$$S_i^2 = \frac{4\pi}{5}\sum_{k=-2}^{2}\langle Y_{2k}^{i}(\Theta,\Phi)\rangle\langle Y_{2k}^{i*}(\Theta,\Phi)\rangle \qquad (1)$$

where the brackets denote integration over the vector directions expressed by the spherical harmonics. To describe molecular

[†] Structural Biology and Biophysics Program, Institute of Biotechnology.
[‡] Department of Physical Sciences.
[§] Department of Biosciences.

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dynamics within the molecule, eq 1 would preferably be inspected in the molecular frame of reference. Nevertheless, when using RDCs as probes of molecular motions, it is instructive to consider eq 1 in the laboratory frame of reference, where the molecular tumbling of a diamagnetic molecule in isotropic solution alone is so effective that S_i^2 vanishes.[18] Therefore, it is necessary to impose at least a small degree of alignment along an average direction that we denote by Θ_o and Φ_o in *i*'s principal axis system (PAS). The alignment manifests itself as nonzero S_i^2 that are, for a completely rigid molecule, the same for every locus and thus denoted by S_o^2. Additional motions, i.e., that within the molecule about the average direction of alignment, manifest themselves after normalization by S_o^2 to give a measure of molecular dynamics in analogy with the earlier derivations.[5,7] The inspection of eq 1 in the laboratory frame of reference is especially useful when there is no obvious common frame of reference for the members of the ensemble, i.e., the molecule undergoes substantial internal motion.

For a pair of nuclei in the same locus, the residual dipolar coupling,

$$D_i = D_i^{\max}\sqrt{\frac{4\pi}{5}}\left\{S_{zz}^i\langle Y_{20}^i(\theta,\varphi)\rangle + \sqrt{\frac{1}{6}}(S_{yy}^i - S_{xx}^i)[\langle Y_{22}^i(\theta,\varphi)\rangle + \langle Y_{22}^{*i}(\theta,\varphi)\rangle]\right\} \quad (2)$$

appears to be a particularly convenient parameter to examine dynamics because it probes the time-average of a vector direction. We give the internuclear vector direction by θ and φ to distinguish it from the direction of the weakly aligned locus *i*, denoted by the capital letters Θ and Φ in eq 1. Usually it takes several RDC values per *i* to determine the components of the Saupe order matrix $\mathbf{S}_i$ and thus also the generalized degree of order[19] squared, S_i^2. The constant $D_i^{\max}$ expresses the absolute strength of the dipole−dipole coupling between the two nuclei. The dipolar coupling does not differentiate the sign of direction, and only even harmonics are detected.

As a flexible molecule traverses through various conformations, RDC values keep changing. According to the ergodic hypothesis, the equivalent description is an ensemble of *N* conformations to give a measured RDC value as a weighted average,

$$\overline{D_i} = \sum_{i=1}^{N} w_i D_i \quad (3)$$

that depends on the molar ratios w_i and via D_i on the vector directions Y_{2k}^i with respect to the alignments $\mathbf{S}_i$. When the motional amplitudes are large, the conformation-specific alignments differ markedly, and in general it becomes difficult to deduce the properties of the ensemble, i.e., the motional modes, from the measurements. However, we are able to illustrate the outcome for a random coil ensemble that can be described with only a few parameters. Most importantly, we will show that, for a flexible system, it is not immaterial how the overall molecular tumbling is perturbed to induce the required alignment. Consequently, an *apparent* order parameter will be measured instead of the true order parameter.

To provide understanding via analytical treatment, we model a completely denatured protein with a valence chain. A valence chain segment executes motion relative to its neighbor in a cone with angle of opening α. For a random coil polypeptide, the valence angle α is approximately $\pi/5$, i.e., 36°.[20] Such an ensemble is axially symmetric, and thus we will also drop the dependence on the azimuth angle in the notation, which leaves us with only the zeroth-order spherical harmonic. Undoubtedly, the valence chain model is too simple to give a precise description of a polypeptide, but it is accurate enough for transparent derivation of the main results. For a more detailed account, we use simulations.

Alignment Due to a Paramagnetic Center. First we consider the alignment due to the coupling of a large anisotropic magnetic susceptibility at a particular locus, e.g., a paramagnetic center, with the external magnetic field.[21] An RDC value at locus *i* is obtained by integration over the continuous and axially symmetric ensemble,

$$D_i = D^{\max}\int_\Omega W_i\langle Y_{20}^i(\theta)\rangle\, d\Omega \quad (4)$$

The locus-dependent weight W_i that is constructed from Gaussian distributions serves to describe the spread of conformations (see Supporting Information). The overall RDC value is thus the weighted sum of the RDC values of each single conformation, $d\Omega$, in the continuous ensemble of conformations Ω. It is important to realize that W_i contains the locus-specific alignment. It depends on how many segments there are from the locus *i* to the particular locus that houses the alignment power. This is the very model presented by Wallach.[22] In the case of a valence chain, W_i is a sum of geometric series dictated by the distance to the paramagnetic site and α (see Supporting Information). The apparent order parameter squared that is available from D_i values is obtained by integration over all of the conformations, The functions in the integral depend on the chain flexibility,

$$S_{\mathrm{app},i}^2 = \frac{4\pi}{5}\int_\Omega W_i\langle Y_{20}^i(\Theta)\rangle W_i\langle Y_{20}^{i*}(\Theta)\rangle\, d\Omega = \int_\Omega W_i^2 S_i^2\, d\Omega = W_i^2 * S_i^2 \quad (5)$$

i.e., α, and also on the variables that describe the ensemble, whereas the result from the integration, i.e., the apparent order parameter, depends only on α. The apparent order parameter squared is not the true measure of the motion but a convolution of S_i^2 with a function W_i^2, which is not a constant. In the spirit of convolution, the weight function and the order parameter are functions of conformations and integrable over the ensemble. Thus, the weight function operates on the order parameter to give the apparent order parameter.

We have calculated $S_{\mathrm{app},i}^2$ in the case of a paramagnetic site in the central segment. The values decrease as one moves farther away from the site of alignment (Figure 1).

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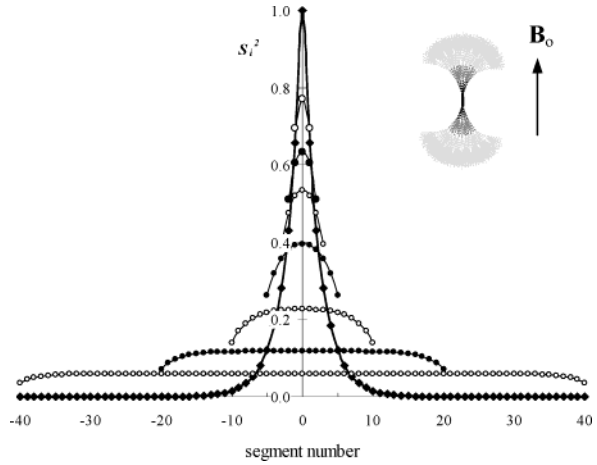

Figure 1. Apparent order parameter squared for valence chains with 1, 3, 5, 7, 11, 21, 41, and 81 segments when the most central segment aligns in a magnetic field (continuous solid line, ♦). For comparison, the unbiased order parameter squared is shown (●, ○). The inset illustrates the mechanism of alignment and the ensuing choice of the local frame of reference.

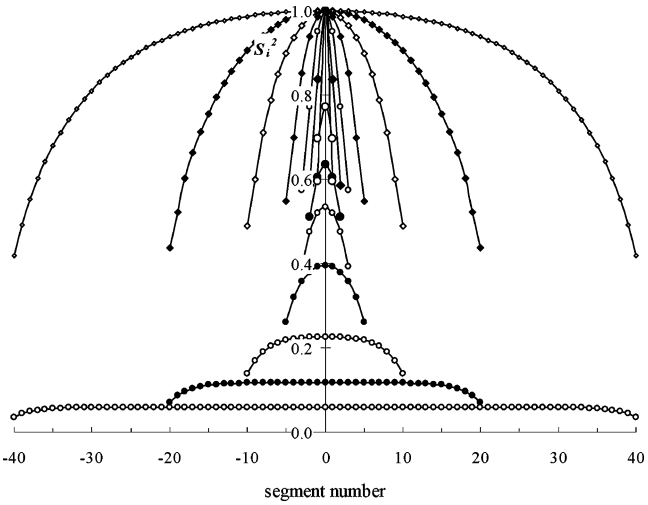

Figure 2. Apparent order parameter squared for valence chains with 1, 3, 5, 7, 11, 21, 41, and 81 segments that are subject to a steric obstruction (♦, ◊), calculated according to eq 7 and normalized to the maximum value. For reference, the unbiased order parameter is shown (●, ○).

The $S^2_{\mathrm{app},i}$ values describe the motion relative to the reference point, but they do not describe faithfully the motion that one expects, e.g., on the basis of relaxation measurements.[23] At least for a long valence chain, one would assume quite uniform motions, i.e., no segment should have a special standing. Furthermore, one does not expect the motional amplitude to vanish altogether but to take a value that depends on the flexibility. When using RDCs, the intuitive quest for an objective frame of reference is, however, fictitious, because such an isotropic frame would abolish the very phenomenon required to generate the alignment and to probe molecular dynamics by RDCs in the first place. Nevertheless, to meet the presentiment of "an objective frame", we computed overall averages of S_i^2 for each locus by moving the locus of alignment from one end of the chain to the other (Figure 1). The S_i^2 values obtained in this way match the intuition. For the one-segment "chain" there is no motion, i.e., $S_i^2 = 1$. For longer chains the S_i^2 values fall as there are more degrees of freedom. In contrast to $S^2_{\mathrm{app},i}$, the S_i^2 values for the central segments are nearly constant and fall first at the chain termini, where the free ends have more degrees of freedom than the central segments. The form of the S_i^2 curve is similar to those of transverse relaxation rates presented for a fully denatured protein.

Alignment Due to a Steric Obstruction. Next we consider the alignment due to the steric obstruction in the form of large liquid crystal particles such as bicells[24] or lamellae.[25] We assume that each member of the ensemble will experience the steric obstruction independently and that the encounter of the solute with the nematogen will not cause conformational changes. As a result, RDCs are weighted averages over the conformations.[26]

$$D_i = D^{\max} \int_\Omega \langle W_i'(\theta) Y^i_{20}(\theta) \rangle \, \mathrm{d}\Omega \qquad (6)$$

In this case the weight, i.e., the spatial probability function $W_i'(\theta)$, is not only locus-specific but also contains a directional part, in analogy to the position−orientation probability density function used to calculate the alignment of a rigid body.[27] The directional dependence serves to approximate the differing Saupe order matrices of the conformations. For the calculation of the order parameter squared, the directional dependence in the weight is included within the brackets that denote averaging over the directions. This "selection of conformations" by the steric obstruction is contained in

$$S^2_{\mathrm{app},i} = \frac{4\pi}{5} \int_\Omega \langle W_i'(\Theta) Y^i_{20}(\Theta) \rangle \langle W_i'(\Theta) Y^{i*}_{20}(\Theta) \rangle \, \mathrm{d}\Omega = W_i'^2 * S_i^2 \qquad (7)$$

to state once again that the apparent order parameter squared is not exactly the true measure of the motion but a convolution of S_i^2 with a function $W_i'^2$.

The $S^2_{\mathrm{app},i}$ values were calculated using the valence chain distribution for the weight function[28] (see Supporting Information) and normalized by the maximum $S^2_{\mathrm{app},i}$ of each chain (Figure 2). The dependence on chain length and residue position is largely in accordance with the expectations. In the case of small-amplitude motions, it makes sense to approximate the hypothetical rigid reference molecule with the largest value of S_i^2 that is available from the measurements. In the case of a highly dynamic system, it is clearly nonsensical to use the maximum value for normalization. For example, a chain with several segments cannot possible have as high values as a chain with one segment which is, per definition, rigid.

The forms of $S^2_{\mathrm{app},i}$ curves do not differ very much from those obtained from the unbiased frame, as is obvious from the data normalized by the unbiased values (Figure 3). It is primarily the data of short chains that show significant convolution effects,

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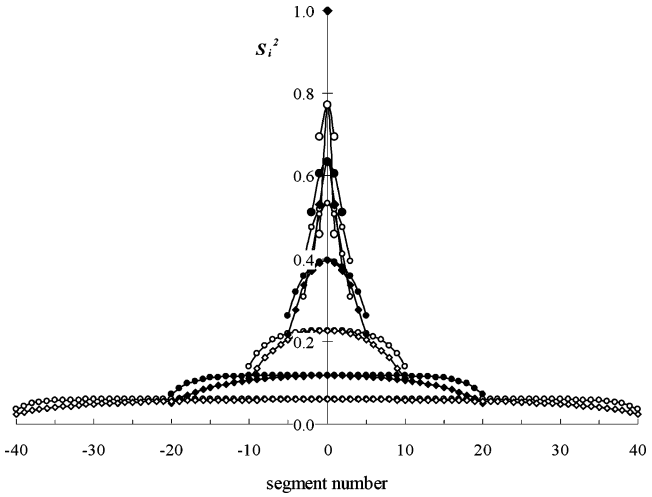

Figure 3. Apparent order parameter squared for valence chains with 1, 3, 5, 7, 11, 21, 41, and 81 segments that are subject to a steric obstruction (♦, ◊), calculated according to eq 7 and normalized to the maxima in the unbiased frame shown as the reference (●, ○).

because elongated conformations are probable and become "selected" by the planar obstruction. The distributions of the long chains are nearly spherical and map more faithfully the fictitious isotropic data, i.e., $W_i'^2$ is flat. In fact, it would be more appropriate to normalize for the chain termini because there the convolution effects are at the smallest. Thus, the motional modes that manifest themselves as elongated conformations are slightly emphasized, whereas collapsed forms are underestimated.

Experiments and Computations

To assess our interpretation of RDCs as weighted averages over the conformations, we have measured and simulated RDCs from a random-coil ensemble of a 21-mer polyglutamate chain subject to a planar steric obstruction. The polyglutamate was synthesized by Cambridge Isotope Laboratories. It contained ^{13}C- and ^{15}N-labeled residues only at the most central and C-terminal sites to avoid spectral overlap. We chose this two-site labeling to avoid spectral overlap that we expected to be indecipherable when labeling for more sites, in hopes of obtaining a more detailed mapping. The alignment was induced by a 6.5% (w/v) DMPC-DHPC liquid crystal[29] prepared in D_2O. RDC data were collected from a slightly basic, approximately 0.5 mM polyglutamate solution. To determine the alignments of the C^α centers, nonredundant $C^\alpha H^\alpha$, $C^\alpha CO$, $C^\alpha C^\beta$, $C^\alpha N$, $H^\alpha C\beta$, $H^\alpha N$, and $H^\alpha CO$ residual dipolar couplings were measured from two-dimensional proton−carbon correlation spectra (as described in Supporting Information) that were acquired at 40 °C with a 600 MHz NMR spectrometer.

Ensembles of all-atom random coil models were generated with the CYANA program[30] and dispatched to the PALES program[31] to obtain RDCs. Finally, average RDCs and alignments were calculated from 2^{14} conformations.

Results

The measured alignments imply that the polyglutamate ensemble is, on the average, an axial system as expected. Therefore, to achieve higher precision of the axial component, only the large $C^\alpha H^\alpha$, $C^\alpha CO$, and $C^\alpha C^\beta$ couplings were used to determine the elements of the traceless rank-2 tensor. The

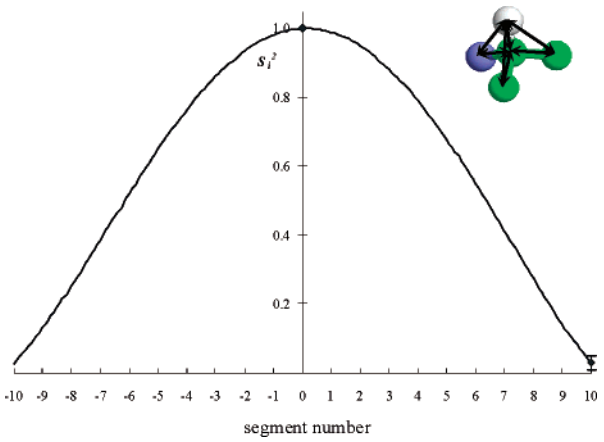

Figure 4. Apparent order parameter squared for the 21-residue homo-glutamate obtained from measured (♦) and simulated (- -) RDCs corresponding to the vector directions at the C^α center shown in the inset.

obtained axial alignments S_{zz} for the central and C-terminal C^α fragments were $(1.8 \pm 0.3) \times 10^{-3}$ and $(0.32 \pm 0.06) \times 10^{-3}$, respectively. The apparent order parameter squared was then assigned according to eq 7 as $S^2_{\mathrm{app},i} = S^2_{zz}$ and normalized by $(S^{\max}_{zz})^2$. After the normalization by the value of the central residue, the standard error of the apparent order parameter squared for the terminal residue was calculated using the law of error propagation[32] (Figure 4). The standard error of the apparent order parameter squared for the noncentral segments using only 2^{12} simulated conformations was no more than 0.03, with the highest precision at the chain termini. The standard error depends inversely on the square-root of the number of samples, which means that the doubling of the number of conformations ought to reduce the error approximately by a factor of $(1/2)^{1/2}$. It is then clear that 2^{14} conformations should be quite enough to give a universal sampling of the available conformation space.

Obviously, the obtained S^2_{app} value at the C-terminus alone does not elucidate the functional form. Nevertheless, the value is consistent with all-atom simulations and in qualitative agreement with those of analytical calculations (Figures 2 and 3). The discrepancy between the simulated values and the analytical curve is most apparent at the chain termini. The calculation yields clearly higher values than the simulations. We understand that most of the inconsistency originates from the infinitely thin locus, i.e., the valence chain segment used in the calculation compared with the finite size of a residue of the all-atom simulation. The results from calculations and simulations are nearly identical with each other when chains composed of very thin elements are simulated. The termini do take high values in the simulations. The remaining small differences can probably be attributed to the continuous exponential function employed in the calculation to approximate the binomial distribution of a discrete chain, and our simple valence chain distribution[33] does not quite precisely describe the ensemble. However, our objective is to elucidate the principal reason for the bias in the order parameter, rather than obtaining an analytical model to give perfect agreement with the measured

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data. Thus, we have not attempted to formulate a more sophisticated analytical model to account for the amino acid residue shapes, since there exist already elaborated models for segmental motions, e.g., as constructed from a sequence of frame transformations.

The eventual differences between the simulated and measured values, that our data are not sufficient to expose, could stem from Coulomb interactions that we have neglected in this simulation.[34] It would take many more site-specifically labeled samples to map the order parameter at every residue. In addition, our assumption that the polypeptide does not experience conformational changes due to the obstruction remains unproven. Nevertheless, it is apparent that the scale of the order parameter squared remains relative only to the chosen frame of reference, i.e., the residue with the maximum value, because no rigid object reference data are obtainable from the measurements to set the absolute magnitude. This seems to be a problem especially for highly flexible polypeptide segments. The absolute scale is only available from the calculations and simulations of a completely rigid object.

Discussion

It is enlightening to compare the generalized order parameter derived from RDC data with that obtained from relaxation measurements, where the total spectral density is modeled as the sum of the rigid-body molecular rotation contribution with the fraction S^2 and the local fluctuation with the fraction $(1 - S^2)$. Spectral densities available from relaxation rates are intrinsically nonzero. RDCs are sensitive to the two contributions as well, however, in the form of the product of the overall molecular sampling of directions attributed to S_o and the local motion assigned to S_i. Consequently, the random molecular sampling of directions must be perturbed by an external coupling to obtain nonzero RDCs, i.e., $S_o \neq 0$, to disclose the local fluctuations. However, this action is potentially problematic because the sampling of directions is contained in the very quantity of the order parameter. The hindered sampling of directions, when imposed on an ensemble of conformations, implicates a convolution.

The alignment due to a paramagnetic site gives the absolute scale of the order parameter relative to the local frame but leads to a bias and associated convolution. The alignment due to the steric obstruction yields an almost unbiased frame because the phenomenon involves the whole molecule, but the absolute scale of the order parameter remains uncertain. The alignment inflicted by filamentous phages deserves also a few words, because of the surface charges carried by them. The alignment due to Coulomb attraction between the solute and nematogen can be regarded as a combination of either of the aforementioned one-site alignments or alignment due to several sites with overall steric obstruction. Consequently, $S^2_{\mathrm{app},i}$ can be a complicated convolution of S_i^2. In general, various alignment mechanisms and media may result in differing values of the apparent order parameters. Another related case is a fatty acid chain in a bilayer whose order parameter along loci has been obtained from deuterium quadrupolar splittings.[35]

The interpretation of RDCs as probes of motion, as presented above, was derived for a highly mobile polypeptide in order to make the outcome more pronounced and tractable, even though the principles used are general. For folded proteins, the convolution effects and problems with referencing the absolute scale are likely to be small. However, analyzes of domain−domain dynamics should benefit from the results. All in all, we expect that the derivations above will find their use in the studies of partially folded proteins such as prions or weakly structured states such as molten globules or folding intermediates.

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Supporting Information Available: Formulas of the weights used for valence chains; figure describing the pulse sequences used for measuring residual dipolar couplings; and figure displaying a spin-state-selective $^{13}C-^{1}H$ correlation spectrum. This material is available free of charge via the Internet at http://pubs.acs.org.

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