

Protein dynamics from NMR

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In the past several years a significant number of new multidimensional NMR methods have been developed to study molecular dynamics spanning a wide range of time scales. Applications involving a large number of biological systems have emerged and correlations with function established. Unique insights are obtained that are not available from structure alone, indicating the importance of dynamics studies for understanding function.

"A picture is worth a thousand words." While this is undoubtedly true of the many beautiful structures that have been produced both by X-ray crystallography and more recently by NMR spectroscopy it is also the case that these static three-dimensional structures alone often do not completely explain results from functional biological assays, nor do they necessarily illuminate the path for protein engineering or rational drug design. This is, of course, not surprising. A three-dimensional static structure provides a description of the ground state of the molecule. Macromolecular function is, in many cases, highly dependent on excursions to excited molecular states and hence intimately coupled to flexibility. In the case of the allosteric molecule hemoglobin, for example, the binding of oxygen results in significant changes in the quaternary structure of the protein. Moreover, in terms of bioenergetics, a significant component of molecular stability derives from motion, even in the context of folded states of molecules. Therefore, a complete and much more useful description of the structure of a molecule will require an understanding of how the structure changes with time. The ultimate goal of dynamics studies is, therefore, to bridge the gap between static and dynamic pictures of molecular structure and to demonstrate how motion relates to function.

The present review focuses on recent developments in solution-based NMR methods to address these issues. Although research efforts are in their initial stages, encouraging results have nevertheless been obtained, and a number of these studies are highlighted. Finally, a summary of methods for relating NMR derived motional parameters to thermodynamics is presented.

Studying dynamics using NMR

Picosecond–nanosecond time scale bond vector motions. Long before advances had made it possible to use NMR to generate

protein structures, ^{13}C and ^{15}N one-dimensional relaxation experiments were devised to study dynamics. The development of isotope labeling strategies in the late 1980s, with the uniform incorporation of ^{15}N and/or ^{13}C nuclei, coupled with multi-pulse, multi-dimensional experiments has facilitated measurement of heteronuclear relaxation parameters at a variety of positions along a protein backbone or side chain. Efforts have largely focused on using ^{15}N spin relaxation techniques¹, which monitor the reorientation of ^{15}N –NH bond vectors, to study backbone dynamics² as well as side chain motions of those residues containing nitrogen³. In addition to obtaining the rate of overall molecular tumbling of the molecule in the case of isotropic tumbling or the components of the diffusion tensor in the case of anisotropic overall motion^{4–7}, a parameter describing the amplitude of internal motions (S^2) and an effective rate constant for internal dynamics is also obtained from a simple analysis of the data⁸.

More recent studies have made use of additional probes of backbone dynamics, including the relaxation of $^{13}\text{C}\alpha$ (refs 9, 10) and carbonyl^{11–14} ($^{13}\text{C}'$) spins in uniformly ^{15}N , ^{13}C labeled proteins. Unfortunately, a complete description of the relaxation properties of these nuclei is somewhat more complicated than for ^{15}N . For example, although in the case of $^{13}\text{C}\alpha$ the dominant contribution to relaxation arises from the attached proton(s), the neighboring carbon spins ($^{13}\text{C}\beta$ and $^{13}\text{C}'$) also are important. Nevertheless, methods and theory have been developed that allow a quantitative interpretation of the relaxation of backbone carbon spins in proteins. It is now possible, therefore, to measure a large number of probes of dynamics and thus distinguish between various motional models. For example, internal motion about an axis parallel to the N–NH bond vector would not contribute to the relaxation of the ^{15}N spin, since this bond is not reoriented by the

motion. If the only probe of dynamics employed derives from ^{15}N relaxation this motion would go unnoticed. By combining experiments that monitor the motions of N–NH and $\text{C}\alpha$ – C' bond vectors, a more complete description of dynamics is thus possible. This important point is underscored in the recent work of Zuiderweg and colleagues¹³. In a study of the backbone dynamics of *Escherichia coli* flavodoxin the N–NH S^2 values were found to be higher than the corresponding $\text{C}\alpha$ – C' values for residues 156–162, part of a helix isolated from the rest of the structure. A motional model, consistent with experiment, was proposed in which there is concerted motion of the entire helix about its axis. Such a picture would not be possible without both N–NH and $\text{C}\alpha$ – C' data sets.

Advances have also been made in the development of methodology to study side chain dynamics in proteins. Recently LeMaster and Kushlan have described a biosynthetic strategy in which ^{13}C is incorporated into alternating carbon sites, thus avoiding the complications in interpretation of ^{13}C relaxation data arising from ^{13}C – ^{13}C dipolar and scalar couplings described above¹⁵. In addition, fractionally deuterated protein is produced and ^{13}C relaxation of methylene or methyl groups containing only a single proton studied. In this way difficulties arising from interference effects between multiple ^{13}C – ^1H bond vectors is avoided. The methods have been applied to *E. coli* thioredoxin providing a description of dynamics at each non-quaternary carbon site in the molecule.

A second approach has also been described for the study of side chain dynamics based on ^2H spin relaxation measurements. The use of deuterium as a probe of molecular motions has had a rich history in solid state NMR spectroscopy of a wide variety of polymers. Extension to high resolution NMR has been limited, however, due to issues involving both sensitivity and resolution. Recently methods have been developed for measuring deu-

terium relaxation properties at methyl¹⁶ (CH₂D) and methylene¹⁷ (CHD) side chain positions in proteins labeled uniformly with ¹³C and fractionally with ²H. The approach involves recording a series of high resolution ¹³C-¹H correlation maps with the intensity of each correlation attenuated by deuterium relaxation.

Many of the relaxation experiments described to date are of the auto-correlation variety in the sense that they monitor how the orientation of a particular bond vector changes in time. It is also possible to measure cross-correlated spin relaxation describing, for example, the correlation between different bond vectors with time. Pioneering studies by Werbelow and Grant¹⁸, Vold and Vold¹⁹, and more recent applications by Daragan and Mayo²⁰ have demonstrated the power of this methodology in obtaining a description of molecular dynamics, at least for small molecules and peptides. Yang *et al.* have recently developed a triple resonance pulse sequence for measuring cross-correlation between the two C-H dipoles in C β -methylene groups in ¹⁵N, ¹³C labeled proteins¹⁷. Measurement of both auto- and cross-correlation S² values provides a more detailed picture of dynamics than would otherwise be obtained from only a single class of experiment²¹.

Millisecond–microsecond time scale motions. Progress has also been made recently in the development of ¹⁵N spin relaxation methods for the measurement of microsecond time scale motions. Although it was recognized early on in ¹⁵N relaxation applications that data were often better fit when an additional parameter was included to account for exchange between two (or more) sites with different chemical shifts, few attempts were made to extract this information rigorously. Palmer and coworkers have described an experiment that measures the effects of chemical exchange on off-resonance rotating frame ¹⁵N relaxation rates²². Assumption of a simple two state model describing the exchange kinetics allows the mean lifetime of the states and a lower limit for the difference in chemical shifts of the exchanging species to be extracted. A study of microsecond conformational fluctuations in the third fibronectin type III domain of the protein tenascin-C demonstrated that significant levels of conformational exchange occur in loops, turns and near ends of β -strands in the domain and that residues with similar exchange rates are part of contiguous regions of the structure²³. This indicates that the motions are locally collective. On

a more technical note the authors discuss that exchange is not restricted to residues that have transverse relaxation rates that are significantly above the mean. This suggests that exchange contributions may well be influencing motional parameters extracted from relaxation data in many proteins and argues the need for a careful analysis of exchange.

In aqueous protein solutions the relaxation rates of ¹⁷O or ²H water nuclei are found to exceed the corresponding rates in solutions where no solute is added. A component of this excess relaxation rate is frequency dependent and in the past several years work by Denisov and Halle have established that this contribution arises from bound water molecules²⁴. This so called relaxation dispersion can be used to measure lifetimes of buried water molecules and because exchange of water occurs as a result of protein conformational fluctuations, these studies provide insight into protein motions. Unlike high resolution NMR methods, however, this technique does not permit the measurement of lifetimes of individual water molecules *a priori*. BPTI, for example, contains four buried water molecules. Denisov *et al.* have determined the lifetime of a specific buried water, W122, by preparing the mutant, G36S, where the buried water molecule W122 is absent, and by recording difference dispersion ¹⁷O and ²H relaxation rates of wildtype and G36S BPTI molecules as a function of temperature²⁵. A lifetime for W122 of $170 \pm 20 \mu\text{s}$ (300 K) is obtained which is significantly longer than for the other three waters (0.01–1 μs). Although the measurements unambiguously determine the exchange lifetimes of the bound waters they do not unequivocally point to an exchange mechanism. As the authors indicate, however, the structure of BPTI reveals that exchange must proceed through significant displacements of both side chain and backbone atoms, involving many conformational substates. Thus, a fit of the temperature dependence of the water lifetime to a simple two-state Arrhenius-type curve to extract the activation enthalpy for the process is likely overly simplistic. A more realistic approach assumes that the energy landscape for the reaction coordinate describing water exchange consists of a superposition of smooth and rugged parts and predicts an activation enthalpy for the process of approximately 10 kJ mol⁻¹.

Dipolar coupling measurements of Prestegard and Tolman on cyanometmyoglobin²⁶, the work of Denisov and Halle²⁴

described above, a large body of data regarding binding kinetics in both myoglobin and hemoglobin²⁷ and the picture emerging from ¹⁵N rotating frame relaxation experiments (see above) suggest that significant millisecond–microsecond fluctuations may well be a general feature of proteins.

From dynamics to function

The first step towards understanding the interplay between dynamics and function is to recognize the range and amplitudes of motions present in the system under investigation. A study that addresses this issue is one by Dahlquist and coworkers who have examined the binding of benzene and indole to engineered hydrophobic cavities in the protein lysozyme²⁸. X-ray studies of mutant lysozymes bound to benzene establish that little structural change occurs upon binding. However, in order for the molecules to penetrate the cavities, the conformation of the protein must change significantly. By monitoring the chemical shift changes of selectively ¹⁵N labeled sites during titration with ligand and the binding constants of benzene and indole have been determined for T4 lysozyme variants L99A, M102A and F104A, and off-rates established by line-shape simulations of resonances that show broadening with binding. On-rates of 10⁶ M⁻¹s⁻¹ are measured for benzene binding to either L99A or M102A mutants, while essentially diffusion limited kinetics are noted for the F104A variant, where binding occurs in a solvent accessible crevice. The binding of indole to L99A proceeds at the same rate as benzene, despite its larger size and the fact that indole binds in a specific orientation in the cavity unlike the smaller benzene molecule. Given the fast binding kinetics observed for both benzene and indole, the authors suggest that binding of polar molecules to proteins may be limited by the burial of dipoles or charges in the low dielectric environment of the protein interior and/or by desolvation steps that precede binding.

Stability and flexibility. An ¹⁵N relaxation study investigating the relation between stability and flexibility was performed by Oas and coworkers on wild type BPTI and a molecule containing the substitution Y35G²⁹. In addition to destabilizing the protein by 5 kcal mol⁻¹ this mutation results in a 250-fold reduction in affinity of the protein for trypsin. Unlike the wild type inhibitor, over a third of the residues in the mutant molecule undergo conformational excursions on

the 5–20 μ s time scale. Crystallographic data on wild type and mutant BPTIs have rationalized the decrease in stability in terms of the significant structural changes that are observed between the molecules³⁰. However, it is also possible that the increased dynamics observed in the mutant may disrupt interactions that would otherwise manifest and contribute to stabilization in the wild type structure. In addition, the decrease in conformational entropy that must accompany binding of the Y35G mutant to trypsin may account for part of the difference in binding affinity observed between wild type and mutant forms of BPTI.

Enzyme function and flexibility. The HIV protease is a homodimeric protein that is essential for viral function. As such the protein has been subject to numerous structural analyses involving both X-ray diffraction and NMR methods. Recently Nicholson, Torchia and coworkers have used ¹⁵N spin relaxation to investigate the backbone dynamics of complexes of the protease with the inhibitors DMP323 and P9941³¹. A number of interesting observations are made by the authors which relate directly to the function of the enzyme. Ile 50 and Gly 51, situated at the tips of the flaps of the protease that control the entry and exit of substrate, experience conformational exchange on a time scale of ~10 μ s. In nearly all of the X-ray derived structures of the protease, these residues display asymmetric β I/ β II hydrogen bond turn conformations (that is, Ile 50/Gly 51 of one monomer is in the β I turn configuration, while the corresponding residues in the second monomer are in a β II conformation). However, the crystal structure of the DMP323 complex shows a symmetric β II/ β II conformation³². Structural data derived from NMR on this complex are consistent with a mixture of β I and β II turns, although a β II/ β II conformation cannot be excluded. Thus, the NMR results from both structural and relaxation studies clearly indicate that this region is flexible. It is particularly noteworthy that both Ile 50 and Gly 51 are conserved in retroviral protease sequences and non-conservative amino acid substitutions at these sites significantly reduce biological activity. The authors argue, therefore, that the multiple conformations available to the tips of the flaps help stabilize the complex and in addition provide the necessary flexibility for product release after catalysis. A second point made by the authors also relates to function. The primary

autolysis site in the HIV-1 protease is the Leu 5–Trp 6 peptide bond and the rate of cleavage at this position may be involved in regulation of the protein *in vivo*. NMR data show that Glu 2 and Val 3 are not flexible but that Thr 4 and Leu 5, located in a solvent exposed loop, are. The dynamics at the Leu 5, Trp 6 site may be important for peptide bond cleavage and hence play an important role in regulation of the life cycle of the virus.

Flexibility of DNA binding domains. The *trp* repressor is a small regulatory protein in *E. coli* that interacts with a number of different operator sequences, including the *trp*, *trpR* and *aroH* operators³³. The considerable sequence diversity associated with the different operators that are under the control of the *trp* repressor suggests that this molecule is flexible. An Ala to Val mutation at position 77 in the sequence creates a super-repressor that is capable of enhanced *in vivo* repression of transcription at the *trp* operator relative to the wild type repressor. In contrast, repression at the *aroH* operator and arguably at the *trpR* site is reduced relative to levels associated with the wild type molecule. The *in vitro* binding constants and the structures of both the wild type and A77V super-repressor are identical. In an effort to understand the origin of the functional differences, Jardetzky and coworkers have measured hydrogen exchange rates in the wild type and the A77V super-repressor in both the absence and presence of the ligand tryptophan³⁴. The data strongly support the notion that the helix-turn-helix DNA binding motif is significantly less flexible in the A77V mutant than in the wild type structure. The authors argue that the decrease in binding at the *aroH* and *trpR* operators that accompanies stabilization of the A77V structure increases the effective concentration of protein available for binding to the *trp* operator, hence converting the molecule into a super-repressor for this site.

A second example illustrating the importance of flexibility for DNA binding derives from dynamics and binding studies of the *c-Myb* DNA binding domain by Ogata and coworkers³⁵. The *c-Myb* domain is comprised of three structurally similar repeats of approximately 50 amino acids, R1, R2 and R3³⁶. In contrast to repeats R1 and R3 which have melting temperatures (T_m) of 61 and 57 °C, the T_m of R2 is only 43 °C. Structural studies of a construct comprising R1R2R3 establishes that only R2 has a cavity in its hydrophobic core and that the cavity is conserved in

mouse and human proteins³⁵. ¹⁵N relaxation experiments were performed on a fragment consisting of R2 and R3 (R2R3), and slow conformational fluctuations on a μ s–ms time scale were noted for the R2 domain but not for R3. The R2 domain was stabilized by removing the cavity through a Val to Leu substitution at position 103 which significantly increased the thermostability of R2 (T_m increased by 20 °C) with concomitant suppression of the slow time scale motions observed in the wild type domain. The NMR results thus indicate a relation between conformational fluctuations and stability which is associated with the presence of a cavity in R2. In order to delineate a functional role for the dynamics and the cavity, Ogata *et al.*³⁵ measured the affinities of wildtype R2R3 and R2R3(V103L) for target DNA as well as the ability of these molecules to activate the *c-myc* promoter *in trans*. Notably, both the affinity for DNA and transactivation were reduced in the case of the mutant, suggesting that the cavity in the hydrophobic core of R2 and the conformational flexibility associated with this domain are important for function. In this regard it is noteworthy that structural studies of a complex of R2R3 with DNA show that Trp 95 reorients upon DNA binding so that its indole ring moves towards the cavity. This reorganization is not possible in the case of a more rigid, well packed structure.

Correlation between binding and dynamics at protein–protein interfaces. SH2 domains are small modules that bind phosphotyrosine containing targets and are involved in a variety of signal transduction processes³⁷. Structural studies of a large number of these molecules have established that, while they all have similar phosphotyrosine (pTyr) binding sites, the regions that interact with residues C-terminal to the phosphotyrosine of the target peptide can vary significantly between different SH2 domains³⁸. In the case of the Src SH2 domain there is a small hydrophobic binding pocket, allowing contacts with essentially only the residue at the +3 position of the pTyr containing peptide³⁸ (+3 refers to the residue three positions C-terminal to the pTyr). Alternatively, in the case of the C-terminal SH2 domain from phospholipase C_{γ1} (PLCC)³⁹ and the N-terminal SH2 domain from the Syp tyrosine phosphatase (NSyp)⁴⁰, a long hydrophobic binding groove contacts residues from the +1 to +5 positions in both molecules.

Despite the structural similarities of the PLCC and the NSyp SH2 domains

and their similar modes of peptide binding, studies suggest that there are important differences in the ways in which each domain interacts with target. C-terminal peptide truncation experiments with a high affinity-binding peptide for the NSyp domain establish that removal of residues up to and including the +3 position decreases binding by a factor of in excess of 300⁴¹. In contrast, in the case of a high affinity-binding target for the PLCC domain, truncation up to the +2 position decreases affinity by less than a factor of five. Given the structural similarities between the two domains it is important to establish the underlying mechanism associated with these different binding results. Insight is provided by a side chain dynamics study probing the dynamics of methyl groups in peptide-free and bound forms of these molecules performed by Forman-Kay and coworkers⁴¹. In the case of the NSyp SH2 domain key residues that line the hydrophobic binding site and are involved in peptide binding have S^2 values (describing amplitudes of ps–ns time scale dynamics) slightly below average. Upon complexation with peptide these order parameters increase. In contrast, the PLCC SH2 domain presents a highly dynamic binding interface that does not change significantly upon addition of peptide. The decreased binding energy arising from residues C-terminal to the pTyr in the case of the PLCC SH2 domain, established by the truncation binding data described above, may be a consequence of the increased dynamics at the interface. In this regard it is noteworthy that electrostatic contributions to the binding energy are likely to be more important in the case of the PLCC SH2 domain since four arginine residues line the pTyr binding site, while in the case of the NSyp SH2 only two positively charged amino acids are present. Since the affinities of these SH2 domains for their cognate targets are similar, an increased binding contribution from the pTyr site (PLCC SH2 domain) must be compensated by a decrease from the hydrophobic binding region. Dynamics may provide a mechanism for modulating binding energy in the case of the PLCC SH2 domain so that the moderate affinities required for biological function are maintained.

From dynamics to thermodynamics

On the basis of the previous discussion it is

clear that structural descriptions of macromolecular interactions alone are often not sufficient for understanding binding and for elucidating key residues in molecular recognition. What is needed is a site-specific description of the contribution that each amino acid makes to the energetics of binding events or folding reactions, for example. Recognizing this, Palmer and coworkers demonstrated that a simple relation between contributions to the change in Gibbs free energy from ps–ns time scale bond vector fluctuations and NMR derived order parameters could be obtained⁴². The methodology was applied to metal binding in calbindin D_{9k} where it was shown that changes in bond vector fluctuations make significant contributions to the cooperativity of calcium binding. Subsequently, Yang and Kay considered how changes in entropy relate to changes in S^2 for a number of different models of bond vector motion and concluded, not surprisingly, that the relation was sensitive to the potential function describing the motion⁴³. The contribution to conformational entropy from ns–ps motions was quantified from a 1 ns molecular dynamics trajectory of the protein RNase HI⁴⁴ and the entropy versus order parameter profile generated was found to be well fit by a function derived on the assumption of bond vector motion in a cone. Using this cone model the contributions to entropy associated with the folding-unfolding transition of the N-terminal SH3 domain from the protein drk⁴⁵ (drkN SH3) arising from rapid NH bond vector motions was found to be 12 J mol⁻¹ K⁻¹ residue⁻¹ (ref. 43). Wand and coworkers have also derived a relation between entropy and order parameter⁴⁶ and concluded on the basis of ¹³C side chain relaxation data on ubiquitin⁴⁷ that there is a significant range in the contribution to entropy from side chain dynamics. Palmer and coworkers⁴⁸ and Yang *et al.*⁴⁹ have noted that the change of backbone order parameters with temperature is much more steep for unstructured regions of proteins or unfolded protein states than for the corresponding folded forms of the molecules. This is consistent with a larger contribution from backbone dynamics to the heat capacity of unfolded proteins. Yang *et al.* have calculated an average heat capacity value of 34 J mol⁻¹ K⁻¹ residue⁻¹ for the unfolded drkN SH3 domain arising

from ps–ns backbone motions based on ¹⁵N relaxation data recorded at 14 and 30 °C⁴⁹. More recently, Alexandrescu and coworkers have conducted an ¹⁵N backbone dynamics study of free and bound forms of the S-peptide from RNase A⁵⁰. The observed decrease in entropy of unfolding towards the center of the peptide has been interpreted in terms of a model whereby the degree of compactness of the molecule varies as a function of the distance of an amino acid from the central residue of the polypeptide chain.

A number of groups have made use of the entropy-order parameter ideas described above in the analysis of ligand binding data. Specifically, Mildvan and coworkers have demonstrated that upon binding inhibitor to 4-oxalocrotonate tautomerase a number of residues show significant increases in order parameter while others show decreases, suggesting partial entropy compensation⁵¹. Sykes and coworkers have compared the order parameters of sites I and II of the apo- form of the N-terminal domain of troponin C and find that the difference between sites I and II ($S_{p,II} - S_{p,I}$) is -3.2 cal mol⁻¹ K⁻¹, corresponding to a free energy difference of about 1 kcal mol⁻¹ (ref. 52). The difference in dynamics between the two sites may account for why site I has a weaker calcium affinity.

Concluding remarks

The experiments described above demonstrate correlations between dynamics and function. Clearly, in order to strengthen our understanding of the relation between the two, more data on a larger number of systems is required. A deep insight into the function of proteins will only be obtained through a combined study of both structural and motional properties of these inherently dynamic molecules.

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New techniques in structural NMR — anisotropic interactions

J.H. Prestegard

Structure determination of biomolecules by NMR has traditionally been based on nuclear Overhauser effects (NOEs). Now there are additional sources of information that can complement NOEs in cases where positioning of remote parts of molecules is important, and where extension to larger and more complex systems is desired.

The traditional NMR approach to structure determination of biomolecules is based on interpretation of rates of magnetization transfer between pairs of protons in terms of distance constraints. The approach requires not only the measurement of magnetization transfer (NOEs or nuclear Overhauser effects), but the resolution and assignment of NMR signals to specific protons, of specific residues, in a known protein sequence. Assignment of resonances and measurement of adequate numbers of NOEs have always been obstacles that made structure determination time consuming and limited to relatively small proteins (<10,000 M_r for early homonuclear studies). The limitations have been pushed back over the years with additional structural information from scalar coupling constants and chemical shifts. Assignment strategies based on the use of through bond connectivities between ^{13}C and ^{15}N sites in isotopi-

cally enriched proteins have also made it possible to assign resonances in increasingly larger proteins. However, full structure determinations have still remained confined to reasonably compact systems of molecular weights less than 30,000–40,000 M_r .^{1,2}

There have been, within the last two years, experiments reported that could dramatically change the range of applicability of NMR structural methods. Interestingly, they share an origin in anisotropic magnetic interactions that are not normally observable in high resolution NMR spectra. One important class of experiments yields structural constraints that are orientational, rather than distance based. The experiments rely on the measurement of residual dipolar couplings, and, in some cases, chemical shift anisotropy (CSA)^{3–5}. The measurements can be made with great efficiency, and when combined with other recent discoveries that take advantage of

interference between the same dipole-dipole and CSA interactions^{6,7,8}, it appears that NMR may be poised to take another large step forward in applicability to larger, more complex systems.

Residual dipolar interactions

The dipole-dipole interaction, the leading term of which is described in equation (1), is actually the basis of the NOE effect:

$$D_{ij} = -\xi_{ij} \frac{(3 \cos^2 \theta - 1)}{2} I_{zi} I_{zj} \quad (1)$$

The interaction constant, ξ_{ij} , contains factors that describe the magnitudes of magnetic moments for a pair of nuclei i and j , and the internuclear distance dependence that shows up in NOE measurements. The spin operator, $I_{zi} I_{zj}$, has the same form as a first order through-bond spin-spin coupling interaction suggest-