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PERSPECTIVE

Prospects for lanthanides in structural biology by NMR

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Abstract The advent of different lanthanide-binding reagents has made site-specific labelling of proteins with paramagnetic lanthanides a viable proposition. This brings many powerful techniques originally established and demonstrated for paramagnetic metalloproteins into the mainstream of structural biology. The promise is that, by exploiting the long-range effects of paramagnetism, lanthanide labelling will allow the study of larger proteins and protein–ligand complexes with greater ease and accuracy than hitherto possible. In particular, lanthanide-induced pseudocontact shifts (PCS) provide powerful restraints and 3D structure determination using PCS as the only source of experimental restraints will probably be possible with data obtained from samples with different lanthanide-tagging sites. Cell-free protein synthesis is positioned to play an important role in this strategy, as an inexpensive source of selectively labelled protein samples and for easy site-specific incorporation of unnatural lanthanide-binding amino acids.

Keywords Lanthanides · Pseudocontact shifts · Residual dipolar couplings · Protein–ligand complexes · Cell-free protein synthesis

Introduction

Paramagnetic NMR spectroscopy has recently lead to spectacular advances of our understanding of the structural species involved in protein folding (Felitsky et al. 2008) and the way in which proteins recognize proteins (Tang et al. 2006; Volkov et al. 2006), DNA (Iwahara and Clore 2006) and small ligand molecules (Tang et al. 2007). All these studies employed paramagnetic relaxation enhancements (PRE) from site-specifically introduced paramagnetic tags (nitroxide radicals or Mn^{2+} chelates) to report on different conformational species in heterogeneous samples. This perspective emphasizes other aspects of paramagnetism, mostly pseudocontact shifts (PCS) but also residual dipolar couplings (RDCs). It will argue that lanthanide tags offer significant scope for expanding the role of solution NMR spectroscopy in structural biology.

For a long time, NMR spectroscopists tended to regard paramagnetic protein NMR as a specialist interest of those working with metalloproteins that happen to contain a paramagnetic metal ion. This view is rapidly changing, as recent advances in protein chemistry have delivered a toolbox of reagents that can be used for site-specific tagging of conventional, diamagnetic non-metalloproteins with paramagnetic metal ions. Thanks to these tools, the door is open to employ lanthanide adducts in 3D structure determinations, a strategy advocated a long time ago by R. J. P. Williams for small biomolecules (Barry et al. 1971) and subsequently extended to Ca^{2+}-binding proteins containing lanthanides instead of Ca^{2+} (e.g., Lee and Sykes 1983; Bertini et al. 2002a).

The great attraction associated with paramagnetic lanthanides lies in their capability of providing long-range structural restraints. In particular, PCS reach as far as 40 Å from the metal ion (Biekofsky et al. 1999; Allegrozzi et al. 2000) and RDCs due to paramagnetically induced alignment in the magnetic field (Tolman et al. 1995) pertain to the entire molecular system. In the following, the methods and merits of tagging a protein with a paramagnetic lanthanide ion are discussed in view of applications in

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structural biology. To start with, it is useful to recall a few simple facts about paramagnetism and lanthanide ions.

PCS and PRE

Paramagnetism arises from unpaired electrons which in the case of the trivalent ions of lanthanides (Ln^{3+}) reside in chemically unreactive f orbitals. Since f orbitals are inner orbitals that are shielded from the ligand field and the interaction of lanthanide ions with ligands is mostly ionic in nature, the unpaired electrons do not nearly as readily delocalize to bound ligand molecules as the electrons of, e.g., paramagnetic heme-iron complexes. As a consequence, contact shifts (the paramagnetic equivalent of scalar couplings) are restricted to nuclei in the immediate vicinity of lanthanides. This leaves pseudocontact shifts, PCS, and paramagnetic relaxation enhancements, PRE, as the two most prominent effects caused by paramagnetic lanthanide ions in the NMR spectra of biological macromolecules.

PCS are manifested by large changes in chemical shifts of the nuclear spins that are exposed to the paramagnetism of the metal ion. PCS arise from through-space dipolar interactions with rapidly relaxing unpaired electrons. The effect is most easily described in terms of the magnetic susceptibility tensor χ which governs the interaction of the paramagnetic dipole moment with the external magnetic field. Pseudocontact shifts are proportional to the anisotropy of the χ tensor ($\Delta\chi$) and independent of the isotropic component of the χ tensor. It is therefore sufficient to describe the PCS as a function of the axial and rhombic components of the $\Delta\chi$ tensor, $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$, in close analogy to the common formulation of residual dipolar couplings (Bertini et al. 2002b):

$$\Delta\delta^{PCS} = \frac{1}{12\pi r^3}\left[\Delta\chi_{ax}\left(3\cos^2\theta - 1\right) + \frac{3}{2}\Delta\chi_{rh}\sin^2\theta\cos 2\varphi\right] \quad (1)$$

where $\Delta\delta^{PCS}$ denotes the difference in chemical shifts measured between diamagnetic and paramagnetic samples and r (the distance between the metal ion and the nuclear spin), θ and φ are the polar coordinates describing the position of the nuclear spin with respect to the principal axes of the $\Delta\chi$ tensor. An intuitive representation of the $\Delta\chi$ tensor is to superimpose isosurfaces of constant PCS (i.e. $\Delta\delta^{PCS}$) values on the structure of the biomolecule (Fig. 1).

Unlike PCS, paramagnetic relaxation enhancements of nuclear spins are proportional to the isotropic magnitude of the $\Delta\chi$ tensor which reflects the magnetogyric ratio of the electronic spin γ_S. In addition, PREs depend on the nuclear spin γ_I and the distance from the paramagnetic centre r. The relaxation enhancements are predominantly due to either dipolar relaxation (for long electronic lifetimes as in the case of Gd^{3+}, Mn^{2+} or nitroxide radicals; Solomon 1955) or Curie-spin relaxation mechanisms (for very short electronic lifetimes as in the case of paramagnetic lanthanides other than Gd^{3+}; Guéron 1975). Both mechanisms display the same distance dependence. It is informative to inspect the predominant term of transverse relaxation enhancement R_2^{PRE} in the limit of slow molecular tumbling as this determines the (mostly undesired) paramagnetic broadening of NMR signals in macromolecules. In the case of dipolar relaxation

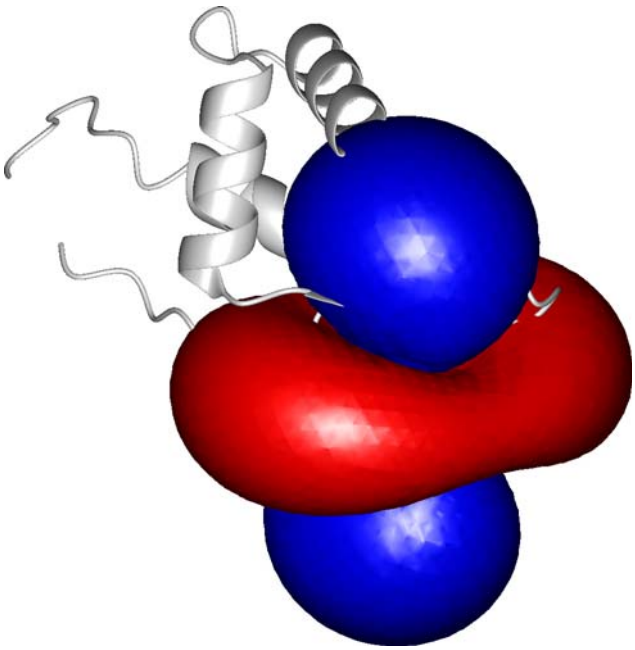

Fig. 1 Isosurfaces marking the location where a Tm^{3+} ion bound to the N-terminal DNA-binding domain of the *E. coli* arginine repressor generates pseudocontact shifts of +1 ppm (blue) and −1 ppm (red). The isosurfaces are superimposed on a ribbon representation of the protein and centred at the Tm^{3+} ion. The figure was created using Numbat (Schmitz et al. 2008) and Molmol (Koradi et al. 1996)

$$R_{2,S}^{PRE} \propto \frac{\gamma_I^2\gamma_S^2\tau_c}{r^6} \quad (2)$$

where $1/\tau_c = 1/\tau_r + 1/\tau_e$ (τ_r is the rotational correlation time of molecular tumbling and τ_e is a measure of the lifetime of the electronic spin state). In the case of Curie-spin relaxation

$$R_{2,C}^{PRE} \propto \frac{\gamma_I^2 B_0^2[g_e S_e(S_e+1)]^2\tau_r}{r^6 T^2} \quad (3)$$

where g_e and S_e are the electronic g-factor and spin, T the temperature and B_0 the magnetic field strength.

A number of fundamental conclusions can be drawn from Eqs. 1–3. (i) The NMR signals of nuclei near the paramagnetic centre are easily broadened beyond detection (depending on the magnitude of the paramagnetic dipole moment of the metal ion) but the PRE decays much more

rapidly with increasing distance from the paramagnetic centre than the PCS. (ii) The best metal ions for PCS measurements are those with a highly anisotropic χ tensor. (iii) PCS are field independent, whereas Curie-spin relaxation (which is the critical mechanism for all PCS-generating lanthanides) increases with the square of the field strength. (iv) The relaxation enhancement is less pronounced for ^{15}N and ^{13}C spins than for ^{1}H spins. (v) In contrast to RDCs, PCS contain distance information with respect to the paramagnetic centre (Eq. 1). Furthermore, PCS are not very sensitive to small displacements of the nuclear spins as they frequently occur in, e.g., flexible loop regions of proteins. These properties make PCS a powerful tool for assessing the global fold of biomolecules and intermolecular complexes. (vi) While restricted motions of a nuclear spin located far from the paramagnetic centre would not change its PCS very much, motions of the paramagnetic tag with respect to the target molecule can be detrimental by leading to much reduced average PCS while PRE remains virtually unabated. Mere reorientation of the $\Delta\chi$ tensor is sufficient to elicit this effect. (vii) Increased rotational correlation times τ_r as encountered in proteins of high molecular weight extend the zone of unobservable nuclear magnetization around the metal ion, but even a 100-fold increase in τ_r (and, hence, molecular weight) increases the radius of this blind zone no more than 2.2-fold (Eq. 3). In a 10 kDa protein at 25 °C, Tm^{3+} broadens the NMR signals of protons at $r = 15$ Å by about 15 Hz on a 800 MHz NMR spectrometer, while PCS can be observed for protons as far as 35 Å from the metal. By extrapolation, PRE in a 1 MDa system would be expected to broaden the ^{1}H-NMR line by about 15 Hz at a distance of 32 Å from the Tm^{3+} ion and by less than 10 Hz at $r = 35$ Å. Therefore, PCS will be measurable for systems of molecular weights at the current limit of solution NMR spectroscopy (Fiaux et al. 2002).

The varied paramagnetism of lanthanides

Placed in the same chemical environment, different lanthanide ions display very different magnitudes of the χ tensor and its associated anisotropy (Bleaney 1972; Bertini et al. 2001a; Fig. 2). Ranked by $\Delta\chi$-tensor magnitude, they may be, somewhat arbitrarily, classified as highly paramagnetic (e.g., Dy^{3+}, Tb^{3+}, Tm^{3+}), moderately paramagnetic (Er^{3+}, Yb^{3+}) and little paramagnetic (Eu^{3+}, Ce^{3+}, Sm^{3+}). Lanthanides with small $\Delta\chi$ tensors also generate little PRE which can be exploited to obtain structural information close to the metal ion. Importantly, there are also diamagnetic lanthanides and other trivalent diamagnetic metal ions with similar ionic radii (La^{3+}, Y^{3+}, Lu^{3+}, Sc^{3+}) which can serve as diamagnetic references. A diamagnetic reference presents

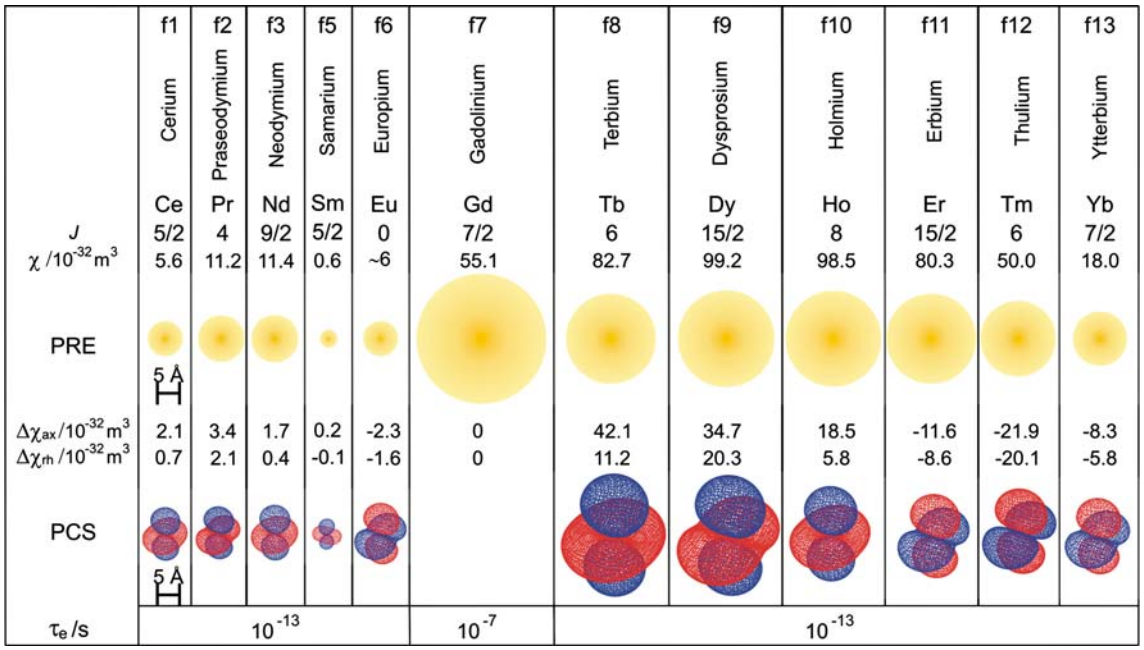

Fig. 2 Paramagnetic properties of Ln^{3+} ions. Only paramagnetic and non-radioactive lanthanides are included. Representative isosurfaces are plotted for PCS by ±5 ppm using tensors reported by Bertini et al. (2001a). The radii of the yellow spheres indicate the distances from the metal ion at which ^{1}H NMR signals of macromolecules with a rotational correlation time of 15 ns broaden by 80 Hz due to PRE at a magnetic field strength of 18.8 T. Typical electronic relaxation times representative for this field strength are indicated at the bottom. The unique paramagnetic properties of gadolinium are the basis for many contrast agents in MR imaging (Bellin 2006). Reproduced with permission from Pintacuda et al. (2007). Copyright 2007 American Chemical Society

the protein–metal complex in its diamagnetic state. This is important because all paramagnetic effects are measured as the difference between data measured in the paramagnetic and diamagnetic state, and the diamagnetic properties (chemical shifts in particular) are sensitive to metal binding.

Gadolinium is special among the paramagnetic lanthanides because, like Mn^{2+} and nitroxide spin-labels, it gives rise to large PREs without noticeable PCS. PREs caused by gadolinium are larger than those caused by nitroxides and comparable to those arising from Mn^{2+}. Lanthanides that generate PCS add another dimension, as PCS deliver orientation information and can be observed over a wide range of distances, ranging from the coordination sphere of the lanthanide (e.g. Sm^{3+}) up to distances greater than 40 Å from the metal ion (e.g. Dy^{3+}). In order to fully exploit lanthanide paramagnetism, however, the molecules of interest must be site-specifically labelled with a lanthanide.

Lanthanide tags

Lanthanides do not play any known role in biology. "Natural" lanthanide binding sites are correspondingly rare, usually requiring the displacement of a different metal ion (e.g. Ca^{2+}, Mg^{2+}, Mn^{2+}) from the metal binding site of a metalloprotein. In most situations therefore, lanthanide-binding tags do not have to compete with natural lanthanide binding sites, ensuring the specificity of lanthanide labelling.

The first lanthanide tags were lanthanide-binding peptides fused to the N- or C-termini of proteins (Ma and Opella 2000; Wöhnert et al. 2003). More freedom of lanthanide positioning is, however, achieved by tags that form a covalent bond with a thiol group of the target molecule. Many different tags have been developed for this purpose (Dvoretsky et al. 2002; Ikegami et al. 2004; Leonov et al. 2005; Haberz et al. 2006; Rodriguez-Castañeda et al. 2006; Su et al. 2006, 2008). In order to avoid PCS averaging due to tag mobility, the lanthanide ion must be rigidly attached to the target molecule. This can be achieved by tethering the lanthanide tag simultaneously to two different attachment sites of the target molecule (Prudêncio et al. 2004; Keizers et al. 2007; Vlasie et al. 2007) or by using a bulky lanthanide tag for which reorientation is prevented by steric hindrance (Su et al. 2006, 2008; Martin et al. 2007). The best tags avoid peak doubling that can arise from metal coordination with different chiralities (Ikegami et al. 2004).

The derivatization of thiol groups with lanthanide tags can be achieved easily and in high yields but is specific only if a single thiol group (or two in the case of bridging reagents) is accessible in the target molecule. Most proteins, however, contain several cysteine residues. In addition, the folding and purification protocols of proteins with disulfide bonds may be compromised if an additional cysteine residue is introduced by site-directed mutagenesis. The advent of metal-binding unnatural amino acids that can be incorporated site-specifically by a modified expression system (Xie et al. 2007) thus presents a great step forward, as this not only delivers site-specific lanthanide labels without any limitation by the amino acid sequence, but also avoids the protein handling associated with chemical derivatization.

The best expression system for incorporation of an unnatural amino acid uses an amber-suppressor tRNA from *Methanococcus jannaschii* and a mutant tRNA synthetase which has been evolved to selectively recognize the suppressor tRNA and charge it with the unnatural amino acid (Wang et al. 2006). Currently available metal-binding unnatural amino acids do not bind lanthanides well (Xie et al. 2007) but this situation is likely to change very soon. Since unnatural amino acids are relatively expensive, it is important to optimise their incorporation into proteins. This can be achieved by cell-free protein synthesis (Ozawa et al. 2005).

Cell-free protein synthesis

A typical cell-free protein synthesis system uses a reaction mixture containing cell extract from *E. coli* in a dialysis bag which is submersed in a dialysis buffer containing amino acids, a source of ATP and other low-molecular weight compounds (Apponyi et al. 2008). Compared to conventional in vivo expression systems, cell-free protein synthesis has the advantage of using amino acids only sparingly because the target protein is the only protein that will be produced. Yields of 1 mg protein per ml reaction mixture are not unusual and isotopic scrambling due to *E. coli* enzymes is greatly suppressed, allowing the inexpensive and clean preparation of selectively ^{15}N-labelled proteins (Ozawa et al. 2004). As an added benefit, the selection of the cross-peaks from ^{15}N-labelled residues afforded by ^{15}N–^{1}H correlation spectra opens the possibility of recording the NMR spectra without chromatographic purification of the protein, i.e. in the presence of the unlabelled proteins from the cell extract (Guignard et al. 2002). This capability makes NMR spectroscopy an ideal detection method for the products of cell-free protein synthesis.

In the context of site-specific introduction of unnatural amino acids into proteins, cell-free protein synthesis has another great advantage up its sleeve: the possibility of producing proteins from PCR-amplified DNA. The advantage lies in the possibility of introducing site-directed amber-stop mutations as part of the PCR-amplification protocol, delivering the mutant protein in about one day without any need of cloning the mutant DNA and without penalty in yield compared to using vector DNA. If the

cell-free extracts can be prepared in-house, the PCR reagents are the most expensive ingredient of the protocol. Therefore, mutant protein samples containing a single ^{15}N-labelled amino acid (and all other amino acids at natural isotopic abundance) can be prepared both quickly and inexpensively, which is advantageous if the integrity of the protein following site-specific incorporation of a lanthanide-binding amino acid is to be explored prior to committing to the more expensive preparation of uniformly isotope-labelled protein (Wu et al. 2007).

Assignment of paramagnetic NMR spectra

In principle, the NMR resonance assignment is harder for paramagnetic than for diamagnetic proteins because many signals are significantly broader or even missing from the NMR spectrum of a paramagnetic protein. While this is less of a problem for paramagnetic species that do not cause PCS (e.g. Gd^{3+}, Mn^{2+}, nitroxides), there are a few strategies that yield resonance assignments very easily also in the presence of PCS.

(i) If the metal ion exchanges sufficiently rapidly between protein molecules to average the chemical shifts of the paramagnetic and diamagnetic species, the NMR signals can simply be tracked in a titration experiment using increasing concentrations of the paramagnetic metal (John and Otting 2007).

(ii) If the lanthanide ion exchanges on the time scale of a NOESY mixing time (up to about 0.5 s), a mixture of paramagnetic and diamagnetic lanthanides can be used to generate exchange cross-peaks between the paramagnetic and diamagnetic signals in the NMR spectrum. This yields the PCS in a single NMR spectrum. Long mixing times can be used because paramagnetism contributes only little to the longitudinal relaxation rates of slowly tumbling molecules (John and Otting 2007). Therefore, the best lanthanide tag often is not one that binds lanthanides very tightly, but one that binds lanthanides with sufficiently rapid exchange kinetics.

(iii) As nuclear spins that are close in space display similar PCS, the cross-peaks of the paramagnetic protein are displaced by similar ppm values in all dimensions of, e.g., a 2D ^{15}N-HSQC spectrum or a 3D HNCO spectrum, compared to the cross-peaks of the diamagnetic reference. Selective isotope labelling can be used to reduce the spectral overlap in the NMR spectra, eliminating any ambiguities in associating paramagnetic cross-peaks with their corresponding diamagnetic cross-peaks (Pintacuda et al. 2004a). For smaller proteins with little spectral overlap in the 2D correlation spectra, cell-free protein synthesis can effectively deliver the residue-type information for all 20 amino acids from only five samples produced with combinatorial isotope labelling (Wu et al. 2006). As a last resort, cell-free protein synthesis also allows resonance assignment by site-directed mutagenesis with acceptable effort (Wu et al. 2007).

(iv) Prior knowledge of the 3D structure of the protein greatly assists the resonance assignment. As a $\Delta\chi$ tensor of a lanthanide ion can be characterized by 8 parameters (3 parameters describing the position of the metal ion, 3 parameters describing the orientation of the $\Delta\chi$ tensor with respect to the molecular coordinates and 2 parameters for the axial and rhombic components), measurement of eight or more PCS is sufficient to fit the tensor parameters and back-calculate the PCS expected for all other nuclei of the protein. If the structure in solution is faithfully represented by the available 3D coordinates of the protein, one can even use a set of experimentally measured PCS to assign both the paramagnetic and the diamagnetic peaks of the protein without any supporting assignments obtained by conventional strategies (Pintacuda et al. 2004a; John et al. 2007).

While it may be efficient and useful for subsequent solution studies to obtain resonance assignments of proteins of known 3D structure from PCS, PCS also present extraordinarily powerful restraints for 3D structure determination.

Exploiting $\Delta\chi$ tensors

Depending on the application, it may not be necessary to measure the PCS of every single nuclear spin. For example, if the 3D structures of two protein molecules are already known and the goal is to model the complex between them by rigid-body docking of the individual proteins, only a very small set of parameters is required. PCS induced in both proteins by a lanthanide attached to one of them can be used to determine the eight $\Delta\chi$-tensor parameters with respect to the first protein and the eight $\Delta\chi$-tensor parameters with respect to the second protein. Straightforward superimposition of the $\Delta\chi$ tensors yields the structure of the complex (Pintacuda et al. 2006). In practice, the accuracy of $\Delta\chi$-tensor determination increases greatly if more than eight experimental PCS values are available for each protein, but complete resonance assignment is certainly not necessary. If a large number of PCS is available, the accuracy of $\Delta\chi$-tensor determination is hardly affected by a small percentage of wrong assignments in the paramagnetic state (Schmitz et al. 2006).

Is it possible to perform 3D structure determinations with PCS as the only restraints? The most favourable situation is perhaps one where the structure to be determined is that of a small ligand molecule bound to a lanthanide-tagged protein of known structure. In this case, the $\Delta\chi$ tensors of paramagnetic lanthanides can be determined using the protein coordinates. If the ligand only has a few rotatable bonds and binds to the protein in the vicinity of the lanthanide-binding site, the conformation of the ligand can indeed be determined using only PCS, especially if different nuclei of the ligand experience PCS of different sign (John et al. 2006).

A much greater challenge is the 3D structure determination of a protein using PCS only. If only a single lanthanide-binding site is available and only PCS of amide protons have been measured, this may not be achievable. The situation may be different, however, if PCS can be measured for multiple samples, where each contains a lanthanide tag at a different position. In principle, four different samples and PCS measured in ^{15}N-HSQC spectra should be sufficient to pinpoint the backbone amides in space. The argument runs as follows. Observing the PCS of different amide groups from 4 different $\Delta\chi$ tensors yields 4 experimental data for each amide. The position of a nuclear spin contains only 3 unknowns (its x, y, z coordinates). Therefore, the 4th PCS measurement yields one extra parameter of information per amide group which is available for the determination of the $\Delta\chi$-tensor parameters. At least $4 \times 8 = 32$ measurements are necessary to define the 4 different tensors. As it is not difficult to measure PCS of 32 ^{15}N-HSQC cross-peaks in a residue-selectively ^{15}N-labelled protein, this strategy should deliver the relative positions of the amides in 3D space without any additional information. Finding a suitable computational approach to solving this high-dimensional problem is still a challenge. If successful, this strategy will act like a molecular zoom, as a region of interest can be picked by the choice of lanthanide-tagging sites and the PCS deliver structural information without the need of having to determine the resonance assignments or structure of the rest of the system.

Unique advantages of PCS

The magnitude and long-range nature of PCS effects make them a powerful tool for the analysis of intermolecular complexes. For example, PCS can readily be measured even if they are scaled down due to incomplete complex formation. This is useful for the structure analysis of transient complexes (Crowley and Ubbink 2003; John et al. 2006). In addition, PCS can reach across interfaces where the NMR resonances may be broadened due to chemical or conformational exchange.

As PCS data are obtained from simple chemical shift measurements, any observable NMR signal can potentially yield a PCS restraint. In principle, therefore, PCS can deliver structural information even for systems of very high molecular weight for which only the most sensitive 2D ^{15}N–^{1}H or ^{13}C–^{1}H correlation spectra yield observable signals, limited only by the increasing efficiency of PRE with increasing molecular weight (Eq. 3). Ultimately, however, even the PCS induced by the strongest lanthanide are limited in range. In situations where the nuclear spins are located beyond the reach of PCS and PRE, it may be attractive to measure RDCs resulting from lanthanide paramagnetism, as RDCs do not depend on the distance from the metal ion.

Paramagnetic alignment for RDC measurements

All lanthanide ions with non-vanishing χ-tensor anisotropy generate weak molecular alignment with the magnetic field and therefore RDCs. The alignment tensor A originating from the $\Delta\chi$ tensor is simply proportional to the $\Delta\chi$ tensor, with the same axes directions

$$A = \frac{B_0^2}{15\mu_0 kT}\,\Delta\chi \tag{4}$$

where B_0 is the magnetic field strength, μ_0 the induction constant, k the Boltzmann constant, and T the temperature (Bertini et al. 2002b). One-bond ^{1}H–^{15}N RDCs greater than 20 Hz have been observed at 18.8 T for a protein with bound Tm^{3+} (Su et al. 2008).

Alignment by paramagnetic lanthanides rather than by alignment media is potentially attractive if interactions with the alignment media could change the structure of the macromolecule and, hence, affect the RDCs. In addition, lanthanide tags placed at different locations of the molecule or different tags placed at the same location readily provide different alignment orientations which are critical for detailed studies of structure and dynamics by RDCs. As a drawback, the paramagnetism invariably leads to line broadening for nuclear spins close to the metal ion, making it harder to measure small RDCs for those nuclei. Interestingly, Eq. 4 does not depend on the molecular weight of the molecule, i.e. a single lanthanide ion should be capable of aligning huge particles! Although the diamagnetic susceptibility increases with increasing molecular size and may become larger than the paramagnetic susceptibility, an experiment subtracting a diamagnetic reference would still isolate the paramagnetic component of molecular alignment. In a large molecule, most of the nuclear spins would hardly be affected by PRE which is helpful for RDC measurements.

Other effects—DSA/DD cross-correlation

A more detailed analysis of paramagnetism in high-resolution NMR reveals additional effects that one has to keep in mind. Some important effects are most readily understood by considering the mathematical analogy of Curie-spin relaxation and chemical shift anisotropy (CSA; Bertini et al. 2001b) which is captured in the term "dipolar shift anisotropy" (DSA). One effect is the cross-correlation between DSA and dipolar (DD) relaxation (Ghose and Prestegard 1997). DSA/DD cross-correlation results in different relaxation rates of individual multiplet components in analogy to the familiar CSA/DD cross-correlation which is the basis of the TROSY effect (Pervushin et al. 1997). Quantitative measurement of DSA/DD cross-correlation effects yields structure restraints complementary to those obtained by PCS, RDC or PRE measurements, but requires good sensitivity and the effects decrease with r^{-3} with increasing distance from the paramagnetic centre (Ghose and Prestegard 1997). More important for studies of large ^{15}N-labelled proteins tagged with a paramagnetic Ln^{3+} ion is the fact that DSA/DD cross-correlation enhances or counteracts the usual TROSY effect depending on the angle between the ^{1}H–Ln^{3+} and the ^{1}H–^{15}N vectors. This can make it harder to acquire TROSY-type NMR spectra of large paramagnetic proteins. Broadband decoupling as usually performed in HSQC spectra not only eliminates DSA/DD cross-correlation effects, but also avoids the RDC contributions which would affect chemical shifts measured in TROSY spectra.

Other effects—DSA/CSA cross-correlation

The analogy between DSA and CSA implies that cross-correlation between both effects can increase or decrease overall relaxation, depending on the relative orientation of the corresponding tensors (Pintacuda et al. 2004b). This means that it is difficult to extract distance restraints from PRE measurements using lanthanide ions that predominantly relax by the Curie relaxation mechanism. Under certain circumstances, the overall relaxation in the paramagnetic molecule can be slower than in the diamagnetic reference! Fortunately, PRE measurements using Gd^{3+} do not suffer from this effect.

Other effects—RACS

Weak molecular alignment in the magnetic field due to paramagnetism not only leads to RDCs, but also to residual anisotropic chemical shifts (RACS) due to non-uniform sampling of the chemical shifts governed by the CSA tensors (John et al. 2005). The effect can affect PCS measurements of nuclei with large CSA tensors, but is significant only at high magnetic field strength (up to 0.2 ppm for ^{15}N at 18.8 T). Like the effects mentioned above, RACS effects can be accurately calculated and taken into account if the structure of the molecule is known a priori.

Other effects—temperature-dependent PCS

As the Curie spin decreases with increasing temperatures (Eq. 3), also the PCS are temperature dependent. This effect can be exploited to support the resonance assignment of paramagnetic proteins, providing the temperature dependence of the diamagnetic chemical shifts can be taken into account (Allegrozzi et al. 2000).

The case for lower magnetic fields

Curie-spin relaxation as well as the paramagnetically induced alignment that gives rise to RDC and RACS effects increase with the square of the external magnetic field, whereas PCS are field independent. The RACS of ^{15}N amide or ^{13}C carbonyl spins due to one of the most strongly paramagnetic lanthanide ions (Dy^{3+}) is less than 0.1 ppm on a 500 MHz NMR spectrometer and an order of magnitude smaller for ^{1}H spins (John et al. 2005). Under the same conditions, the maximal paramagnetically induced RDC between two ^{1}H spins separated by 2.5 Å is about 7 Hz. Higher magnetic fields are thus barely useful for macromolecules tagged with the most paramagnetic lanthanides, unless the intention is to use them for molecular alignment. Contrary to conventional wisdom, lower magnetic fields than the highest available can increase the spectral resolution for PCS measurements by avoiding excessive line broadening due to unresolved ^{1}H–^{1}H RDCs as well as PRE (Eq. 3).

Concluding remark

The use of lanthanides as tools for the 3D structure analysis of proteins and biomolecular aggregates has barely begun. Proofs of principle have been established, mathematical descriptions of the paramagnetic effects are in place and software for data analysis is available. The toolbox is completed by the recent advent of reagents and techniques for easy site-specific incorporation of lanthanide tags. The possibility of extracting 3D structural information from simple 2D correlation spectra is certainly alluring! Ample opportunities in structural biology are beckoning.

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