Perspectives in paramagnetic NMR of metalloproteins

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NMR experiments and tools for the characterization of the structure and dynamics of paramagnetic proteins are presented here. The focus is on the importance of ^{13}C direct-detection NMR for the assignment of paramagnetic systems in solution, on the information contained in paramagnetic effects observed both in solution and in the solid state, and on novel paramagnetism-based tools for the investigation of conformational heterogeneity in protein–protein complexes or in multi-domain proteins.

Introduction

A significant share of the proteome is constituted by metalloproteins.[1] Among them, a large number contains paramagnetic metal ions, or metal ions which cycle between different oxidation states, one of which is paramagnetic.

Proteins containing diamagnetic metal ions can in principle be studied by NMR like any non-metal-containing protein. The metal nucleus–proton scalar connectivities can be exploited to prove the existence of a metal–protein bond. This is the case for ^{113}Cd and ^{199}Hg, for which metal–ligand couplings can be detected by heteronuclear 2D experiments such as metal–proton correlation spectra.[2–5] Histidine ligands can be easily recognized through ^{1}H–^{15}N HSQC spectra optimized for the detection of $^{2}J_{NH}$ couplings.[6] For the identification of other ligands, such as cysteines, one can rely on NMR experiments that provide the assignment of ^{13}C nuclei, the chemical shift of which is generally very sensitive to the presence of a bond to a metal ion.[7] The application protocols are well established and we do not expect relevant innovations in this area for the near future.

Paramagnetic proteins are more challenging, and were believed not to be suitable for NMR structure determination until the mid-nineties,[8] but eventually novel experiments and software protocols were developed with the specific purpose of making these systems as friendly as possible. Most importantly, the presence of the paramagnetic center can be a precious source of structural information.[9] It affects longitudinal and transverse relaxation rates of the observed nuclei, as well as their chemical shifts, *via* different mechanisms that are now well understood and exploited.[9,10] Furthermore, self-orientation of the protein due to the intrinsic magnetic anisotropy of a metal ion induces additional splitting of coupled nuclei without the need for external orienting media like liquid crystals.[11] As each paramagnetic metal ion has peculiarities, to exploit its potential of information it is necessary to tailor the approach accordingly. To the end of obtaining structural information, NMR spectroscopy can be complemented with other techniques targeted at the metal ion, such as electronic spectroscopy, extended X-ray absorption fine structure (EXAFS) and the like.[12]

We will present here some experiments and novel tools in paramagnetic NMR for the characterization of protein structure and dynamics. In particular, our attention will be focused on the importance of ^{13}C direct detection for the characterization of paramagnetic systems in solution, on the information contained in paramagnetism-based effects observed both in solution and in the solid state, and on novel paramagnetism-based tools for the investigation of conformational heterogeneity in protein–protein complexes or in multi-domain proteins. Some of these experiments may require instrumentation not available in all laboratories; in such cases researchers can take advantage of the availability of research infrastructures for NMR offering access to their instrumentation, such as those in the EU-NMR consortium in Europe (www.eu-nmr.eu) or NHMFL (http://www.magnet.fsu.edu) and NMRFAM (http://www.nmrfam.wisc.edu) in the USA.

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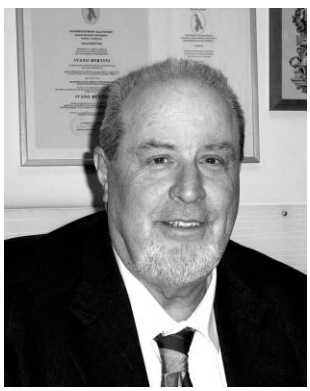

Ivano Bertini

Ivano Bertini was born in 1940, obtained the Italian degree of Doctor in Chemistry at the University of Florence in 1964 and then the Libera Docenza in 1969. He became full professor in general and inorganic chemistry in 1975 at the University of Florence where he is now. He received Honoris Causa Degrees from the University of Stockholm in 1998, Ioannina in 2002 and Siena in 2003. He is a physical inorganic chemist who has cultivated NMR since 1965. He is founder and director of the Center of Magnetic Resonance (CERM), an NMR-based infrastructure operating in the field of life sciences.

Enhancing the detectability of NMR signals in paramagnetic systems

The magnitude of the paramagnetic effects on nuclear relaxation rates depends on the nature of the metal ion and on its electronic

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relaxation rate.[9,13] Depending on the metal–nucleus distance, the electron–nucleus coupling effects may prevent the observation of proton signals of the residues close to the metal site due to induced broadening of the NMR lines.[9] As the paramagnetic dipolar contributions to nuclear relaxation depend on the square of the gyromagnetic ratio of the observed nucleus, going from ^{1}H ($\gamma_H = 2.67 \times 10^8$) to ^{13}C ($\gamma_C = 6.73 \times 10^7$) detection produces a decrease in relaxation rates of a factor of about 16. This reduction enables the identification of nuclei in the proximity of the paramagnetic center.[14,15] A further step toward the metal ion can be accomplished by exploiting ^{15}N direct-detection ($\gamma_N = -2.71 \times 10^7$).[16,17]

Starting from the available building blocks for triple-resonance experiments,[18] it is possible to implement a set of sequences enabling the complete assignment of a protein using ^{13}C direct detection without involving ^{1}H transfers.[19–21] A peculiarity of ^{13}C direct-detection experiments is the presence of signal splitting in the acquisition dimension due to the strong $^{1}J_{CC}$ coupling. For a C' signal the main coupling is with the C^{α}, which gives a relatively constant splitting of about 54 Hz. Such a splitting can be removed using a "trick", based on spin-state-selective methods, developed taking advantage of the uniformity of the splitting value.[22] C^{α} nuclei generally present two large couplings, with C' ($\approx$ 54 Hz) and with C^{β} ($\approx$ 35 Hz) nuclei. In the case of spectra based on C^{α} acquisition, a double spin-state-selective scheme can thus be implemented to remove the double splitting.[19,22] The removal of the splitting can be obtained, even with some compromise in the signal-to-noise ratio of the spectra, with the use of band-selective homodecoupling.[14,23] This could be convenient for those cases in which ^{13}C lines have a linewidth comparable to the J_{CC} coupling. As a further step, the final building block of the NMR pulse sequence (in which in general the anti-phase coherence is refocused) can be eliminated and the anti-phase component directly detected.[24,25] This leaves the signal splitting, but considerably reduces the relaxation losses in case of highly paramagnetic proteins.

The ^{13}C direct-detection experiments can be further optimized for paramagnetic proteins by selecting the most efficient coherence transfer pathways and identifying those that are least affected by fast relaxation. In principle, the most sensitive experiments are those based on coherence transfer mechanisms mediated by large scalar couplings; however, when transverse relaxation is much faster than longitudinal relaxation, dipolar-based transfers (*e.g.* nuclear Overhauser effect spectroscopy, NOESY) can be usefully exploited, especially at higher fields.[14]

A nice example of the potential of ^{13}C direct-detection is provided by the study of oxidized monomeric copper–zinc superoxide dismutase (SOD).[14,26] This enzyme catalyzes the dismutation of superoxide to O_2 and H_2O_2, and it contains a copper ion which alternates between Cu(II) and Cu(I) during the catalytic cycle. Cu(II) is coordinated by four histidines, one of which also coordinates to Zn(II), which in turn is bound to three histidines and an aspartate (Fig. 1). Copper(II) in SOD is type II, and as such provides much stronger line broadening than type I copper(II) or mixed valence copper.[14,27–32] For the monomeric form of SOD it was observed that ^{1}H NMR signals within a 11 Å sphere centred on Cu(II) are not detectable in standard ^{1}H–^{15}N HSQC experiments due to paramagnetic broadening.[14] The assignment of C' and C^{α} nuclei of this protein was thus accomplished by using a set of ^{13}C direct-detection NMR experiments such as C^{α}–C' correlation (COCA) and C^{β}–C^{α}–C' correlation (CBCACO) experiments,[20–22]

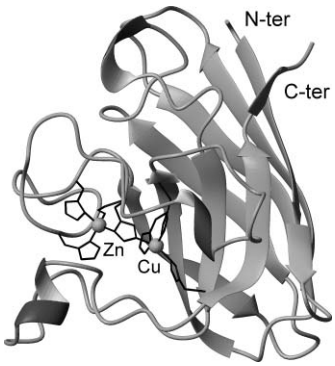

Fig. 1 Ribbon display of the structure of the monomeric unit of Cu–Zn SOD. The metal ions are indicated as spheres of arbitrary radius. The metal ion ligands are depicted by black sticks.

as well as ^{13}C–^{13}C NOESY experiments.[14,33] Only the four copper ligands could not be identified because contact relaxation, due to the presence of unpaired spin density delocalized through chemical bonds onto the resonating nuclei, broadens the NMR lines beyond detectable limits.

Calbindin D_{9k} (Cb), a calcium binding protein where one calcium ion can be easily replaced with paramagnetic lanthanide ions, can be used as an example to show the different performances of various type of NMR experiments for the assignment purpose. This is graphically summarized in Fig. 2 for the highly paramagnetic thulium(III)-substituted derivative. In this case the sphere for which signals are broad beyond detection has a radius as large as 17.5 Å for standard ^{1}H experiments, that can be reduced to 14 Å for standard ^{13}C-detected experiments, 9 Å for ^{13}C-detected experiments optimized to fast relaxing signals, 6 Å for ^{13}C 1D spectra and 4.5 Å for 1D ^{15}N spectra.[34] Thus, the use of low-γ nuclei direct-detection provided the identification of all residues while only about 50% of amino acids were assigned with standard ^{1}H-detected experiments. In the much less paramagnetic cerium(III)-substituted Cb, the NMR signals of carbon atoms from carbonyl

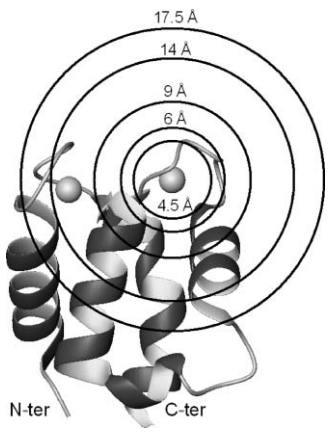

Fig. 2 Radii of the spheres within which signals cannot be observed in CaTmCb. Different spheres are projected onto the ribbon display of the structure for each different set of NMR experiments. Radii are as follows: 17.5 Å for standard ^{1}H-detected NMR experiments, 14 Å for "standard" ^{13}C-detected experiments, 9 Å for ^{13}C-detected experiments optimized to fast relaxing signals, 6 Å from ^{13}C 1D spectra and 4.5 Å for 1D ^{15}N spectra.

and carboxylate groups in the loop hosting the Ce(III) ion could be detected and assigned, thus making it possible to characterize in solution the first coordination sphere of the metal ion.[35]

Carbon-13 direct-detection experiments have already proved to be a valuable tool to detect and assign signals and to obtain information on regions of the protein that cannot be enlightened through ^{1}H direct detection due to paramagnetism. Efforts are being made to optimize experimental protocols in this promising field.

Solution structures determination and refinement

Having overcome the limitations posed by the assignment of the NMR spectrum, one can proceed with the calculation of the solution structure of the protein based on the use of the available ^{1}H–^{1}H NOE, *J*-coupling and H-bond restraints as well as of paramagnetism-based restraints (longitudinal relaxation rates, pseudocontact shifts and residual dipolar couplings, in particular). The former restraints are effective for computing the structure of the part of the protein far enough from the metal ion to be treated as a diamagnetic protein. The paramagnetism-based restraints are precious for refining the regions surrounding the metal ions, especially if obtained through ^{13}C direct-detection experiments as they are also available for residues for which ^{1}H signals are broadened beyond detectable limits.

In the case of copper(II) proteins like SOD, longitudinal relaxation rates[9,36] can be conveniently used as they are relatively large. The relaxation-rate enhancements due to the presence of a paramagnetic ion can be extracted from the measured longitudinal relaxation rates after subtraction of the diamagnetic contribution, which can be safely estimated from the set of lower relaxation rates corresponding to nuclei far from the paramagnetic ion. Such enhancements can be related to the metal–nucleus distance according to the Solomon equation,[37] and can thus provide useful restraints for structure calculation. Thanks to the different observability properties of ^{1}H and ^{13}C, relaxation-rate measurements of both nuclei provide complementary distance restraints for nuclei in two shells at different distances from the paramagnetic metal ion. Transverse relaxation-rate enhancements can also be used, but in general they provide redundant information with respect to longitudinal relaxation-rate enhancements and the latter are easier to measure.

In order to appreciate the importance of ^{13}C restraints in paramagnetic proteins, in Fig. 3 the comparison of the structure of SOD obtained using only conventional ^{1}H restraints (a) and additional paramagnetism-based restraints derived from both ^{1}H and ^{13}C NMR data (b) is reported. The quality of the latter is dramatically increased, the backbone root-mean-square deviation (RMSD) to the mean being 1.2 Å compared to a value of 2.4 Å obtained using only conventional restraints (and linking the metal ions to their ligands *a priori*).[26]

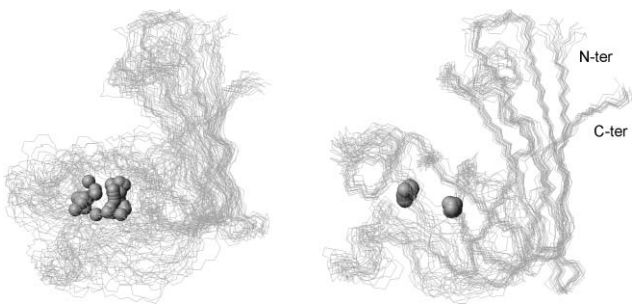

Fig. 3 Family of 20 conformers of monomeric oxidized SOD obtained with (a) *diamagnetic* restraints only, and (b) *paramagnetic*-derived restraints.

Additional paramagnetic contributions to relaxation, besides Solomon relaxation, can arise from the dipolar interaction of the nuclear spin with the thermal equilibrium magnetic moment, oriented along the magnetic field, caused by the difference in population of the electron spin levels according to the Boltzmann distribution (Curie relaxation).[38,39] This effect is important for metals with efficient electron relaxation combined with a large Zeeman splitting. The simultaneous presence of both Solomon and Curie relaxation mechanisms provides cross-correlation effects, which can also result in additional structural restraints.[40–44] Furthermore, cross correlation between Curie and chemical shielding anisotropy (CSA) relaxation can have a significant effect on nuclear relaxation when the latter is predominantly driven by the Curie relaxation mechanism.[45]

Chemical-shift information for both proton and carbon nuclei should also be used to derive further restraints. In the case of copper(II) proteins like SOD, paramagnetic contributions to the chemical shifts are also relatively small at short distances from the metal ion because of the small magnetic susceptibility anisotropy of the metal ion, so that the chemical shifts could be used to obtain restraints on the protein-backbone dihedral angles[46,47] as in any non-metal-containing protein. In the case of proteins containing metal ions with large magnetic susceptibility anisotropy, such as low-spin iron(III), high-spin iron(II), cobalt(II) or lanthanides (except gadolinium(III)), chemical shifts should be used to extract the pseudocontact shift (pcs) contribution affecting a large number of nuclei sensing the paramagnetic center. Pcs's are obtained from the difference in chemical shift with a diamagnetic analogue of the protein.[48] Pcs's are related to the position of the nucleus with respect to the metal ion and to the magnetic susceptibility anisotropy tensor.[9,11,49]

Finally, ^{1}J splittings can be used to obtain residual dipolar couplings (rdc). In case the metal ion induces measurable self-alignment in the magnetic field[11] they can be immediately derived from the difference with the ^{1}J splittings of a diamagnetic analogue of the protein. In copper(II) proteins and in all cases where paramagnetic rdc are negligibly small, like manganese(II) or gadolinium(III) containing proteins, rdc can be induced by external orienting agents.[50–54]

Carbon-13 direct-detection experiments have also been recently exploited for the measurement of rdc. Protocols for the measurement of C^{α}–C^{β} and C^{α}–C' rdc are based on the inphase antiphase (IPAP) scheme since the homonuclear $^{1}J_{C\alpha C'}$ ($\approx$ 54 Hz) and $^{1}J_{C\alpha C\beta}$ ($\approx$ 35 Hz) coupling constants are large enough to be easily measured from the doublet splitting.[55,56] New pulse sequences for the measurement of H^{α}–C^{α} and H^{N}–N rdc have been proposed based on the fact that $^{1}J_{HN}$ and $^{1}J_{H\alpha C\alpha}$ are not measurable by ^{1}H experiments due to the fact that the broadening can be recovered by heteronuclear detection and proton-recoupled ^{13}C direct-detected experiments.[57] Such experiments are thus able to provide rdc values with a good precision also for very broad ^{1}H resonances. The protocol for the measurement of ^{13}C direct-detected rdc was tested on Cb where one calcium ion has been substituted by thulium(III).

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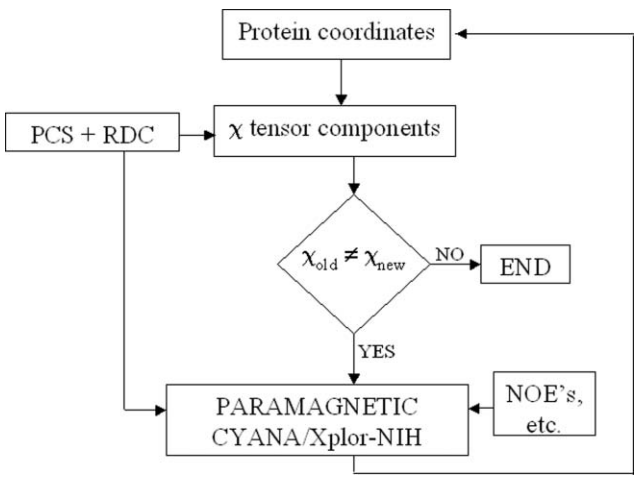
Paramagnetic broadening prevents signal detection for about 50% of amino acids; ^{13}C-detected experiments gave about 30% increase in the number of observed rdc.[57]

Protocols for the use of pcs and rdc as structural restraints have been developed for two of the most used programs for solution structure determination of proteins, Xplor-NIH[58] and Cyana,[59] where additional routines dealing with the paramagnetic terms have been implemented.[57,60,61] Pcs's depend on the position of the observed nuclei and rdc's on the orientation of the observed nuclear pair with respect to the magnetic susceptibility anisotropy tensor; both of them also depend on the axial and rhombic anisotropy parameters of the magnetic susceptibility tensor of the metal, $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$. The protocols are based on cycling between the simulated annealing structural calculation, performed with all classical restraints together with the pcs/rdc restraints, where fixed $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ values are used, and determination of the $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ values, performed using the present coordinates for protein nuclei, until convergence is reached (Scheme 1). A pseudoresidue is introduced in the protein sequence to place the metal ion and its magnetic susceptibility anisotropy tensor into the protein structure.



Scheme 1 Flow-chart of the cycle to be repeated during the calculation of protein solution structures with pcs and rdc restraints. A protein structure is needed to obtain the values of the magnetic susceptibility anisotropy. The values of the magnetic susceptibility anisotropy are needed to calculate the coordinates of the atoms in the protein. The cycle can be initiated from a rough estimation of the magnetic susceptibility anisotropy parameters, or from a model for the protein structure.

Interestingly, pcs depend neither on the applied magnetic field nor on the observed nucleus, but only on its position in the magnetic susceptibility tensor. On the contrary, rdc do not depend on the distance of the observed coupled nuclei from the paramagnetic ion, but only on the orientation of the vector connecting the two nuclei with respect to the magnetic susceptibility tensor, *i.e.* to the same tensor relevant for pcs. For these reasons, pcs and rdc nicely complement each other and can be conveniently used together. Pcs are particularly effective in determining the nuclear distances from the paramagnetic ion, the orientation of the tensor and its anisotropy values, rdc are particularly effective in defining the orientation of the coupled nuclei, *i.e.* of the substructure to which they belong.[62]

Unfortunately, the solution of the equations describing pcs and rdc is not unique, *i.e.* different positions/orientations for each nucleus can satisfy both equations. Degeneracy can, however, be removed if pcs and rdc relative to different metal ions are available, for instance after substitution of the paramagnetic ion with a different one. In fact, in this case the magnetic susceptibility anisotropy tensor responsible for the observed pcs and rdc is different. As a consequence, only in the presence of data acquired for more than one paramagnetic ion may pcs and rdc be used to guide the global fold of the protein. In the case of Cb, at least three sets of pcs and rdc are necessary to correctly provide the fold of the protein when only a minimal number of H^N–H^N NOE, hydrogen bonds and backbone dihedral-angle restraints are available.[63] On the other hand, pcs and rdc can be always used to refine a protein solution structure efficiently. The increase in the precision and accuracy of the calculated structure that can be achieved is dramatic, as has been demonstrated for Cb.[64]

As pcs and rdc are long-range restraints, they may represent a quite valuable source of information not only for refining protein structures but also for determining and validating the global fold of a protein with the addition of few other further restraints.[63,64] This has been demonstrated in the case of a low-spin iron(III)-containing protein, cytochrome c_{556}.[65] For this cytochrome the structure has been modelled on the structure of another cytochrome sharing with it 35% homology in the primary sequence. The model obtained has been validated using pcs and rdc data obtained with a restricted set of NMR experiments, unable by themselves to provide the structure of the protein. Structures obtained with this approach are expected to represent in the future a useful complement to the *de novo* determined structures.

Solid-state NMR applications

Solid-state NMR that has been developed on ^{13}C direct detection provides a further tool for the investigation of paramagnetic metalloproteins. Indeed, the observation of NMR correlations is little affected by paramagnetic relaxation, and most of the signals arising from nuclei close to the paramagnetic center can be detected with standard solid-state NMR experiments.[66] In particular, a source of line-broadening of NMR lines often operative for paramagnetic molecules in solution, Curie relaxation,[38,39] is largely suppressed in solid-state NMR.[67]

The 2D proton driven spin diffusion (PDSD)[68,69] spectrum of dimeric oxidized SOD is shown in Fig. 4. The resolution of the spectrum is impressive and the assignment of large part of the resonances can be transferred by the solution NMR assignment of its diamagnetic analogue (*i.e.* SOD with Cu(II) reduced by the addition of ascorbate) very easily. In the absence of assignment in solution, experiments such as N–C^α–C^β correlation (NCACB) and N–C'–C^α–C^β correlation (NCOCACB) provide, as in solution NMR, the sequential assignment of the polypeptide.[70–79]

As already seen for proteins in solution, pcs of ^{13}C nuclei can also be used as structural restraints in the solid state. These are particularly valuable, as the restraints available for structure determination in solid-state NMR are usually few.[80–85] In Fig. 5, two spectra of the catalytic domain of the matrix metalloproteinase 12 (MMP) are reported. This MMP is a Zn-containing protein of molecular mass 17.6 kDa. The zinc(II) ion can be easily substituted with cobalt(II),[86] providing a paramagnetic complex. In the

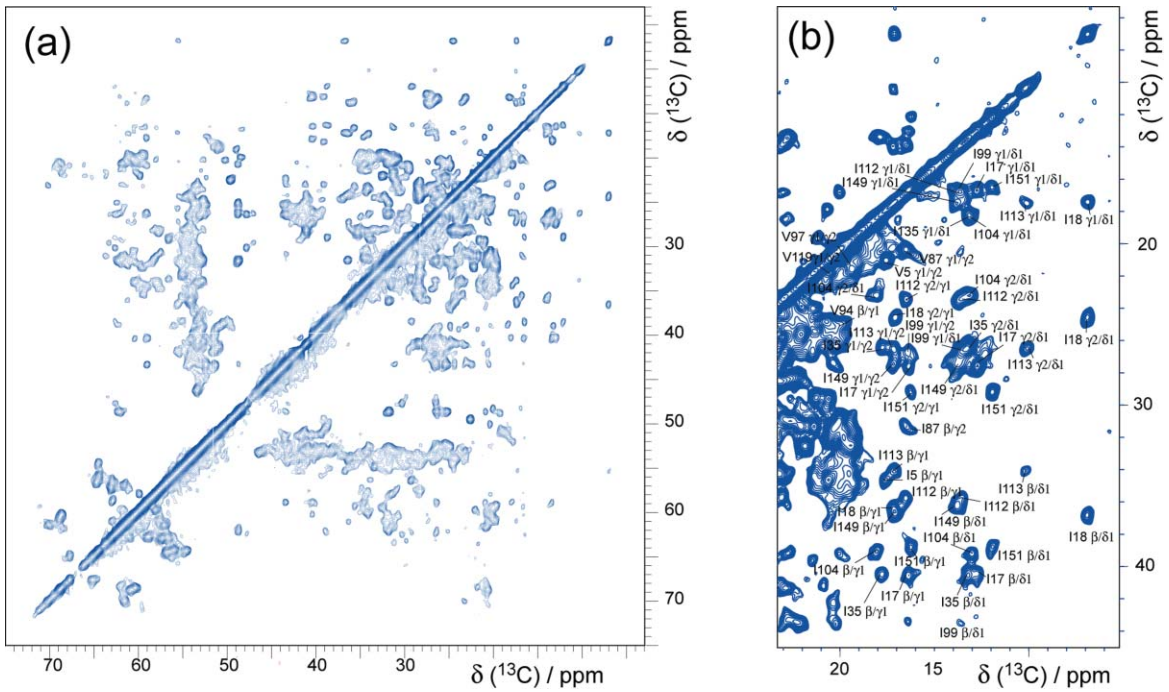


Fig. 4 Aliphatic region (a) and expansion of the methyl-aliphatic region with the assignment (b) reported of a ^{13}C–^{13}C PDSD spectrum of oxidized dimeric SOD recorded at 15 kHz MAS at 16.4 T with a mixing time of 50 ms. The sample of 10 mg of ^{13}C,^{15}N-labeled SOD, was crystallized from a PEG solution in citrate buffer at pH 5.

superimposition of the CoMMP and ZnMMP[87] spectra shown in Fig. 5, the presence of significant pcs is evident.[88] The most noticeable are marked with arrows. Since microcrystal samples are often used, where protein molecules are closely packed, attention must be paid to distinguish between intramolecular contributions arising from the dipolar interaction between the nuclei and the metal ion belonging to the same molecule, and intermolecular contributions arising from the dipolar interaction between the nuclei in one protein molecule and metal ions belonging to different protein molecules.[88] These two contributions can be easily separated and used to provide different kinds of information. Pcs due to the intramolecular interactions can be used as structural restraints through the very same approach already described for proteins in solution. Therefore, pcs together with few further restraints are expected to be effective in solving the protein structure. The intermolecular contribution to the observed pcs can instead be used to determine the position of neighbouring protein molecules. In fact, once the structure of the protein is known, and the orientation of the magnetic susceptibility anisotropy tensor with respect to the structure itself has been determined by the intramolecular contributions to the observed pcs, such intermolecular pcs provide the position of the metal ions located in neighbouring molecules, and the orientation of the main axes of the magnetic susceptibility anisotropy tensor of the latter. Such tensor axes provide the information necessary to orient the molecule themselves.

Solid-state NMR is developing very fast. Progress in methodology and instrumentation is expected to rapidly expand the possibilities of studying molecular systems of increased size and complexity. As solid-state NMR does not require crystalline and soluble samples, experiments can be conducted under a variety of experimental conditions, from microcrystals and powders to frozen solutions and gels. Eventually, intact protein complexes in membranes will become affordable. In all these cases, paramagnetism-derived information will be as precious as it is in solution NMR.

Orientation of different domains of a single protein and protein–protein interactions

The paramagnetism-based pcs and rdc restraints represent a valuable source of information for the investigation of the relative position of protein domains when one of the domains contains a paramagnetic ion, or for protein–protein complexes in all cases when one of the two proteins contains a paramagnetic ion.[89–94] Actually, exploitation of the pcs and rdc represents nowadays one of the most promising tools for obtaining information on the relative position and orientation of interacting molecules having some degrees of freedom, or of partially independent domains of proteins.[95–97] If no metal ions are present in the system, a paramagnetic tag rigidly attached to the protein can be conveniently used,[49,90,98–103] so that this technique can be profitably applied in most cases.

Considering a multidomain system, let us call A the protein domain containing a paramagnetic ion and B any other domain. Similarly, for a protein–protein complex, A is the protein containing a paramagnetic ion and B the partner protein. Because rdc do not depend on the distance of the observed nuclei from the paramagnetic ion, two cases may arise: (1) rdc values for the nuclei in B have the same distribution than those observed for A, or (2) rdc values measured for B have a (much) smaller distribution with

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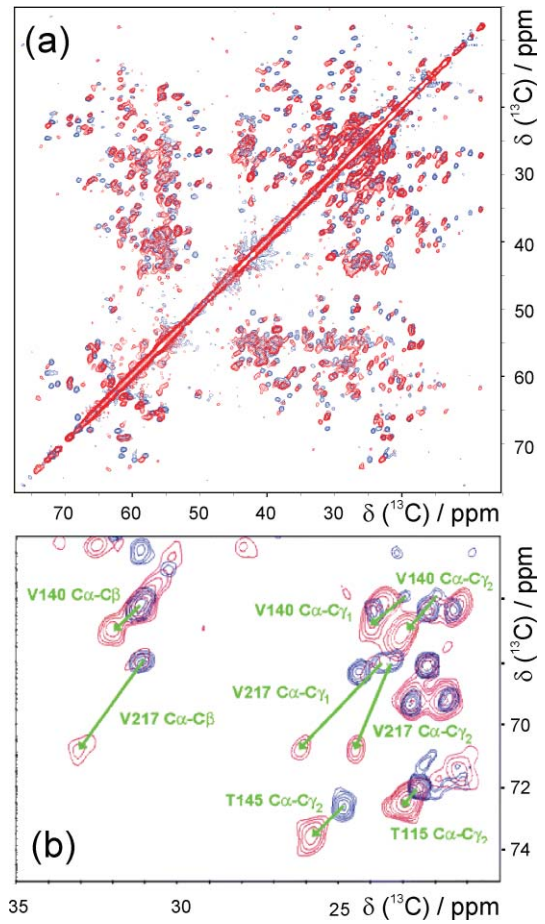

Fig. 5 Superimposition of the PDSD spectra of the diamagnetic ZnMMP (dark-grey contours) and of the paramagnetic CoMMP (light-grey contours) proteins. In (a) the aliphatic region of the two spectra is shown. In the expansion (b) some notable pcs are indicated by arrows, whose assignment is reported. The spectra were recorded at 11.5 kHz MAS at 16.4 T, mixing times of 60 ms for CoMMP and 15 ms for ZnMMP. The samples, of about 15 mg of ^{13}C,^{15}N-labeled MMP, were crystallized from a PEG solution in tris buffer at pH 7.

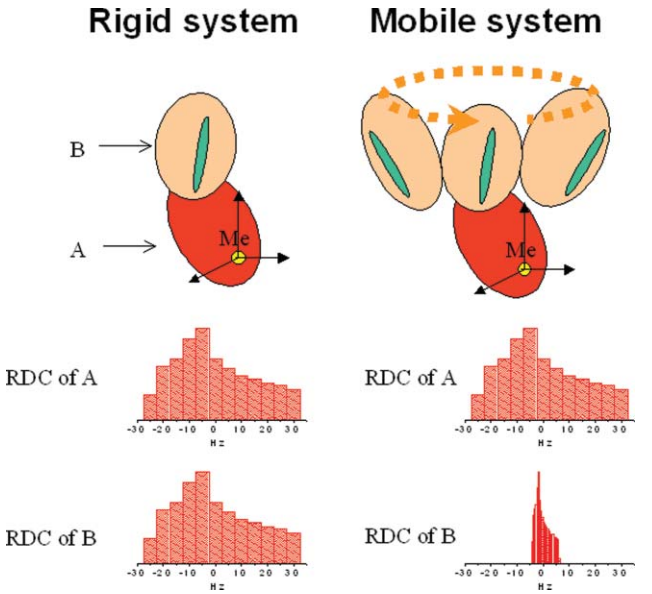

Fig. 6 Inspection of the relative distribution of observed rdc in the different domains of a protein or of a protein–protein complex provides information on the possible conformational heterogeneity experienced by the system.

respect to those of A (see Fig. 6). In fact, the measured rdc values are the average of the rdc values corresponding to the ensemble of the orientations with respect to the reference frame provided by the principal axes of the metal magnetic susceptibility tensor experienced by the system in a ms time scale. In case of motion, the range of the observed rdc values is reduced, and it can collapse to zero for cases of overall isotropic reorientation of B with respect to A.

Similarly, in case of motion, the observed pcs values are the average of the pcs values corresponding to the ensemble of the positions experienced by the system in a ms timescale with respect to the reference frame provided by the principal axes of the metal magnetic susceptibility tensor. However, differently from the rdc values, the averaged pcs values also depend on the distances of the observed nuclei from the metal ion.

If the rdc distribution measured for nuclei in B is basically the same as that measured for nuclei in A, it can be concluded that the two systems form a rigid unit. The relative orientation of the two systems can be obtained by imposing that the magnetic susceptibility anisotropy tensors obtained from A and B coincide. Because of the simultaneous availability of pcs, the relative position of the two systems can be determined as well, from the calculated position of the paramagnetic metal ion. Such information would not be available if external orienting devices are used to induce rdc.[104,105]

The degeneracy in the solution, intrinsic in the use of pcs and rdc, can be removed by acquiring data referred to more than one paramagnetic metal, unless further experimental restraints of different nature are available and are effective in removing the "ghost" solutions, or the latter can be excluded due to the presence of steric clashes.[106]

One of the most direct ways to assess the presence of conformational variability in protein systems is the observation that the range of the experimentally obtained paramagnetic rdc values of nuclei in B is significantly smaller than that of nuclei in A. Rdc measurements, however, provide only average information from which it is difficult to recover the conformations actually experienced by the system. In order to investigate such preferred conformations of B with respect to A, the following strategy has been conceived.[94] The pcs and rdc values observed on A are used to obtain position, orientation and anisotropy of the magnetic susceptibility tensor. The preferred positions of B can then be investigated by looking for the ensemble of positions (each of them with a weighing factor) providing pcs and rdc values that average to the experimentally obtained values, using the magnetic susceptibility anisotropy tensor calculated from A data. An efficient algorithm has been developed for the determination of the maximum allowed probability (MAP) that any position of B can attain with respect to A while maintaining the agreement between observed and calculated pcs and rdc data.[94] The different positions can then be ranked according to their MAP value, those with the largest MAP value likely corresponding to the preferential conformations of the complex.

One comment is due on the paramagnetic ions which should be preferentially selected to perform the measurements. Since pcs decrease with the third power of the distance of the nuclei from the metal, ions with large magnetic susceptibility anisotropy are preferable for obtaining pcs values of nuclei at relatively large distance with a sufficiently large signal-to-noise ratio. On the other hand, the presence of the paramagnetic ion does not permit the measurement of pcs and rdc of proton nuclei close to the ion itself, *i.e.* it may prevent an accurate estimate of the magnetic susceptibility anisotropy tensor on A. This difficulty can be overcome through ^{13}C direct detection, able to provide pcs and rdc values at short distances from the paramagnetic ion.

The above approach was applied to calmodulin (CaM), both free and ligated to α-synuclein.[94] CaM is a protein constituted by two rigid domains (N-terminal and C-terminal domains) of known structure, the relative orientation of which is not fixed. Pcs and rdc data acquired for terbium(III), thulium(III) and dysprosium(III) CaM were used, because they are the ions with the largest magnetic anisotropy. The magnetic susceptibility anisotropy tensors were obtained from the pcs data of the N-terminal domain, and their anisotropies were found one order of magnitude larger than the anisotropy values calculated from the rdc of the C-terminal domain.[91] The conformations that have the largest MAP values were then obtained from the conformational averaged pcs and rdc values measured on the C-terminal domain. They are reported in Fig. 7 for free CaM and for the CaM–α-synuclein complex. The MAP value for such conformations calculated for CaM when free in solution is about 0.36. The conformation with largest MAP value (0.35) obtained for CaM when bound to α-synuclein resembles the "canonical" closed conformation of calmodulin. In this case CaM can thus experience multiple conformations, the weight of the canonical closed conformation being not larger than 0.35.[94]

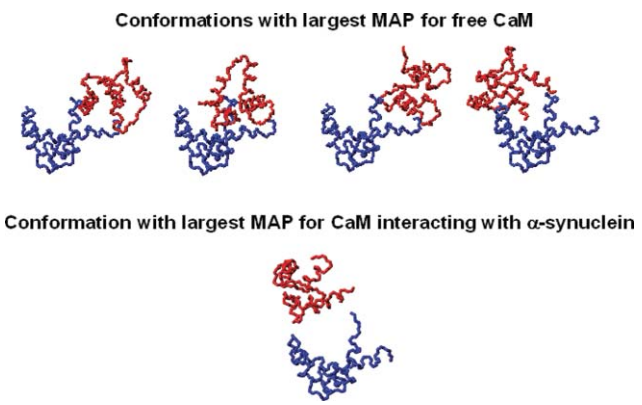

Fig. 7 Preferred conformations of CaM when free in solution or in the presence of α-synuclein.

Remarkably, this procedure cannot be easily applied using rdc data induced by external orienting media because (1) the external media orients both A and B, and thus the partial orientation observed for B is not transferred from A but it is mainly induced by direct interaction of B with the orienting materials, and (2) pcs are not available, and they are the only source of information to obtain not only the relative orientation between the two systems but also their position. Thus, the exploitation of paramagnetic metal ions is key for the success of this approach.

As a perspective, it would be highly desirable to establish methods for obtaining information on the actual weight that all possible conformations in one region of space should have. Efforts in this direction are being made. Furthermore, information from relaxation data could be combined with MAP data to further improve the description of the conformational heterogeneity of the system.

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References

1 W. Shi, C. Zhan, A. Ignatov, B. A. Manjasetty, N. Marinkovic, M. Sullivan, R. Huang and M. R. Chance, *Structure*, 2005, **13**, 1473–1486.
2 J. E. Coleman, *Methods Enzymol.*, 1993, **227**, 16–43.
3 E. Wörgötter, G. Wagner, M. Vasak, J. H. Kägi and K. Wüthrich, *J. Am. Chem. Soc.*, 1988, **110**, 2388–2393.
4 L. M. Utschig, J. G. Wright, G. Dieckmann, V. L. Pecoraro and T. V. O'Halloran, *Inorg. Chem.*, 1995, **34**, 2497–2498.
5 G. L. Oz, D. L. Pountney and I. M. Armitage, *Biochem. Cell. Biol.*, 1998, **76**, 223–234.
6 A. P. Eijkelenboom, F. M. Van den Ent, A. Vos, J. F. Doreleijers, K. Hard, T. D. Tullius, R. H. Plasterk, R. Kaptein and R. Boelens, *Curr. Biol.*, 1997, **7**, 739–746.
7 G. J. Kornhaber, D. Snyder, H. N. Moseley and G. T. Montelione, *J. Biomol. NMR*, 2006, **34**, 259–269.
8 L. Banci, I. Bertini, L. D. Eltis, I. C. Felli, D. H. W. Kastrau, C. Luchinat, M. Piccioli, R. Pierattelli and M. Smith, *Eur. J. Biochem.*, 1994, **225**, 715–725.
9 I. Bertini, C. Luchinat, G. Parigi and R. Pierattelli, *ChemBioChem*, 2005, **6**, 1536–1549.
10 I. Bertini, C. Luchinat and M. Piccioli, *Methods Enzymol.*, 2001, **339**, 314–340.
11 I. Bertini, C. Luchinat and G. Parigi, *Prog. Nucl. Magn. Reson. Spectrosc.*, 2002, **40**, 249–273.
12 L. Banci, I. Bertini and S. Mangani, *J. Synchrotron Radiat.*, 2005, **12**, 94.
13 I. Bertini, C. Luchinat and G. Parigi, *Adv. Inorg. Chem.*, 2005, **57**, 105–172.
14 W. Bermel, I. Bertini, I. C. Felli, R. Kümmerle and R. Pierattelli, *J. Am. Chem. Soc.*, 2003, **125**, 16423–16429.
15 C. Caillet–Saguy, M. Delepierre, A. Lecroisey, I. Bertini, M. Piccioli and P. Turano, *J. Am. Chem. Soc.*, 2006, **128**, 150–158.
16 T. E. Machonkin, W. M. Westler and J. L. Markley, *Inorg. Chem.*, 2005, **44**, 779–797.
17 M. John, A. Y. Park, N. E. Dixon and G. Otting, *J. Am. Chem. Soc.*, 2007, **129**, 462–463.
18 M. Sattler, J. Schleucher and C. Griesinger, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1999, **34**, 93–158.
19 W. Bermel, I. C. Felli, M. Matzapetakis, R. Pierattelli, E. C. Theil and P. Turano, *J. Magn. Reson.*, 2007, **188**, 301–310.
20 W. Bermel, I. Bertini, I. C. Felli, R. Kümmerle and R. Pierattelli, *J. Magn. Reson.*, 2006, **178**, 56–64.
21 W. Bermel, I. Bertini, I. C. Felli, M. Piccioli and R. Pierattelli, *Prog. Nucl. Magn. Reson. Spectrosc.*, 2006, **48**, 25–45.
22 W. Bermel, I. Bertini, L. Duma, L. Emsley, I. C. Felli, R. Pierattelli and P. R. Vasos, *Angew. Chem., Int. Ed.*, 2005, **44**, 3089–3092.
23 B. Vögeli, H. Kovacs and K. Pervushin, *J. Biomol. NMR*, 2005, **31**, 1–9.
24 I. Bertini, B. Jiménez and M. Piccioli, *J. Magn. Reson.*, 2005, **174**, 125–132.

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25 N. Shimba, H. Kovacs, A. S. Stern, A. M. Nomura, I. Shimada, J. C. Hoch, C. S. Craik and V. Dötsch, *J. Biomol. NMR*, 2005, **30**, 175–179.
26 I. Bertini, I. C. Felli, C. Luchinat, G. Parigi and R. Pierattelli, *ChemBioChem*, 2007, **8**, 1422–1429.
27 I. Bertini, K. L. Bren, A. Clemente, J. A. Fee, H. B. Gray, C. Luchinat, B. G. Malmström, J. H. Richards, D. Sanders and C. E. Slutter, *J. Am. Chem. Soc.*, 1996, **46**, 11658–11659.
28 I. Bertini, C. O. Fernández, B. G. Karlsson, J. Leckner, C. Luchinat, B. G. Malmström, A. M. Nersissian, R. Pierattelli, E. Shipp, J. S. Valentine and A. J. Vila, *J. Am. Chem. Soc.*, 2000, **122**, 3701–3707.
29 I. Bertini, S. Ciurli, A. Dikiy, C. O. Fernández, C. Luchinat, N. Safarov, S. Shumilin and A. J. Vila, *J. Am. Chem. Soc.*, 2001, **123**, 2405–2413.
30 A. Donaire, B. Jiménez, C. O. Fernandez, R. Pierattelli, T. Niizeki, J. M. Moratal, J. F. Hall, T. Kohzuma, S. S. Hasnain and A. J. Vila, *J. Am. Chem. Soc.*, 2002, **124**, 13698–13708.
31 L. Banci, R. Pierattelli and A. J. Vila, *Adv. Protein Chem.*, 2002, **60**, 397–449.
32 F. Arnesano, L. Banci, I. Bertini, I. C. Felli, C. Luchinat and A. R. Thompsett, *J. Am. Chem. Soc.*, 2003, **125**, 7200–7208.
33 I. Bertini, I. C. Felli, R. Kümmerle, D. Moskau and R. Pierattelli, *J. Am. Chem. Soc.*, 2004, **126**, 464–465.
34 S. Balayssac, B. Jiménez and M. Piccioli, *J. Biomol. NMR*, 2006, **34**, 63–73.
35 I. Bertini, Y.–M. Lee, C. Luchinat, M. Piccioli and L. Poggi, *ChemBioChem*, 2001, **2**, 550–558.
36 G. Pintacuda, A. Moshref, A. Leonchiks, A. Sharipo and G. Otting, *J. Biomol. NMR*, 2004, **29**, 351–361.
37 I. Solomon, *Phys. Rev.*, 1955, **99**, 559–565.
38 M. Gueron, *J. Magn. Reson.*, 1975, **19**, 58–66.
39 A. J. Vega and D. Fiat, *Mol. Phys.*, 1976, **31**, 347–355.
40 R. Ghose and J. H. Prestegard, *J. Magn. Reson.*, 1997, **128**, 138–143.
41 J. Boisbouvier, P. Gans, M. Blackledge, B. Brutscher and D. Marion, *J. Am. Chem. Soc.*, 1999, **121**, 7700–7701.
42 G. Pintacuda, K. Hohenthanner, G. Otting and N. Muller, *J. Biomol. NMR*, 2003, **27**, 115–132.
43 I. Bertini, G. Cavallaro, M. Cosenza, R. Kümmerle, C. Luchinat, M. Piccioli and L. Poggi, *J. Biomol. NMR*, 2002, **23**, 115–125.
44 I. Bertini, J. Kowalewski, C. Luchinat and G. Parigi, *J. Magn. Reson.*, 2001, **152**, 103–108.
45 G. Pintacuda, A. Kaikkonen and G. Otting, *J. Magn. Reson.*, 2004, **171**, 233–243.
46 D. S. Wishart and D. A. Case, *Methods Enzymol.*, 2001, **338**, 3–34.
47 G. Cornilescu, F. Delaglio and A. Bax, *J. Biomol. NMR*, 1999, **13**, 289–302.
48 M. John and G. Otting, *ChemPhysChem*, 2007, **8**, 2309–2313.
49 G. Pintacuda, M. John, X. C. Su and G. Otting, *Acc. Chem. Res.*, 2007, **40**, 206–212.
50 J. J. Chou, S. Li and A. Bax, *J. Biomol. NMR*, 2000, **18**, 217–227.
51 J. J. Chou, S. Li, C. B. Klee and A. Bax, *Nat. Struct. Biol.*, 2001, **8**, 990–997.
52 J. H. Prestegard, C. M. Bougault and A. I. Kishore, *Chem. Rev.*, 2004, **104**, 3519–3540.
53 P. Bernado, C. W. Bertoncini, C. Griesinger, M. Zweckstetter and M. Blackledge, *J. Am. Chem. Soc.*, 2005, **127**, 17968–17969.
54 J. H. Prestegard, K. L. Mayer, H. Valafar and G. C. Benison, *Methods Enzymol.*, 2005, **394**, 175–209.
55 M. Ottiger, F. Delaglio and A. Bax, *J. Magn. Reson.*, 1998, **131**, 373–378.
56 P. Permi, P. R. Rosevear and A. Annila, *J. Biomol. NMR*, 2000, **17**, 43–54.
57 S. Balayssac, I. Bertini, C. Luchinat, G. Parigi and M. Piccioli, *J. Am. Chem. Soc.*, 2006, **128**, 15042–15043.
58 C. D. Schwieters, J. Kuszewski, N. Tjandra and G. M. Clore, *J. Magn. Reson.*, 2003, **160**, 65–73.
59 T. Herrmann, P. Güntert and K. Wüthrich, *J. Mol. Biol.*, 2002, **319**, 209–227.
60 L. Banci, I. Bertini, G. Cavallaro, A. Giachetti, C. Luchinat and G. Parigi, *J. Biomol. NMR*, 2004, **28**, 249–261.
61 I. Bertini, C. Luchinat and G. Parigi, *Concepts Magn. Reson.*, 2002, **14**, 259–286.
62 B. A. Fowler, F. Tian, H. M. Al–Hashimi and J. H. Prestegard, *J. Mol. Biol.*, 2000, **304**, 447–460.
63 R. Barbieri, C. Luchinat and G. Parigi, *ChemPhysChem*, 2004, **21**, 797–806.
64 I. Bertini, A. Donaire, B. Jiménez, C. Luchinat, G. Parigi, M. Piccioli and L. Poggi, *J. Biomol. NMR*, 2001, **21**, 85–98.
65 I. Bertini, J. Faraone–Mennella, B. H. Gray, C. Luchinat, G. Parigi and J. R. Winkler, *JBIC, J. Biol. Inorg. Chem.*, 2004, **9**, 224–230.
66 G. Pintacuda, N. Giraud, R. Pierattelli, A. Böckmann, I. Bertini and L. Emsley, *Angew. Chem., Int. Ed.*, 2007, **46**, 1079–1082.
67 G. Kervern, S. Steuernagel, F. Engelke, G. Pintacuda and L. Emsley, *J. Am. Chem. Soc.*, 2007, **129**, 14118–14119.
68 N. Bloembergen, *Physica*, 1949, **15**, 386.
69 N. M. Szeverenyi, M. J. Sullivan and G. E. Maciel, *J. Magn. Reson.*, 1982, **47**, 462–475.
70 M. Hong, *J. Biomol. NMR*, 1999, **15**, 1–14.
71 J. Pauli, M. Baldus, B. van Rossum, H. de Groot and H. Oschkinat, *ChemBioChem*, 2001, **2**, 272–281.
72 A. Böckmann, A. Lange, A. Galinier, S. Luca, N. Giraud, M. Juy, H. Heise, R. Montserret, F. Penin and M. Baldus, *J. Biomol. NMR*, 2003, **27**, 323–339.
73 A. T. Petkova, M. Baldus, M. Belenky, M. Hong, R. G. Griffin and J. Herzfeld, *J. Magn. Reson.*, 2003, **160**, 1–12.
74 S. Luca, H. Heise and M. Baldus, *Acc. Chem. Res.*, 2003, **36**, 858–865.
75 T. I. Igumenova, A. J. Wand and A. E. McDermott, *J. Am. Chem. Soc.*, 2004, **126**, 5323–5331.
76 A. Detken, E. H. Hardy, M. Ernst, M. Kainosho, T. Kawakami, S. Aimoto and B. Meier, *J. Biomol. NMR*, 2004, **20**, 203–221.
77 T. I. Igumenova, A. E. McDermott, K. W. Zilm, R. W. Martin, E. K. Paulson and A. J. Wand, *J. Am. Chem. Soc.*, 2004, **126**, 6720–6727.
78 K. Seidel, M. Etzkorn, H. Heise, S. Becker and M. Baldus, *ChemBioChem*, 2005, **6**, 1638–1647.
79 N. P. Wickramasinghe and Y. Ishii, *J. Magn. Reson.*, 2006, **181**, 233–243.
80 C. P. Jaroniec, B. A. Tounge, J. Herzfeld and R. G. Griffin, *J. Am. Chem. Soc.*, 2001, **123**, 3507–3519.
81 A. Lange, S. Luca and M. Baldus, *J. Am. Chem. Soc.*, 2002, **124**, 9704–9705.
82 F. Castellani, B. van Rossum, A. Diehl, M. Schubert, K. Rehbein and H. Oschkinat, *Nature*, 2002, **420**, 98–102.
83 L. Sonnenberg, S. Luca and M. Baldus, *J. Magn. Reson.*, 2004, **166**, 100–110.
84 A. Lange, K. Giller, S. Hornig, M. F. Martin–Eauclaire, O. Pongs, S. Becker and M. Baldus, *Nature*, 2006, **440**, 959–962.
85 A. Grommek, B. H. Meier and M. Ernst, *Chem. Phys. Lett.*, 2007, **427**, 404–409.
86 I. Bertini, M. Fragai, Y.–M. Lee, C. Luchinat and B. Terni, *Angew. Chem., Int. Ed.*, 2004, **43**, 2254–2256.
87 S. Balayssac, I. Bertini, K. Falber, M. Fragai, S. Jehle, M. Lelli, C. Luchinat, H. Oschkinat and K. J. Yeo, *ChemBioChem*, 2007, **8**, 486–489.
88 S. Balayssac, I. Bertini, M. Lelli, C. Luchinat and M. Maletta, *J. Am. Chem. Soc.*, 2007, **129**, 2218–2219.
89 J. A. R. Worrall, U. Kolczak, G. W. Canters and M. Ubbink, *Biochemistry*, 2001, **40**, 7069–7076.
90 G. Pintacuda, A. Y. Park, M. A. Keniry, N. E. Dixonj and G. Otting, *J. Am. Chem. Soc.*, 2006, **128**, 3696–3702.
91 I. Bertini, C. Del Bianco, I. Gelis, N. Katsaros, C. Luchinat, G. Parigi, M. Peana, A. Provenzani and M. A. Zoroddu, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 6841–6846.
92 I. Diaz–Moreno, A. Diaz–Quintana, M. A. De la Rosa, M. Ubbink, *J. Biol. Chem.* 2005.
93 A. N. Volkov, J. A. R. Worrall, E. Holtzmann and M. Ubbink, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 18945–18950.
94 I. Bertini, Y. K. Gupta, C. Luchinat, G. Parigi, M. Peana, L. Sgheri and J. Yuan, *J. Am. Chem. Soc.*, 2007, **129**, 12786–12794.
95 J. A. Lukin, G. Kontaxis, V. Simplaceanu, Y. Yuan, A. Bax and C. Ho, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 517–520.
96 A. N. Volkov, J. A. R. Worrall, E. Holtzmann and M. Ubbink, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 18945–18950.
97 X. Wang, S. Srisailam, A. A. Ye, A. Lemak, C. Arrowsmith, J. H. Prestegard and F. Tian, *J. Biomol. NMR*, 2007, **39**, 53–61.
98 J. Wöhnert, K. J. Franz, M. Nitz, B. Imperiali and H. Schwalbe, *J. Am. Chem. Soc.*, 2003, **125**, 13338–13339.
99 L. J. Martin, M. J. Hähnke, J. Wöhnert, N. R. Silvaggi, K. N. Allen, H. Schwalbe and B. Imperiali, *J. Am. Chem. Soc.*, 2007, **129**, 7106–7113.

100 T. Ikegami, L. Verdier, P. Sakhaii, S. Grimme, P. Pescatore, K. Saxena, K. M. Fiebig and C. Griesinger, *J. Biomol. NMR*, 2004, **29**, 339–349.
101 M. Prudencio, J. Rohovec, J. A. Peters, E. Tocheva, M. J. Boulanger, M. E. Murphy, H. J. Hupkes, W. Koster, A. Impagliazzo and M. Ubbink, *Chem.–Eur. J.*, 2004, **5**, 3252–3260.
102 X. C. Su, T. Huber, N. E. Dixon and G. Otting, *ChemBioChem*, 2006, **7**, 1599–1604.
103 J. Y. Suh, C. Tang and G. M. Clore, *J. Am. Chem. Soc.*, 2007, **129**, 12954–12955.
104 M. W. Fischer, J. A. Losonczi, J. L. Weaver and J. H. Prestegard, *Biochemistry*, 1999, **38**, 9013–9022.
105 G. M. Clore, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 9021–9025.
106 I. Bertini, M. Longinetti, C. Luchinat, G. Parigi and L. Sgheri, *J. Biomol. NMR*, 2002, **22**, 123–136.

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