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PERSPECTIVE

# Paramagnetic labelling of proteins and oligonucleotides for NMR

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**Abstract** Paramagnetic effects offer a rich source of long-range structural restraints. Here we review current methods for site-specific tagging of proteins and oligonucleotides with paramagnetic molecules. The paramagnetic tags include nitroxide radicals and metal chelators. Particular emphasis is placed on tags suitable for site-specific and rigid attachment of lanthanide ions to macromolecules.

**Keywords** Lanthanides · Nitroxide Radicals · Paramagnetic NMR · Paramagnetic protein and oligonucleotide tags · Site-specific labelling

## Introduction

Site-specific labelling of proteins and oligonucleotides with paramagnetic tags introduces pronounced paramagnetic effects in the macromolecules. At greater distances from the paramagnetic centre where through-bond effects are unimportant, four different effects can be exploited to obtain long-range structural information: (i) paramagnetic relaxation enhancement (PRE), (ii) pseudocontact shifts (PCS), (iii) residual dipolar couplings (RDC) induced by anisotropic paramagnetic centres and (iv) cross-correlated relaxation (CCR) effects between anisotropic paramagnetic centres and anisotropic parameters of the nuclear spins. Paramagnetic centres with isotropic electron spin distribution (nitroxide radicals, $Mn^{2+}$ and $Gd^{3+}$ ions) are characterized by slow electron relaxation and produce large PREs that decay with $r^{-6}$ ($r$ = distance of the nuclear spin from the paramagnetic centre) without complicating PCS, RDC or CCR effects. In contrast, paramagnetic centres with anisotropic electron spin distributions (most paramagnetic metal ions, including most of the lanthanides) create all four long-range paramagnetic effects, with a corresponding increase in structural information (Otting 2008).

Site-specific tagging of proteins and nucleic acids with nitroxide radicals has long been used in electron paramagnetic resonance (EPR) for studies of the chemical and dynamic environment of the tag and for distance measurements between two tags (Hustedt and Beth 1999). The labelling strategies developed for EPR have also successfully been applied in high-resolution NMR spectroscopy. One of the most powerful recent applications of PREs is the detection of rare conformational species in systems undergoing chemical exchange, which is possible if the rare species experiences much stronger PRE than the predominant species (e.g. Clore et al. 2007). Complexes with $Mn^{2+}$ or $Gd^{3+}$ act in a similar way as nitroxide radicals except that they deliver stronger PREs.

Site-specific tagging with metal ions associated with anisotropic electron density distribution is less attractive for EPR applications because of short electron relaxation times. For NMR applications, however, these metal ions are of great interest as their paramagnetic effects contain very useful angular and distance information that can be used to refine protein structures (Bertini et al. 2002a), rapidly determine the structure of protein-protein and protein-ligand complexes (Pintacuda et al. 2006, 2007; John et al. 2006), explore inter-domain orientations in proteins (Bertini et al. 2004), and assign protein NMR resonances automatically by reference to the known three-dimensional structure of the protein (Pintacuda et al. 2004a; Schmitz et al. 2006, 2008; John et al. 2007a).

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For example, a pseudocontact shift $\Delta\delta^{PCS}$ depends on the polar coordinates $r$, $\theta$ and $\varphi$ of the nuclear spin with respect to the principal axes of the $\Delta\chi$ tensor:

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

where $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ denote, respectively, the axial and rhombic components of the $\Delta\chi$ tensor which is the anisotropy component of the magnetic susceptibility tensor $\chi$ describing the magnetic moment of the paramagnetic centre. The $1/r^3$ distance dependence of $\Delta\delta^{PCS}$ leads to measurable effects at distances of up to 40 Å and longer (Table 1; Allegrozzi et al. 2000) which is greater than the reach of PRE. In addition, a non-vanishing $\Delta\chi$ tensor leads to partial molecular alignment in the magnetic field and, consequently, RDCs which are independent of the distance from the metal (Tolman et al. 1997; Bertini et al. 2002b). Finally, a non-vanishing $\Delta\chi$ tensor also causes CCR effects, some of which contain valuable structural information (Ghose and Prestegard 1997; Boisbouvier et al. 1999), while others break the link between PREs and distance information (Pintacuda et al. 2004b).

Pseudocontact shifts are particularly easy to measure, as they simply correspond to the difference in chemical shifts between paramagnetic and diamagnetic samples (provided contact shifts and residual anisotropic shifts due to alignment in the magnetic field (John et al. 2005) can be disregarded). For PCSs, as for all measurements of paramagnetic effects, reference experiments performed with suitable diamagnetic samples are of critical importance.

A sample with a diamagnetic tag presents a better diamagnetic reference than a sample without tag, as the mere presence of a tag will change the chemical shifts in its vicinity. In the case of nitroxide radicals, reduction with ascorbate leads to diamagnetic hydroxylamine compounds which can serve as a diamagnetic reference. Paramagnetic metal ions can often be substituted by diamagnetic metal ions of similar ionic radius and chemical properties. In this regard and in many other aspects, lanthanide ions present ideal paramagnetic labels. (i) They are chemically very similar, i.e. a lanthanide binding site can bind any of the lanthanide ions. (ii) Different lanthanides have very different $\Delta\chi$ tensors (Table 1), with $La^{3+}$, $Y^{3+}$ and $Lu^{3+}$ presenting suitable diamagnetic references. (iii) The unpaired electrons of lanthanides are shielded from the ligand field, so that contact shifts are much less prominent than for transition metal ions (Shelling et al. 1984). (iv) The electron relaxation times of lanthanides are very short (except for $Gd^{3+}$), minimizing nuclear relaxation enhancements. (v) Lanthanides play no biological role, i.e. proteins do not naturally contain designated lanthanide binding sites. As a result, even an excess of lanthanide ions usually produces only relatively minor paramagnetic effects (Campbell et al. 1973; Sattler and Fesik 1997). This is an advantage because the absence of competing natural binding sites facilitates the use of lanthanide tags. Usually, only some metalloproteins in their apo form and the phosphate backbone of oligonucleotides can bind lanthanides with significant affinity, placing more stringent requirements on the lanthanide affinity of the tags.

With the recent advent of new lanthanide tags (for a review, see Rodriguez-Castañeda et al. 2006), paramagnetic NMR with lanthanides has gained strongly in interest. Some of the lanthanide tags also confer useful luminescent properties to the protein (Chen and Selvin 1999; Nitz et al. 2003).

**Table 1** Paramagnetism of metal ions with significantly anisotropic magnetic susceptibility tensors

| | $\Delta\chi_{ax}/(10^{-32}\ m^3)$[a] | PCS/ppm $r = 15$ Å | PCS/ppm $r = 20$ Å | PCS/ppm $r = 30$ Å | PCS/ppm $r = 40$ Å |
|---|---|---|---|---|---|
| $Co^{2+}$ (high spin) | 7.0[b] | 1.10 | 0.46 | 0.14 | 0.06 |
| $Ce^{3+}$ | 2.1 | 0.33 | 0.14 | 0.04 | 0.02 |
| $Pr^{3+}$ | 3.4 | 0.53 | 0.23 | 0.07 | 0.03 |
| $Nd^{3+}$ | 1.7 | 0.27 | 0.11 | 0.03 | 0.01 |
| $Tb^{3+}$ | 42.1 | 6.62 | 2.79 | 0.83 | 0.35 |
| $Dy^{3+}$ | 34.7 | 5.46 | 2.30 | 0.68 | 0.29 |
| $Ho^{3+}$ | 18.5 | 2.91 | 1.23 | 0.36 | 0.15 |
| $Er^{3+}$ | 12.2 | 1.92 | 0.81 | 0.24 | 0.10 |
| $Tm^{3+}$ | 26.0 | 4.09 | 1.72 | 0.51 | 0.22 |
| $Yb^{3+}$ | 8.5 | 1.34 | 0.56 | 0.17 | 0.07 |

The PCS values are listed for $\Delta\chi_{rh} = 0$ and $\theta = 0$ (Eq. 1) at the metal-nuclear spin distances indicated

[a] The $\Delta\chi_{ax}$ values are those of Bertini et al. (2001), except that the $\Delta\chi_{ax}$ values of $Er^{3+}$, $Tm^{3+}$ and $Yb^{3+}$ were recalculated such that the principal axes components follow the convention of $|z| > |y| > |x|$. This definition results in the z axes of these three lanthanides being approximately orthogonal with respect to the z axes of the other lanthanides. In a given ligand field, most pseudocontact shifts induced by $Er^{3+}$, $Tm^{3+}$ and $Yb^{3+}$ have the opposite sign from those generated by the other lanthanides

[b] $\Delta\chi_{ax}$ value from Arnesano et al. (2005)

This review focuses on the design of lanthanide and other metal tags, following a brief overview over nitroxide tags.

## Discussion

### General strategies for site-specific tagging

The principal strategies available for site-specific tagging are the same for fluorescent tags, nitroxide tags and metal tags. There are three principal strategies, including production of fusion proteins, site-specific chemical modification and engineering of specific tag sites.

Fusion with green fluorescent protein (GFP) is perhaps the most successful example of any tagging of macromolecules. Also synthetic peptides containing nitroxide radicals can, in principle, be fused to proteins in enzymatic reactions using inteins or sortase (Mao et al. 2004; Muralidharan and Muir 2006). Metal-binding peptide motives are as straightforward to use in fusions as GFP (Donaldson et al. 2001; Ma and Opella 2000; Wöhnert et al. 2003; Martin et al. 2007; Zhuang et al. 2008).

Peptide fusions by in vivo biosynthesis are limited to proteins and invariably restrict the tagging site to the N- and C-termini. A more generally applicable strategy employs site-specific chemical modification. Most reagents target free thiol groups as the chemically most reactive moiety in proteins or (suitably chemically modified) oligonucleotides.

In order to circumvent the requirement of single solvent exposed thiol groups, unnatural amino acids have recently become available that bind metal ions and can be site-specifically incorporated into a polypeptide chain using amber stop codons together with orthogonal tRNA/aminoacyl-tRNA-synthetases (Xie et al. 2007; Lee et al. 2009).

### Nitroxide tags

Proteins have been labelled with nitroxide tags for almost 50 years (Berliner 1976) and analysed by EPR. The first nitroxide labelled protein studied by NMR was hen egg white lysozyme with a spin label attached to His15 (Schmidt and Kuntz 1984). Site-specific labelling of proteins with nitroxide tags has been extensively reviewed for EPR and NMR applications (Kosen 1989; Hubbell and Altenbach 1994; Hubbell et al. 1996; Fanucci and Cafiso 2006; Margittai and Langen 2008; Qin and Dieckmann 2004; Sowa and Qin 2008). Figure 1 presents an overview over some of the commercially available reagents.

The most popular nitroxide tags target free thiol groups and have been chemically activated as methanethiosulfonates (MTS) (Smith et al. 1975) or by the pyridylthio group (Zecherle et al. 1992). These tags react with thiol groups with formation of disulfide bonds according to Scheme 1.

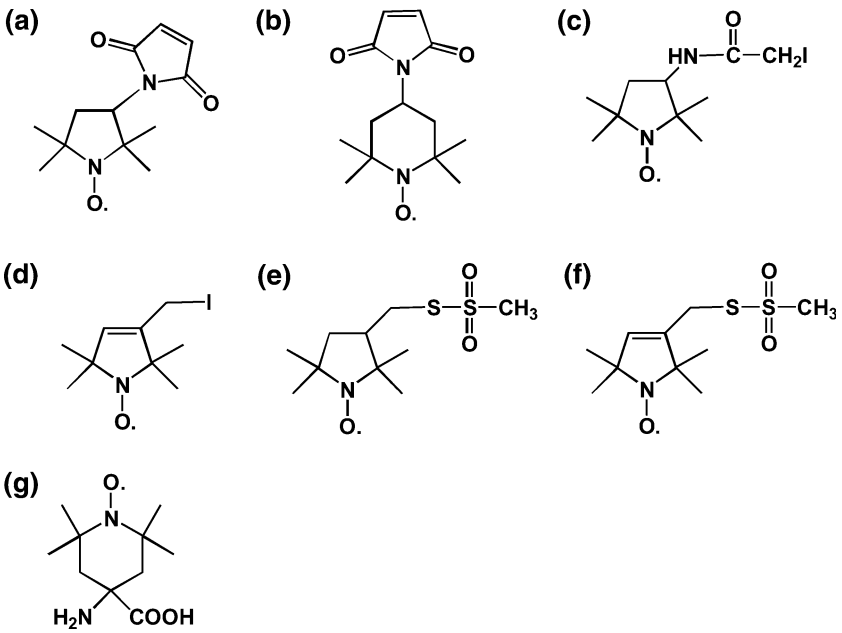
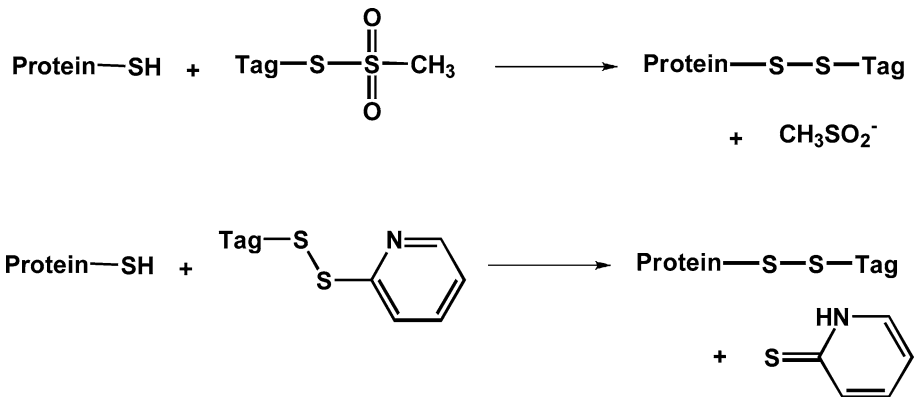



**Fig. 1** Various commercially available nitroxide radical tags. **a** 3-maleimido-2,2,5,5-tetramethyl-1-pyrolinyloxyl or maleimido-proxyl (Griff and McConnell 1966), **b** 4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxyl (4-maleimido-TEMPO; Griff and McConnell 1966), **c** iodoacetamido-proxyl (Ogawa and McConnell 1967), **d** iodomethyl-2,2,5,5-tetramethyl-2,5-dihydropyroli-1-oxyl (Hankovsky et al. 1980), **e** methanethiosulfonate of the proxyl spin label (Mchaourab et al. 1999), **f** 1-oxy(-2,2,5,5-tetramethyl-3-pyrroline-3-methyl) methanethiosulfonate (MTSL; Berliner et al. 1982), **g** 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl-4-carboxylic acid (TOAC) which corresponds to TEMPO functionalised into an α-amino acid (Marchetto et al. 1993; Karim et al. 2007; Lindfors et al. 2008)

**Scheme 1**




The release of methanesulfinate or pyridine-2-thione, respectively, makes these reactions practically irreversible. Nonetheless, disulfide bonds can readily be broken by reducing agents. Therefore, it can be advantageous to use iodide tags that react with thiol groups under formation of stable thioether bonds. Iodides are more difficult to synthesize than methanethiosulfonates and correspondingly fewer iodide tags are commercially available.

Maleimides react with thiol groups by simple addition to the double bond. Both maleimides and iodides react not only with thiol groups but also, more slowly, with amino groups (Berliner 1976; Berliner et al. 1982).

## Tagging with paramagnetic metal ions–natural metal binding sites

Since lanthanide ($Ln^{3+}$) ions have similar ionic radii as $Ca^{2+}$ (Table 2), they can often replace $Ca^{2+}$ in calcium binding proteins. The additional positive charge and larger preferred coordination number, however, can lead to different stoichiometric ratios. For example, the two $Ca^{2+}$ ions in the EF hand motif of parvalbumin can be replaced by a single $Yb^{3+}$ ion (Lee and Sykes 1981). A similar situation has been observed for a large number of calcium binding proteins (Pidcock and Moore 2001), whereas in the case of Calbindin $D_{9k}$ (Allegrozzi et al. 2000) the calcium at one of the two EF-hand $Ca^{2+}$ binding sites can be replaced by a lanthanide without displacing the other calcium ion. As a rule, however, the differences in charge and preferred coordination number compromise simple $Ca^{2+}$–$Ln^{3+}$ replacement (Biekofsky et al. 1999). The replacement can be promoted by introduction of an additional charged residue by site-directed mutagenesis (Bertini et al. 2003).

**Table 2** Coordination numbers and iron radius of $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$ and $Ln^{3+}$

| | Ion radius/Å | Coordination numbers |
|---|---|---|
| $Ca^{2+}$ | 1.00 | 6–7 |
| $Mg^{2+}$ | 0.65 | 6 |
| $Mn^{2+}$ | 0.78 | 6 |
| $Ln^{3+}$ | 0.98–1.20 | 8–9 |

Another group of proteins capable of binding lanthanides are enzymes with two divalent metal ions in the active site. For example, the exonucleases of T4 DNA polymerase and of the $\varepsilon/\theta$ complex of the *E. coli* DNA replisome have two $Mg^{2+}$ or $Mn^{2+}$ ions in their active sites which can be replaced by a single lanthanide ion (Frey et al. 1996; Brautigam et al. 1999; Pintacuda et al. 2004a).

## Fusion tags

Different groups have proposed fusions with metal binding tags. The ATCUN tag is a tripeptide sequence which binds $Cu^{2+}$ with high affinity. It is restricted to the N-terminus of proteins, as the amino terminus participates in the $Cu^{2+}$ coordination (Donaldson et al. 2001).

Lanthanide-specific fusion tags were developed from the metal binding motives found in calcium binding proteins. Fusion of the EF-hand calcium binding motif DNDGDGKIGADE to the N-terminus of a membrane protein has been shown to endow the protein with a lanthanide binding site (Ma and Opella 2000). As this motif strips the lanthanide ion of any hydration water, the complex with $Tb^{3+}$ can also serve as a fluorescent tag, in particular if a sensitising tryptophan side chain is positioned nearby for energy transfer to the lanthanide (MacManus et al. 1990). Directed evolution for improved lanthanide affinity and fluorescent properties led to the lanthanide binding tag LBT1 (Fig. 2) which boasts lanthanide affinities in the low micromolar to nanomolar range (Nitz et al. 2003). N-terminal fusion to ubiquitin was shown to produce RDCs in the presence of $Tb^{3+}$ (Wöhnert et al. 2003). A 32-residue peptide tag containing two LBTs produced even larger RDCs but also enhanced the relaxation of the protein resonances (Martin et al. 2007).

While chemical reactions for paramagnetic tagging are difficult to perform with 100% yield, the tag is always

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Troponin C | | I | F | **D** | K | **N** | A | **D** | G | **F** | I | D | I | E | **E** | L | G | E |
| LBT1 | | Y | I | **D** | T | **N** | N | **D** | G | **W** | Y | **E** | G | D | **E** | L | L | A |
| LBT2 | C | Y | V | **D** | T | **N** | N | **D** | G | **A** | Y | **E** | G | D | **E** | L | | |
| LBT3 | | Y | V | **D** | T | **N** | N | **D** | G | **A** | Y | **E** | G | D | **E** | L | C | |

**Fig. 2** Amino acid sequences of lanthanide binding peptides. The troponin C sequence is from Rao et al. (1996) and the LBT1 sequence from Nitz et al. (2003). Lanthanide-coordinating residues are shown with bold letters. Cysteine residues of tags designed for disulfide bond formation with proteins (Su et al. 2008a) are highlighted in black. The W9A mutation enhances the solubility of the peptide. Leu16 and Ala17 of LBT1 show multiple conformations in solution and are not required for lanthanide binding

present in a fusion. Optimization of the length of the linker peptide between tag and protein, however, is usually necessary in order to minimize averaging of the anisotropic effects due to motions of the tag with respect to the protein.

## Peptide tags attached to cysteine residues

Lanthanide-binding peptide tags can also be site-specifically attached to proteins if tag and protein contain a cysteine residue. As methanethiosulfonate derivatives of peptides are difficult to prepare, disulfide bond linkages are more easily established by first activating the protein thiol group with Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), to form a reactive mixed disulfide. Excess DTNB is removed by dialysis. Subsequently, the tag is added in stoichiometric quantities. The activated mixed disulfide spontaneously reacts with the thiol group of the tag to yield the mixed disulfide with the tag. Progress of the reactions can be monitored by the release of 5-thio-2-nitro-benzoate (TNB) which is intensely yellow (Fig. 3). The yields of tagged protein are usually very high (>70%; Su et al. 2006; note that excess of tag can give rise to disulfide exchange reactions leading to reduced protein and a disulfide bond between two tag molecules.) As the LBTs are very acidic, they significantly change the pI values of the protein. Therefore, purification by ion exchange chromatography readily separates the ligated product from the unligated protein.

Chemical derivatization of a cysteine residue by a peptide tag has a number of advantages over fusion proteins. (i) The tag can be placed at strategically chosen sites of the protein. (ii) Peptide tags are relatively bulky and a disulfide bond often presents a shorter tether than the polypeptide linker in a fusion protein. Therefore, the position and orientation of the metal ion is easily restricted by steric hindrance between tag and protein, limiting averaging of the paramagnetic anisotropic effects induced in the protein. (iii) The peptide tag can be unlabelled while the protein is labelled with stable isotopes so that the NMR resonances of the tag can readily be eliminated by isotope selected experiments. (iv) The orientation and magnitude of $\Delta\chi$ tensors with respect to LBT have been determined (Su et al. 2008a; Fig. 4). A protein with a free cysteine residue can thus be derivatized with tags containing cysteines at different sites (e.g. LBT2 and LBT3 in Fig. 2) to achieve predictably different PCS and RDC effects.

Peptide tags are not universally suitable for all proteins even if they contain only a single cysteine residue. If the cysteine is located at a highly solvent exposed site, the steric hindrance between tag and protein may not be sufficient to immobilize the tag, leading to reduced PCS and RDC effects. Conversely, if the cysteine side chain is insufficiently exposed, the tag may be unable to fold around the lanthanide ion which prevents lanthanide binding.

## Chemical metal tags

Figure 5 shows a representative selection of the molecular structures of published low molecular weight tags that bind

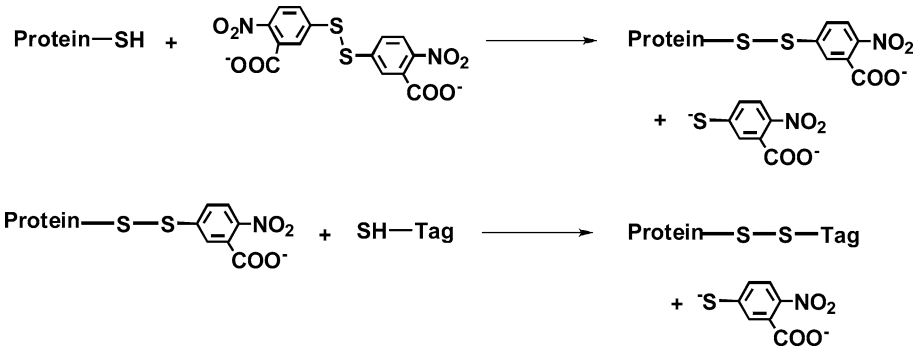



**Fig. 3** Strategy for formation of a disulfide bond between the cysteine residue of a protein and the thiol group of a tag

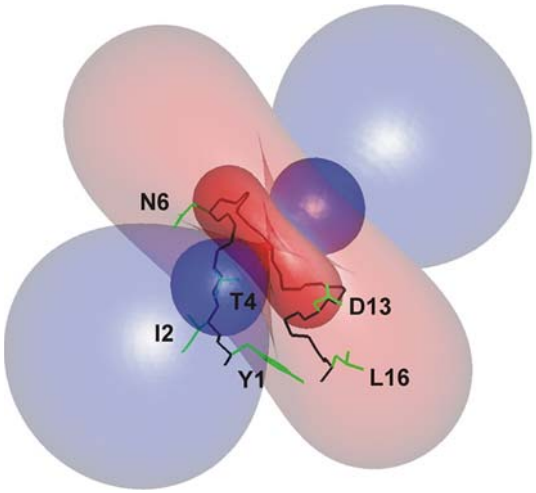



**Fig. 4** PCS isosurfaces of the $Tm^{3+}$-LBT complex (PDB code 1TJB; Nitz et al. 2004). The isosurfaces identify the locations of PCSs of ±20 and ±1 ppm (*blue*: positive PCS; *red*: negative PCS). The peptide backbone is drawn in *black* and solvent exposed side chains of the tag that could potentially be mutated to Cys are shown in *green*. Note that the PCS values of the backbone atoms of the N- and C-terminal residues (Tyr1 and Leu16) have the opposite sign. Consequently, attachment to a protein via the N- or C-termini produces PCS data of opposite sign in the protein

metal ions and can be attached to proteins via one or two disulfide bonds. Almost all of them are activated as methanethiosulfonates, except for the tag T8 (4-mercaptomethyldipicolinic acid, 4-MMDPA) which has a free thiol group. T8 is attached to cysteine residues of proteins using the strategy of Fig. 3.

The ethylenediaminetetraacetic acid (EDTA) derivate T1 is commercially available. Complexes with $Co^{2+}$ or $Yb^{3+}$ generated significant PCS and RDC effects in the N-terminal domain of the protein STAT4 (Gaponenko et al. 2002; Dvoretsky et al. 2002). Complexes with $Tb^{3+}$, $Tm^{3+}$ or $Yb^{3+}$ also produced sizeable RDCs in a membrane protein (Table 3) which is of special interest as detergents are incompatible with most alignment media (Kamen et al. 2007). The main problem associated with T1 is the fact that coordination to a metal ion generates two enantiomers which, following attachment to a protein, give rise to diastereomers. The formation of diastereomers is of little consequence for metal ions with isotropic $\chi$ tensor ($Mn^{2+}$ and $Gd^{3+}$; Pintacuda et al. 2004c; Clore et al. 2007). In the case of metal ions causing PCSs, however, two different PCS values result for each nuclear spin of the protein (Ikegami et al. 2004; Pintacuda et al. 2004c) unless the enantiomers are in fast chemical exchange with each other or one of the enantiomers is much more populated in the presence of the protein. The fast chemical exchange regime can also be established, if the tag is bound to a peptide that binds to the target protein, while undergoing rapid exchange between free and bound peptide (Balogh et al. 2009).

The formation of diastereomers can be prevented by the use of chiral ligands that preferentially select a single metal binding chirality. The EDTA derivates T2 and T3 were synthesized with a chiral carbon centre (Leonov et al. 2005; Haberz et al. 2006). Indeed, the $^{15}$N-HSQC spectra of trigger factor and apo-calmodulin showed only one set of peaks following derivatization with the $Dy^{3+}$ complexes

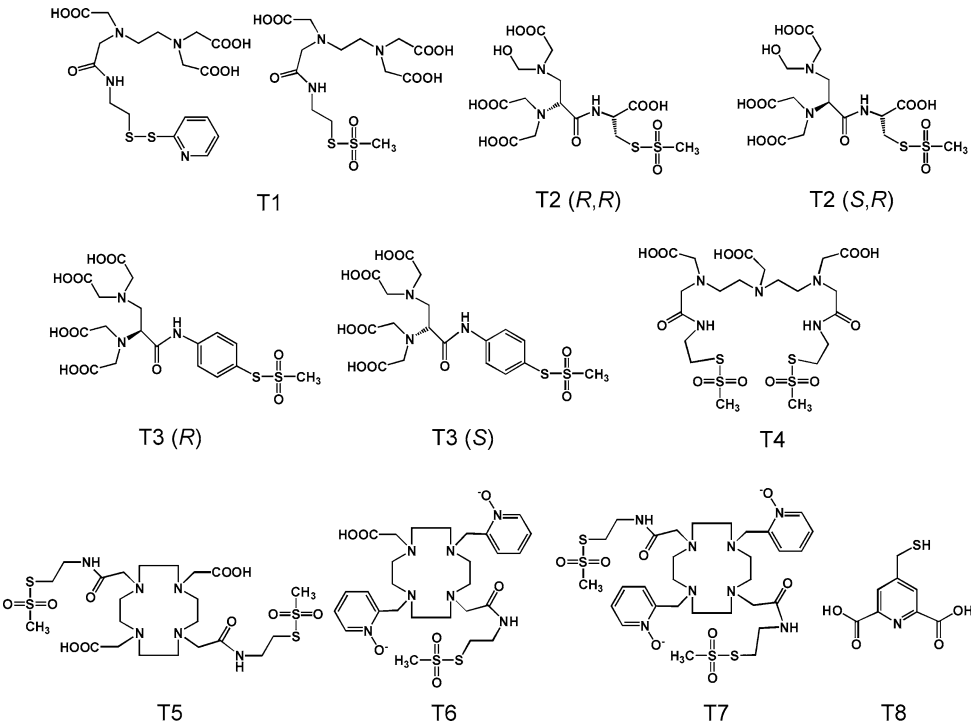



**Fig. 5** Lanthanide binding tags for site-specific labelling of proteins with metal ions. The tags T1 and T8 are commercially available

**Table 3** Maximal residual dipolar couplings of protein backbone amides, $^1D_{NH}$, obtained with different lanthanide binding tags

| Lanthanide binding tag | Protein | Metal ion | Magnetic field/Tesla | Maximal[a] ($^1D_{NH}$/Hz) | Reference |
|---|---|---|---|---|---|
| sLBT[b] | Ubiquitin | Tm | 18.8 | About 5 | Martin et al. (2007) |
| dLBT[c] | Ubiquitin | Tm | 18.8 | About −17 | Martin et al. (2007) |
| LBT[d] | Galectin-3 | Dy | 21.1 | ±15 | Zhuang et al. (2008) |
| LBT2[e] | Arginine repressor | Tm | 18.8 | 21 | Su et al. (2006) |
| T1[f] | $F_1F_o$ ATP synthase | Tm | 18.8 | −10.6 | Kamen et al. (2007) |
| T1[f] | $F_1F_o$ ATP synthase | Tb | 21.1 | −8.1 | Kamen et al. (2007) |
| T2[f] | Trigger factor | Dy | 18.8 | 10.5 | Ikegami et al. (2004) |
| T3[f] | Trigger factor | Dy | Not reported | About 9 | Haberz et al. (2006) |
| T7[f] | Pseudoazurin | Dy | 21.1 | ±17 | Keizers et al. (2008) |
| T8[f] | Arginine repressor | Tm | 18.8 | 12.7 | Su et al. (2008b) |
| CaM(Tb)$_4^g$ | DHFR-M13 | Tb | 14.1 | −7.4 | Feeney et al. (2001) |

[a] The largest numerical value is reported

[b] The peptide GPGYIDTNNDGWYEGDELLA fused to the N-terminus

[c] The peptide GPGYIDTNNDGWIEGDELYIDTNNDGWIEGDELLA fused to the N-terminus

[d] The peptide YIDTNNDGWYEGDELLA fused to the C-terminus

[e] Tag shown in Fig. 2

[f] Tags T1 to T8 are depicted in Fig. 5

[g] Calmodulin loaded with four $Tb^{3+}$ ions

of these tags. In addition, the T2 and T3 tags bind lanthanides very tightly with dissociation constants as low as $10^{-18}$ M (Leonov et al. 2005).

Diethylene triamine pentaacetic acid (DTPA) has an even higher affinity for lanthanide ions than EDTA by matching the preferred ligand coordination number of lanthanide ions (eight to nine). Reaction of the bis-anhydride of DTPA with S-(2-methylaminomethyl)methanethiosulfonate provides a straightforward synthetic route to the tag T4. T4 has two thiol active groups and is designed for ligation to two cysteines that are located 8–10 Å apart in the target protein. In the case of a dicysteine mutant of the protein pseudoazurin, however, the NMR spectrum revealed at least five different diastereomeric species (Prudêncio et al. 2004).

Tags T5 to T7 are based on 1,4,7,10-tetraazacyclododecane-N,N′,N″,N‴-tetraacetic acid (DOTA) derivatives and analogues. The macrocyclic ring binds lanthanides tightly but in different enantiomeric forms (Parker et al. 2002). Indeed, NMR spectra of the double mutant E51C/E54C of pseudoazurin derivatized with tag T5 and $Yb^{3+}$ showed evidence for diastereomers (Vlasie et al. 2007). In contrast, the same protein derivatized with T6 or T7 showed only a single set of peaks for the $Yb^{3+}$ complex (Keizers et al. 2007). The advantage of the two-armed tag T7 over the single-armed tag T6 is its reduced mobility with respect to the protein, preventing averaging of the paramagnetic effects. The positions of the cysteine residues in the protein, however, need to be planned carefully to ensure a suitable distance between the thiol groups and sufficient solvent exposure, while avoiding steric incompatibilities between tag and protein (Keizers et al. 2008).

Tag T8 is based on pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA). It is the smallest lanthanide tag and commercially available. Even though it is only a tridentate ligand, it binds lanthanides with nanomolar affinity (Grenthe 1960). As lanthanide ions have up to nine coordination sites, the tag allows the lanthanide ion to bind to additional ligands. Intramolecular coordination to carboxyl groups of the protein is particularly favoured, resulting effectively in two-armed attachment of the lanthanide ion to the protein. In this way, the lanthanide can be immobilized close to the protein surface. In our experience, distances of about 5–6 Å between protein thiol group and the nearest carboxyl group yield good lanthanide binding sites for the T8 tag (Fig. 6; Su et al. 2008b). By binding lanthanide ions in a non-chiral fashion, the tag circumvents the problem of diastereomer formation with the protein. Additional coordination by a protein carboxyl group not only immobilizes the lanthanide ion but also enhances the lanthanide binding affinity. In at least one example, the alignment tensor produced by the tag showed the theoretical proportionality with the underlying $\Delta\chi$ tensor (Su et al. 2008b). This highlights the rigidity of lanthanide attachment achieved in this way, as RDCs tend to be more sensitive to averaging caused by tether flexibility than PCS data.

## Engineering metal binding sites into proteins

Metal binding sites can be engineered into proteins by site-directed mutagenesis (Lu et al. 2001). This strategy very much depends on the protein. Even if the structure of the

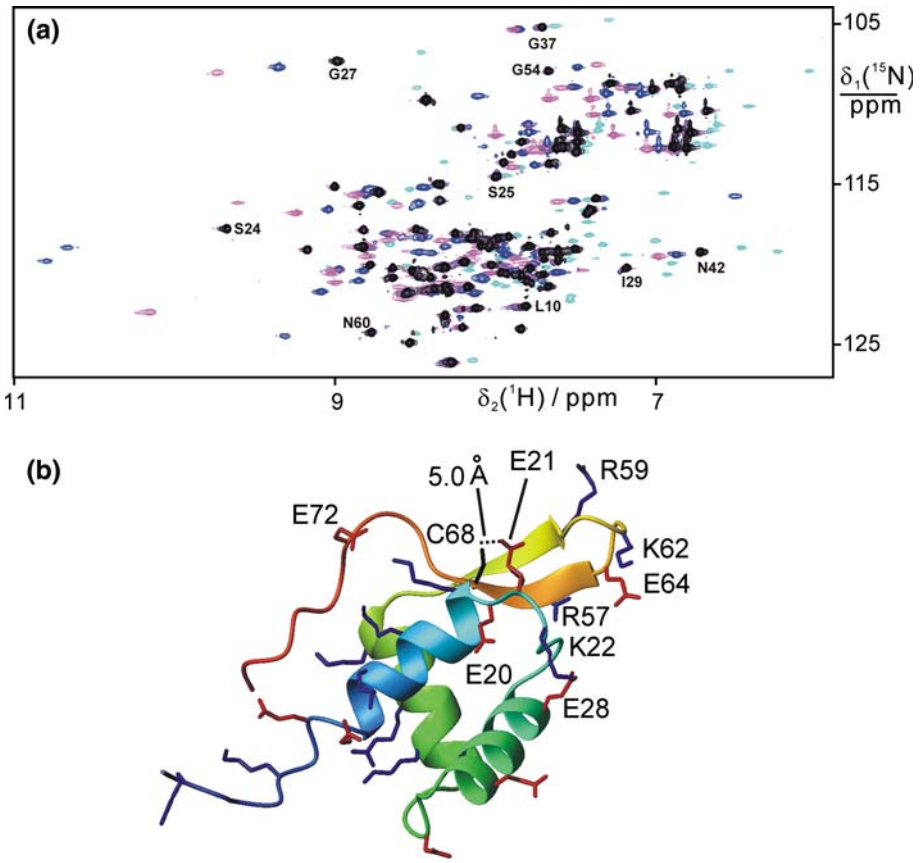



**Fig. 6** Tagging of the N-terminal domain of the *E. coli* arginine repressor derivatized with tag T8 at Cys68. **a** Superimposition of $^{15}$N-HSQC spectra of the uniformly $^{15}$N-labelled protein with tag T8. The samples were prepared with 1:1 mixtures of $Lu^{3+}$ and $Tb^{3+}$ (*cyan*), $Lu^{3+}$ and $Tm^{3+}$ (*magenta*), $Lu^{3+}$ and $Yb^{3+}$ (*blue*), or with $Lu^{3+}$ only (*black*). The simultaneous presence of paramagnetic and diamagnetic lanthanides allows extraction of the paramagnetic shifts from a single spectrum, ensuring identical conditions for the paramagnetic and diamagnetic samples. For example, one of the two cross-peaks of Gly37 in the cyan spectrum arises from the diamagnetic protein but does not overlap with the corresponding peak in the black spectrum, illustrating the sensitivity of chemical shifts to slightly different sample conditions. The spectra were recorded at 25°C and pH 6.5 at a $^{1}$H NMR frequency of 800 MHz. **b** Ribbon representation of the NMR structure of the protein (Sunnerhagen et al. 1997). The side chains of Glu and Asp (*red*), Arg and Lys (*blue*) and Cys68 (*black*) are shown as sticks. The distance between the side chain oxygen of Glu21 and sulphur of Cys68 is indicated with a dashed line. A fit of the lanthanide position using the experimental PCS data positions the metal within hydrogen bonding distance of the Glu21 carboxyl oxygens (Su et al. 2008b)

protein is known, significant challenges remain in designing a lanthanide binding site with the affinity and rigidity of binding required for NMR. In a recent example, the mutations R31D and K43D of the N-terminal domain of the cell adhesion protein CD2 led to an unfolded structure, presumably because the mutations disrupted salt bridges present in the wild-type protein (Fig. 7; Li et al. 2008). Complexation with lanthanide or calcium ions, however, restored the chemical shift dispersion characteristic of the folded protein. As lanthanides prefer high coordination numbers and coordination by carboxylate groups, destabilization of the native protein structure is a likely side effect of the design of lanthanide binding sites with at least three negatively charged amino acid side chains.

Introduction of unnatural metal-binding amino acids can circumvent the requirements of single cysteine residues (as used by most paramagnetic tags) or a cluster of negatively charged residues (as in the design of lanthanide binding sites using natural amino acids). Aminoacyl-tRNA-synthetases specific for over 30 different unnatural amino acids have been evolved in the group of Peter G. Schultz (Xie and Schultz 2005). Two of these amino acids carry bispyridyl and 8-hydroxyquinoline groups, respectively, which bind transition metal ions with high affinity (Xie et al. 2007; Lee et al. 2009). In complex with $Co^{2+}$, these amino acids would be expected to generate useful PCS data, but NMR results have not yet been published.

## Tagging of oligonucleotides

Paramagnetic tags can be attached to DNA and RNA oligonucleotides using the same strategies as for proteins.

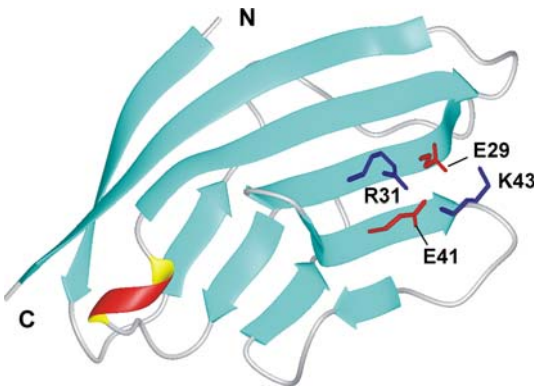



**Fig. 7** Ribbon presentation of the N-terminal domain of the cell adhesion protein CD2 (PDB code 1HNG; Jones et al. 1992). The side chains of Glu29 and Glu41 are coloured in *red* and the side chains of Arg31 and Lys43 are coloured in *blue*. The R31D/K43D double mutant can bind calcium and lanthanide ions with dissociation constants of 90 and 0.3 μM, respectively (Li et al. 2008)

Methods for site-specific labelling are available that achieve selective tagging of the 3′ or 5′ ends, the ribose ring, the phosphate backbone and the nucleotide base. In order to target an individual phosphoester group, it is usually introduced as a thiophosphate. This presents a thiol group that is more reactive towards, e.g., iodomethylproxyl than any other functional group in oligonucleotides. Synthetic strategies are available to introduce thiophosphate groups selectively at the 3′ or 5′ ends (Caron and Dugas 1976; Macosko et al. 1999; Grant and Qin 2007) or the phosphate backbone (Qin et al. 2001). Selective attachment of a nitroxide radical to thiouracil relies on the same principle.

Nitroxide labelling of ribose rings is possible by reacting a 2′-aminoribose nucleotide with a spin label that is reactive towards amino groups (e.g. TEMPO-isocyanate; Edwards et al. 2001; Kim et al. 2004; Edwards and Sigurdsson 2007). Unnatural nucleotides can be chemically synthesised with metal binding sites at many different positions (Ramos and Varani 1998; Iwahara et al. 2003; Qin et al. 2003; Piton et al. 2007). Figure 8 shows the nucleotide base-linker-EDTA moiety of a commercially available thymidine derivative that has been used in NMR studies (Iwahara et al. 2003).

### Tags for PCSs and RDCs

Different considerations apply if a lanthanide tag is to be used for measurements of PCSs or RDCs. If the primary goal is the measurement of RDCs, positioning the metal ion far from the surface of the protein has the advantage of minimizing paramagnetic relaxation enhancements of nuclear spins in the protein. For RDC measurements, limited flexibility of the tether between the metal binding site and the protein can be acceptable, as the resulting

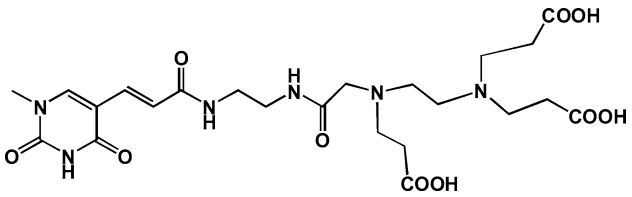



**Fig. 8** Part of a commercially available thymidine derivative with EDTA tag

average alignment tensor can be accurately determined from the RDCs. For PCS measurements, however, only a superimposition of different $\Delta\chi$ tensors can accurately describe the PCSs if the metal position varies. Therefore, if PCS data are the primary aim, rigid attachment of the metal ion is of critical importance. This is most easily achieved if the metal ion is positioned in the core or close vicinity of the protein. In addition, protein spins surrounding a metal ion on all sides offer a more comprehensive sampling of the PCS isosurfaces and, hence, a more accurate fit of the $\Delta\chi$ tensor and metal position than if the metal ion is positioned far from the protein. For many applications, this advantage may outweigh the drawback that protein spins near a paramagnetic centre are subject to pronounced PRE effects.

Table 3 provides an overview of the largest RDCs reported for a selection of proteins modified with different lanthanide binding tags. In comparing the RDCs, one must keep in mind that the magnitudes of the alignment tensors and RDCs resulting from paramagnetic molecular alignment are proportional to the square of the magnetic field strength (Bertini et al. 2002b). The examples show that paramagnetic alignment can produce sizeable RDCs. Furthermore, the variability in RDC magnitudes obtained with very similar lanthanide-binding peptide tags indicates that minimizing the movements of a tag relative to the protein is more important for the observation of large RDCs than the use of increased magnetic field strengths or more highly paramagnetic metal ions.

## Concluding remarks and future directions

Recent efforts have delivered strategies by which lanthanide-binding tags can be site-specifically positioned in proteins and oligonucleotides with a covalent tether. Less invasive strategies could use non-covalently binding tags. Examples include specifically binding ligands with nitroxide (Jahnke et al. 2000) or metal tags (Balogh et al. 2009). In each case, the suitably tagged ligands have to be synthesized chemically. In a related approach, calmodulin loaded with a paramagnetic lanthanide ion has been used to confer paramagnetism to a protein fused to a calmodulin-binding

peptide (Feeney et al. 2001; Table 3). Conceptually, non-covalently binding lanthanide tags are reminiscent of the long-established use of lanthanide reagents as shift reagents for the analysis of small organic compounds. Non-covalent binding allows for chemical exchange to occur between bound and free tag which can considerably assist in resonance assignments (John and Otting 2007; John et al. 2007b; Su et al. 2008a). The challenge is to find lanthanide reagents that offer site-selective complementarity of molecular shape and charge to out-compete non-selective intrinsic affinities to functional groups in proteins and oligonucleotides.

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