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Calmodulin tagging provides a general method of using lanthanide induced magnetic field orientation to observe residual dipolar couplings in proteins in solution

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Abstract

A general method is presented for magnetic field alignment of proteins in solution. By tagging a target protein with calmodulin saturated with paramagnetic lanthanide ions it is possible to measure substantial residual dipolar couplings (RDC) whilst minimising the effects of pseudocontact shifts on the target protein. A construct was made consisting of a calmodulin-binding peptide (M13 from *sk*-MLCK) attached to a target protein, dihydrofolate reductase in this case. The engineered protein binds tightly to calmodulin saturated with terbium, a paramagnetic lanthanide ion. By using only a short linker region between the M13 and the target protein, some of the magnetic field alignment induced in the CaM$(Tb^{3+})_4$ is effectively transmitted to the target protein (DHFR). ^{1}H-^{15}N HSQC IPAP experiments on the tagged complex containing ^{15}N-labelled DHFR-M13 protein and unlabelled CaM$(Tb^{3+})_4$ allow one to measure RDC contributions in the aligned complex. RDC values in the range $+4.0$ to -7.4 Hz were measured at 600 MHz. Comparisons of ^{1}H-^{15}N HSQC spectra of ^{15}N-DHFR-M13 alone and its complexes with CaM$(Ca^{2+})_4$ and CaM$(Tb^{3+})_4$ indicated that (i) the structure of the target protein is not affected by the complex formation and (ii) the spectra of the target protein are not seriously perturbed by pseudocontact shifts. The use of a relatively large tagging group (CaM) allows us to use a lanthanide ion with a very high magnetic susceptibility anisotropy (such as Tb^{3+}) to give large alignments while maintaining relatively long distances from the target protein nuclei (and hence giving only small pseudocontact shift contributions).

Abbreviations: CaM, calmodulin; DHFR, dihydrofolate reductase; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulphonate; IPAP, in phase-anti phase; HSQC, heteronuclear single quantum coherence spectroscopy; Ln^{3+}, trivalent lanthanide ion; M13, the CaM binding peptide (26-residues) from sk-MLCK; MTX, methotrexate; NOE, nuclear Overhauser enhancement; RDC, residual dipolar coupling; sk-MLCK, skeletal muscle myosin light chain kinase.

Introduction

Measurements of residual dipolar coupling constants in NMR spectra of proteins partially aligned in solution have become a well-established source of restraints for obtaining long-range structural information. Most of these alignment studies have used bicelles (Tjandra and Bax, 1997) and filamentous phages (Clore et al., 1998; Hansen et al., 1998) as the agents to provide the molecular orientation. An alternative approach has been to take advantage of the anisotropic paramagnetic susceptibility of paramagnetic ions to induce alignment of proteins in magnetic fields. This approach was initially demonstrated by Tolman et al. (1995) for the case of cyanometmyoglobin (using the alignment induced by the paramagnetic iron in the heme group). Other workers have shown that paramagnetic lanthanides bound to calcium-binding

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proteins can provide a useful method of producing magnetic field alignments (Contreras et al., 1999; Biekofsky et al., 1999; Bertini et al., 2000, 2001). For example, the partial replacement of diamagnetic Ca^{2+} by paramagnetic Tb^{3+} in the Ca^{2+}/calmodulin system resulted in magnetic alignment of the molecule such that significant residual dipolar couplings could be measured (Biekofsky et al., 1999). This approach works well for metal-binding proteins but in order to generalise the method to other proteins it is necessary to introduce a metal-binding group into the protein. Ideally one would like to introduce a metal ion in such a manner as to produce substantial magnetic field orientation without causing major pseudocontact shifts and paramagnetic line broadening. Tüchelmann and coworkers (Tüchelmann et al., 1998) have suggested various approaches for tagging biomolecules with Ln^{3+} ions. Examples of such studies have been described including introduction of a zinc-finger capable of binding to paramagnetic cobalt ions (Gaponenko et al., 2000) and the metal-binding loop of a single 'EF hand' for binding to paramagnetic lanthanide ions (Ma and Opella, 2000). One way of achieving a substantial magnetic field orientation whilst minimising pseudocontact shifts and paramagnetic line broadening would be to use a fairly large metal-binding protein to tag the protein of interest. In this case, the linkage between the two proteins would need to have restricted motion to ensure effective transmission of the alignment of the metal-bound tag to the protein of interest. Here we report that terbium-saturated calmodulin ($CaM(Tb^{3+})_4$) can be used for such a purpose by arranging for it to bind to a CaM-binding target sequence expressed as a fusion peptide linked to the protein of interest (Figure 1).

Materials and methods

Expression of DHFR-M13 protein

A DNA fragment comprising the entire sequence of *L. casei* dihydrofolate reductase was prepared by the polymerase chain reaction (PCR) on plasmid NFI/PMT 702 (Andrews et al., 1985). This was inserted into the pCal-C vector digested with NheI and KpnI to give the sequence for the adduct DHFR-M13. The M13 sequence (a 26-residue CaM-binding peptide from *sk*-MLCK (Ikura et al., 1992) is attached to the DHFR C-terminal residue 162 (see Figure 1). The resulting plasmid was maintained in the *E. coli* strain DH5α. For protein production the plasmid was transformed into the *E. coli* strain BL21/DE3/pLysS and grown in Spizizens medium containing one gram of $(^{15}NH_4)_2SO_4$ per litre together with 100 μg/ml of Ampicillin and 50 μg/ml of chloramphenicol. Cultures were grown at 30 °C, induced at an OD_{600} between 0.6 and 0.8 with 1 mM IPTG and harvested 4 h after induction. Cells from a 1-l culture were freeze-thawed to induce lysis and 10 ml of ice-cold binding buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM β-mercaptoethanol, 1mM magnesium acetate, 1 mM imidazole and 2 mM $CaCl_2$ as specified by Stofko-Hahn and coworkers (Stofko-Hahn et al., 1992)) were added together with protease inhibitors (1 Boehringer mini-protease tablet without EDTA), DNAse (20 μl of a 10 mg/ml solution), 100 μl of 1M $MgC1_2$ and 10 μl of 1 M $MnCl_2$, and then incubated on ice for 5–10 min until the suspension became mobile. After a brief sonication, the suspension was centrifuged and the supernatant stored at −80 °C prior to purification. Two further extractions involving sonication with binding buffer alone were performed to increase the recovery of product. The pH of the combined supernatants was raised to pH 8 and the extract loaded onto a column of calmodulin-agarose (12 × 1.6 cm), and eluted with binding buffer until no further A_{280} absorbing material emerged. Elution of the DHFR-M13 was achieved with elution buffer (50 mM Tris-HCl pH 8.0, 10 mM β-mercaptoethanol, 2 mM EGTA, 150 mM NaCl) and the fractions containing DHFR activity were combined.

An amount of methotrexate equimolar to the DHFR was added and the solution concentrated to a volume of approximately 1 ml in an Amicon stirred cell (YM 10 membrane), after which an equimolar amount of NADPH was added.

The concentrate was loaded onto a column of G75 (53 × 1 cm) eluted with 25 mM KCl and 5 mM bis-Tris pH 6.5 and the fractions containing the DHFR-M13 combined. Various column fractions were analysed on a 15% SDS polyacrylamide gel and visualised by a modification of the silver stain described by Nesterenko and coworkers (Nesterenko et al., 1994) replacing steps 1–5 by fixing for 1 hour in 50% methanol. The product was concentrated to 1.2 ml and lyophilised. A 1-l culture typically produced 15–20 mg of purified product.

Reagents were purchased from Sigma or BDH unless otherwise stated. Acrylamide was from National Diagnostics. Restriction enzymes were from New England Biolabs. The pCal-C vector and calmod-

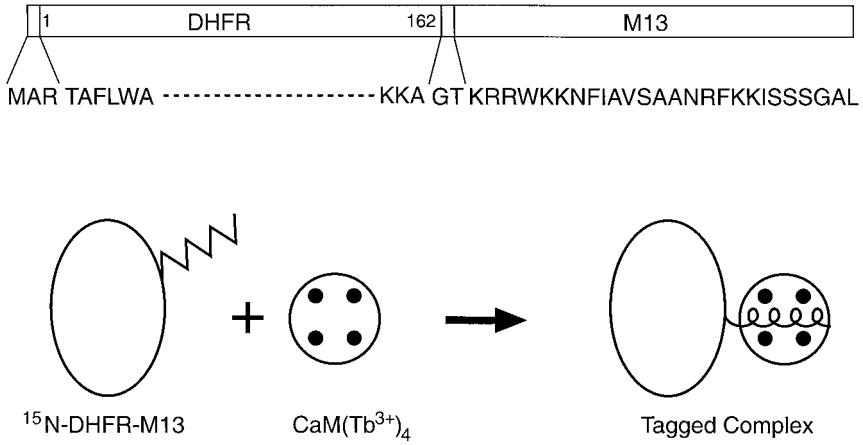

Figure 1. Top: schematic structure of the construct of the DHFR-M13 target protein. The M13 peptide sequence is attached at the C-terminus (residue Ala^{162}) of *L. casei* DHFR via a two residue linker (Gly-Thr). Bottom: schematic representation of the CaM tagging of the target protein. The ^{15}N-DHFR-M13 binds via its M13 peptide extension to the $CaM(Tb^{3+})_4$ thus providing a complex which aligns in the magnetic field. The CaM is not labelled with ^{15}N.

ulin affinity resin were obtained from Stratagene. G75 was from Pharmacia. The compositions of the binding and elution buffers for the calmodulin affinity column were those specified by Stratagene, and Stofko-Hahn and coworkers (Stofko-Hahn et al., 1992).

Calmodulin

The cDNA coding for *Drosophila melanogaster* calmodulin, supplied by K. Beckingham in the pOT-SNco12 vector (Maune et al., 1992), was transformed into the *E. coli* cell line AR58 (Ikura et al., 1990). After the bacterial cell culture was grown, the calmodulin was isolated and purified to homogeneity as described elsewhere (Browne et al., 1997). The apo protein was prepared by treating with 10 mM EDTA as described previously (Biekofsky et al., 1999). An apo-CaM stock solution (1.2 mM) was prepared containing 50 mM KCl and 15 mM bis-Tris pH 6.5.

Titrations with metal ions

Stock solutions of 50 mM $CaCl_2$ and $TbCl_3$ in 90% H_2O/10% D_2O were prepared from standard 1 M solutions. The $CaM(Ca^{2+})_4$ was prepared by adding 4 equivalents of $CaCl_2$ (a $[Ca^{2+}]$/[CaM] ratio of 4) and the $CaM(Tb^{3+})_4$ was prepared by titrating with 4 equivalents in steps of 0.5 equivalents and the metal-binding monitored using ^{1}H NMR spectroscopy. Some samples were prepared containing 10% of added ^{15}N-Ile CaM and these allowed the Tb^{3+} titrations to be monitored more precisely using ^{1}H-^{15}N HSQC spectra (Fefeu et al., 2000). This made it easier to prepare $CaM(Tb^{3+})_4$ species without any excess free Tb^{3+} ions, whose non-specific binding to the target protein can cause line broadening.

Preparation of the CaM-tagged protein complex

Several protocols for making the complexes were explored. The most successful approach involved adding the lyophilised target protein to the terbium-saturated CaM. The complexes were thus prepared by dissolving lyophilised samples of ^{15}N-DHFR-M13(MTX-NADPH) in solutions of either $CaM(Ca^{2+})_4$ or $CaM(Tb^{3+})_4$. The final concentrations of the complexes were 1.0 mM and 0.83 mM respectively in 100 mM KCl and 15 mM bis-Tris pH 6.5 in 90% H_2O/10% D_2O. The samples had a 10–20% excess of the CaM species (complexes formed in this tagging process would be expected to be in slow exchange with any free species present).

NMR spectroscopy

All NMR experiments were performed on Varian Unity or Unity Plus spectrometers operating at ^{1}H Larmor frequencies of 600 or 500 MHz, respectively. All data were collected in phase sensitive mode using the method of States and co-workers (States et al., 1982) and using WATERGATE (Sklenar et al., 1993) for water suppression. Spectra from the Tb^{3+} and Ca^{2+} titrations of the protein were acquired at 25 °C. Spectra used for the measurement of residual dipolar coupling values were acquired at 35 °C.

^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectra (Bodenhausen and Ruben, 1980; Mori et al., 1995) were acquired typically with 1275 complex points in t_2 (0.150 s acquisition time) with a spectral width of 8500 Hz and the ^{1}H carrier set to the water frequency at 4.77 ppm (at 25 °C); 128 complex points were collected in t_1 (0.053 s acquisition time) with a spectral width of 2431 Hz and the ^{15}N carrier at 118 ppm. Residual dipolar couplings were extracted from 2D IPAP ^{1}H-^{15}N HSQC spectra (Ottiger et al., 1998) Spectra were acquired with 1275 complex points in t_2 (0.150 s acquisition time with a spectral width of 8500 Hz, and 292 complex points were collected in t_1 (0.120 s acquisition time) with a spectral width of 2431 Hz.

The signal assignments in the ^{15}N-labelled DHFR-M13 (MTX-NADPH) complexed with non-labelled CaM were made by using ^{15}N-^{1}H 3D NOESY-HSQC (Marion et al., 1989) experiments in conjunction with the previously assigned ^{1}H and ^{15}N resonances of DHFR in the ternary complex DHFR-MTX-NADPH (B. Birdsall, P.M. Nieto, V.I. Polshakov and J. Feeney, unpublished results). These experiments were carried out on the complexes containing $CaM(Ca^{2+})_4$ and $CaM(Tb^{3+})_4$. The NOESY mixing times were 0.075–0.150 s and the acquisition times were 0.150, 0.018 and 0.013 s in the indirect ^{1}H, indirect ^{15}N and real time ^{1}H dimensions.

All spectra were processed using VNMR (Varian Associates) and Felix software (Felix, version 2.3, 1993, Biosym Technologies, Inc., San Diego). Spectra were analysed using the program XEASY (Bartels et al., 1995). The ^{1}H-^{15}N splittings were measured in the F1 dimension of the IPAP spectra.^{1}H chemical shifts were referenced to sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) and ^{15}N chemical shifts referenced to liquid NH_3 (Wishart et al., 1995). 10% D_2O was added to all the samples to provide a lock signal.

Results and discussion

The CaM tagging method

The method involves first cloning the target protein with a CaM-binding peptide at either the N- or C-terminus of the protein and then expressing it as an ^{15}N-labelled protein. In the example considered here (Figure 1) the target protein, *L. casei* dihydrofolate reductase is attached at its C-terminus to the M13 peptide. This peptide and related sequences are known to bind very tightly to $CaM(Ca^{2+})_4$ ($Ka > 10^9\ M^{-1}$ (Ikura et al., 1992; Bayley et al., 1996; Martin et al., 1999) and also to lanthanide-bound calmodulin, ($CaM(Ln^{3+})_4$) (Biekofsky et al., 1999). Thus the modified target protein with its attached M13 peptide is expected to bind efficiently to a paramagnetic lanthanide-saturated CaM (see Figure 1). Tagging the target protein with the paramagnetic CaM species in this way offers the potential for inducing magnetic field alignment in order to observe residual dipolar couplings. The modified target protein is ^{15}N-labelled whereas the added CaM is unlabelled thus simplifying the spectral analysis of the 2D ^{1}H-^{15}N HSQC spectra used to extract the residual dipolar couplings from the complexes. Miyawaki and coworkers have successfully prepared constructs of proteins fused to CaM-binding peptides for other purposes (Miyawaki et al., 1997).

Choice of lanthanide

Because the ionic sizes and binding characteristics of trivalent lanthanide ions are similar to those of the Ca^{2+} ion (Snyder et al., 1990), lanthanide ions have frequently been used as paramagnetic probes by replacing the Ca^{2+} ion in NMR studies (Lee and Sykes, 1983; Bentrop et al., 1997; Biekofsky et al., 1999). Lanthanide ions are known to bind very tightly to CaM both in the absence and presence of target peptide sequences (Bentrop et al., 1997, Biekofsky et al., 1999). An ideal lanthanide ion for the CaM tagging method should bind selectively and tightly to CaM and should give rise to substantial RDC values from alignment of the molecule in the magnetic field. It should not produce excessive pseudocontact shifts and paramagnetic line broadening for the target protein signals. The degree of alignment of a paramagnetic species in an external magnetic field depends on the degree of anisotropy of the magnetic susceptibility tensors (Bothner-By, 1996) and the Tb^{3+} ion is known to have one of the highest magnetic susceptibility anisotropies within the lanthanide series (Horrocks and Sipe, 1972). A previous study has shown that Tb^{3+} binds tightly to CaM and results in substantial magnetic field alignment leading to large residual dipolar coupling contributions (Biekofsky et al., 1999). While Tb^{3+} can give rise to substantial pseudocontact shifts and line broadenings these are minimised at large distances from the metal ion. The linker region and the size of the CaM should result in relatively large distances between the target

protein nuclei and the metal ions. The present work exploits the use of Tb^{3+} bound to the CaM tag in order to achieve a substantial magnetic field orientation of the target protein.

In initial experiments to prepare metal-saturated calmodulin, $CaM(Tb^{3+})_4$, four equivalents of Tb^{3+} ions were titrated into the sample using NMR to follow the titration. However, if the concentrations of the components of the system are accurately known there is no need to carry out the titration and all four equivalents can be added as one aliquot. It is important to avoid excess Tb^{3+} ions because they can bind weakly to the target protein and cause complications because of paramagnetic line broadening. When a small excess of Tb^{3+} was present, we observed metal binding involving adjacent pairs of acidic surface residues in DHFR. The NH signals of these residues (Glu^{66} and Asp^{67}; Asp^{108} and Asp^{109}; Asp^{134} and Asp^{135}) and a few of their neighbours became too broad to observe. Such line broadening could be removed by back titration with apo-CaM (or EDTA).

Some experiments were carried out using CaM species with a combination of Ca^{2+} and Tb^{3+} present in different ratios to give the various combinations of Ca^{2+}/Tb^{3+} occupation of the four sites (Biekofsky et al., 1999). Complexes containing different numbers of Tb^{3+} ions would each result in a different induced alignment in the field and this could be useful for removing ambiguities in the orientational data. However, because the pseudocontact shifts on the DHFR signals are small, the resulting overlapping signals for the four possible species cannot be resolved thus making it impossible to measure the individual residual dipolar coupling constants for each of the species. It is therefore essential to use a single $CaM(Tb^{3+})_4$ (or other paramagnetic lanthanide CaM complex) to achieve the necessary spectral simplicity in this tagging method.

The linker region

For the construct reported here, the linker region between the target protein and the M13 peptide comprises only two residues (Gly-Thr). However, because the last two residues of *L. casei* DHFR (Lys^{161}-Ala^{162}) are known to be flexible, the effective flexible linkage is a four-residue sequence. This linkage is sufficiently flexible to allow the M13 peptide to bind effectively to the CaM while retaining enough restricted motion to ensure that the DHFR target protein experiences some of the magnetic field alignment of the bound paramagnetic CaM component. This linkage is also sufficiently long to prevent large paramagnetic pseudocontact shifts and line broadenings on signals from the target protein. Any flexibility in the linkage will also further reduce such effects. A longer linkage (LVPRGSGSGDDDDK) connecting the M13 to the N-terminus of DHFR was also investigated. However, this construct did not provide a significant alignment of the DHFR molecule.

NMR spectra of the tagged complexes

Figure 2a shows part of the 2D ^1H-^{15}N HSQC IPAP spectrum of the complex formed by combining DHFR-M13 (MTX-NADPH) with $CaM(Tb^{3+})_4$. The measured ^1H-^{15}N splittings given on the spectrum are in the range -89.3 to -101.6 Hz with many of them showing substantial differences from the corresponding scalar couplings (~ -94 Hz). The values of the ^1H-^{15}N scalar splittings were obtained in a separate experiment on the complex formed by combining DHFR-M13 (MTX-NADPH) with $CaM(Ca^{2+})_4$ (spectrum shown in Figure 2b). The residual dipolar coupling contributions range from $+4.0$ to -7.4 Hz. Thus it is clear that this tagging method leads to substantial magnetic field alignment and provides residual dipolar coupling constants that can be use used for structure refinement.

It is also necessary to demonstrate that this tagging method does not perturb the structure of the target protein. This has been confirmed by showing that no major chemical shift perturbations are observed in the spectrum of the target protein when the protein is tagged with the diamagnetic $CaM(Ca^{2+})_4$. This is illustrated by the comparisons of the 2D ^1H-^{15}N HSQC spectra (same spectral regions as in Figure 2) for ^{15}N-DHFR-MTX-NADPH (Figure 3a) and for ^{15}N-DHFR-M13 (MTX-NADPH) in its complex with $CaM(Ca^{2+})_4$ (Figure 3b). The comparison of the spectra of these diamagnetic samples (DHFR with and without $CaM(Ca^{2+})_4$) showed only small shift perturbations on DHFR signals (<0.1 ppm in ^1H shifts) thus indicating that there have been no substantial changes in the overall DHFR structure. Additional signals for the residues in the M13 peptide sequence were detected in the sample containing the DHFR-M13 construct: two such signals are enclosed in boxes in Figure 3b. A comparison of the spectra of the DHFR-M13 (MTX-NADPH) and its $CaM(Ca^{2+})_4$ tagged complex (not shown) again revealed no substantial chemical shift differences. However, the linewidths of

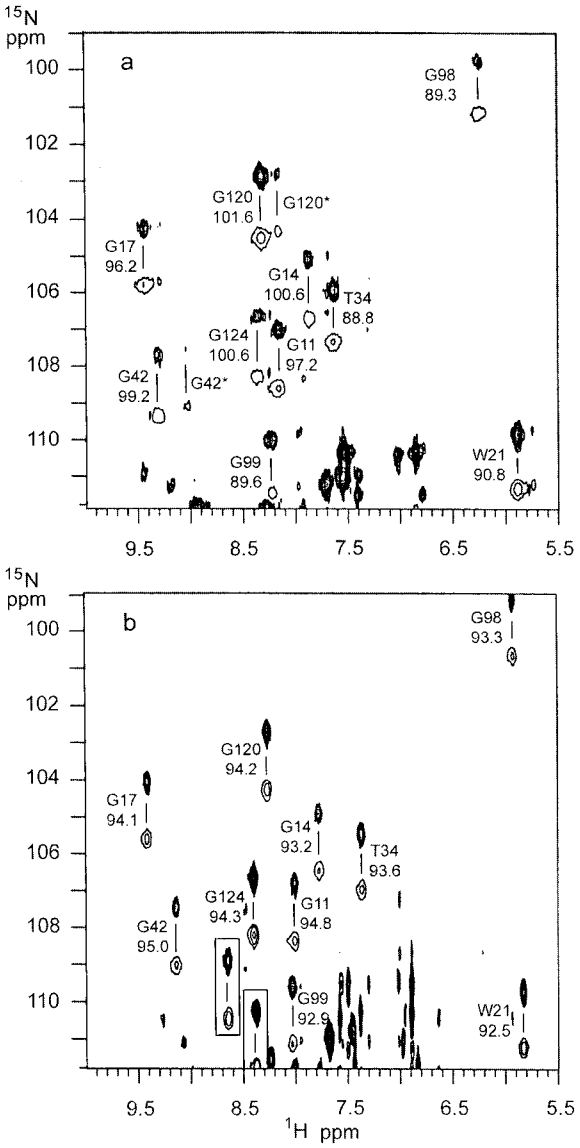

Figure 2. (a) Part of the 600 MHz 2D ^{1}H-^{15}N HSQC IPAP spectra of the complex formed by combining ^{15}N-DHFR-M13 (MTX-NADPH) with CaM(Tb^{3+})$_4$ (35 °C). The assignments and the ^{1}H-^{15}N splittings (negative signs not shown) are indicated in the figure. These splittings are composed of scalar couplings ($^{1}J_{HN} \approx -94$ Hz) and ^{1}H-^{15}N residual dipolar couplings and the values range from -89.3 to -101.6 Hz and are given with a minimum uncertainty of ± 0.5 Hz. A minor species present in the sample gives small signals at the chemical shifts of unbound ^{15}N-DHFR-M13 (MTX-NADPH): the ^{1}H-^{15}N splittings on these signals correspond to the scalar coupling constants (negative signs not shown) with no contribution from residual dipolar coupling. The minor form signals (indicated by an asterisk) correspond to DHFR that is not attached to CaM(Tb^{3+})$_4$ and the signals all show $^{1}J_{HN}$ values ~ -94 Hz. (b) The same spectral region of the 600 MHz 2D ^{1}H-^{15}N HSQC IPAP spectra of the complex formed by combining ^{15}N-DHFR-M13 (MTX-NADPH) with CaM(Ca^{2+})$_4$ (25 °C). The scalar ^{1}H-^{15}N couplings are indicated on the signals (negative signs not shown). Signals from the tethered M13 peptide are enclosed by boxes.

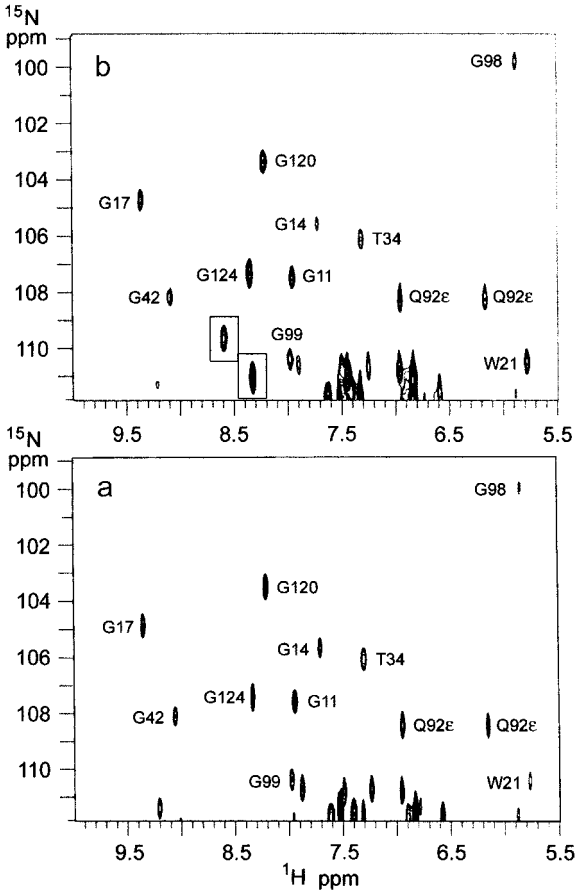

Figure 3. The same spectral region of the 2D ^{1}H-^{15}N HSQC spectra as in Figure 2 for (a) ^{15}N-DHFR (MTX-NADPH) (500 MHz, 25 °C). (b) The complex formed by combining ^{15}N-DHFR-M13 (MTX-NADPH) with CaM(Ca^{2+})$_4$ (600 MHz, 25 °C). Signals from the tethered M13 peptide are enclosed by boxes.

the CaM(Ca^{2+})$_4$ tagged complex increase because of the increase in effective size of the complex.

The spectra of the CaM(Ca^{2+})$_4$ and CaM(Tb^{3+})$_4$-tagged complexes with the ^{15}N-DHFR-M13 (MTX-NADPH) (Figures 2a and 2b) were compared to assess the overall paramagnetic perturbations on the spectrum. Only small pseudocontact shifts (< 0.4 ppm in ^{1}H) are measured for the DHFR signals. The addition of CaM(Tb^{3+})$_4$ causes some paramagnetic line broadening but all the DHFR signals could still be detected and their positions measured accurately. Because the pseudocontact shift contributions are small many of the signal assignments could be transferred between the two complexes without ambiguity. However, a 3D ^{1}H-^{15}N NOESY-HSQC experiment was used to verify the assignments in crowded spectral regions.

The M13 residue signals observed in the spectra of CaM(Ca^{2+})$_4$-tagged complex in Figures 2b and 3b (boxed signals) are not seen in the spectrum of the CaM(Tb^{3+})$_4$-tagged complex (Figure 2a) because of the close proximity of the M13 to the Tb^{3+}. Some samples contained a small amount of untagged target protein and this species could be detected in the experiments on DHFR-M13 (MTX-NADPH) with CaM(Tb^{3+})$_4$. Thus, in Figure 2a additional small signals were observed that showed no pseudocontact shifts and only the scalar ^{1}H-^{15}N couplings (the signals labelled G42* and G120* in Figure 2a are typical examples).

Concluding remarks

This tagging approach provides a general method for orienting any protein in a magnetic field. The use of a relatively large tagging group containing paramagnetic lanthanide ions can effectively transmit some of its magnetic field alignment to the protein of interest providing that a fairly short linker is used. Because of the relatively large size of the CaM tagging group, the effects of its bound paramagnetic ions on the shifts and linewidths of the target protein are minimised. In this method it is easy to avoid excess lanthanide in the final solution because of the tightness of binding of the paramagnetic ion to CaM. An additional bonus of the CaM tagging method is that it also provides a very convenient general method of purifying the target protein using standard CaM affinity column procedures.

We anticipate that the method could work equally well with a CaM construct containing only one of its binding domains since the individual domains also bind very tightly to M13 related peptides (Bayley et al., 1996). For example, a construct of the target protein attached to the first 10 residues of the M13 peptide would bind tightly to the C-terminal domain of calmodulin (Mr $\sim$ 8 kDa) with its two metal-binding sites saturated with Tb^{3+}. This would enable this tagging method to be applied to proteins of even higher molecular weight. It is also likely that lanthanide ions with weaker paramagnetic properties than Tb^{3+} could be used successfully with this method, especially for studies at magnetic fields higher than 14.1 T (600 MHz).

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