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Site-Specific Labelling of Proteins with a Rigid Lanthanide-Binding Tag

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This paper describes a generic method for the site-specific attachment of lanthanide complexes to proteins through a disulfide bond. The method is demonstrated by the attachment of a lanthanide-binding peptide tag to the single cysteine residue present in the N-terminal DNA-binding domain of the Escherichia coli *arginine repressor. Complexes with Y^{3+}, Tb^{3+}, Dy^{3+}, Ho^{3+}, Er^{3+}, Tm^{3+} and Yb^{3+} ions were formed and analysed by NMR spectroscopy. Large pseudocontact shifts and residual dipolar couplings were induced by the lanthanide-binding tag in the protein NMR spectrum, a result indicating that the tag was rigidly attached to the protein. The axial components of the magnetic susceptibility anisotropy tensors determined for the different lanthanide ions were similarly but not identically oriented. A single tag with a single protein attachment site can provide different pseudocontact shifts from different magnetic susceptibility tensors and thus provide valuable nondegenerate long-range structure information in the determination of 3D protein structures by NMR spectroscopy.*

Introduction

Lanthanide ions are attractive tools in the NMR analysis of biomolecular macromolecules because many of these ions are highly paramagnetic, thereby generating pronounced effects in the ^{1}H NMR spectrum. Those ions associated with a magnetic susceptibility anisotropy ($\Delta\chi$) tensor generate pseudocontact shifts (PCS) and partially align the molecule with respect to the magnetic field, which results in residual dipolar couplings (RDC).[1] Some of the lanthanide (Ln^{3+}) ions generate measurable PCS over distances of 40 Å[2] and this property has been used for the 3D structure determination of a protein–protein complex containing a natural Ln^{3+}-binding site.[3] An additional benefit of lanthanides arises from their chemical similarity, which allows the filling of a binding site with Ln^{3+} ions of very different paramagnetic properties. However, proteins with natural lanthanide-binding sites are rare. In order to broaden the application of paramagnetic Ln^{3+} ions in structural biology, ways must be found for their site-specific attachment to diamagnetic proteins.

The site-specific attachment of Ln^{3+} ions to proteins has been achieved in two principally different ways: 1) N- or C-terminal fusion of the protein to a Ln^{3+}-binding peptide[4,5] and 2) chemical derivatisation of a cysteine side chain with a lanthanide complex.[6–8] Fusion with a Ln^{3+}-binding peptide limits the number of possible attachment sites to two. Furthermore, fusion to highly flexible N and C termini decreases the magnitude of the measurable PCS and RDC values due to conformation averaging.[4,5] Similarly, PCS and RDC values are reduced when the Ln^{3+} ion is attached by means of a small organic Ln^{3+}-binding compound to a cysteine SH group through a flexible linker. A further complication arises when the metal coordination generates two enantiomeric forms of the complex, since these combine with a protein to form diastereomers, which results in peak doubling in the NMR spectrum.[6,7,9] Here we show that all of these problems can be addressed by attaching an enantiomerically pure Ln^{3+}-binding peptide (LBP) to a cysteine side chain of the protein. The ultimate goal of the project is the use of PCS in the determination of the three-dimensional structures of proteins and for the structure determination of protein–protein and protein–ligand complexes.

Results and Discussion

NMR analysis of lanthanide-binding peptides

The present study primarily used a 16-residue Ln^{3+}-binding peptide with the sequence CYVDTNNDGAYEGDEL, henceforth referred to as LBP2. Its sequence was based on the Ln^{3+}-binding sequence YIDTNNDGWYEGDELLA, henceforth referred to as LBP1, which has been reported to bind Tb^{3+} ions with 50 nM binding affinity[10] and for which a crystal structure has been determined.[11] The N-terminal cysteine residue was added to provide a site for attachment of LBP2 to a cysteine residue of the protein through a disulfide bond. The two C-terminal residues of LBP1 were omitted from LBP2, since NMR spectra recorded for the mutant peptide LBP1(T4C) in complex with Lu^{3+} ions showed that the C-terminal residues assumed two different slowly exchanging conformations, a fact suggesting

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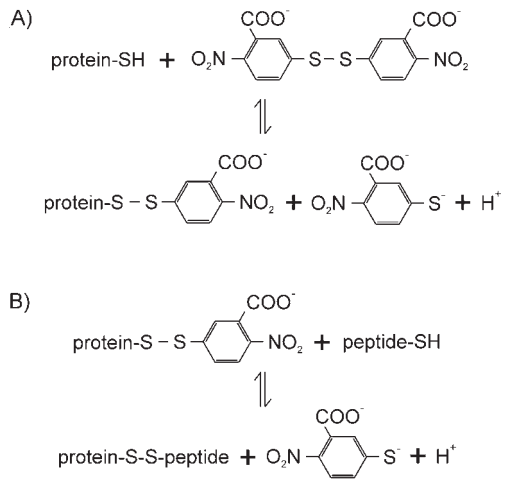
Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author: assignments of the ^{15}N-HSQC spectra of ArgN–LBP2 in complexes with Y^{3+} and paramagnetic Ln^{3+} ions.

that they are not important for Ln^{3+} binding. Similarly, the tryptophan residue in LBP1 was replaced by alanine in LBP2, since two indole NH resonances observed in the 1D ^{1}H NMR spectra suggested the presence of alternative side-chain conformations of this residue. This substitution also markedly improved the solubility of the peptide in aqueous buffers.

LBP1(T4C) and LBP2 assumed random-coil conformations in solution at neutral pH values and displayed narrow signals with little chemical shift dispersion in their ^{1}H NMR spectra (data not shown). Titration of LBP1(T4C) and LBP2 with diamagnetic Lu^{3+} and Y^{3+} ions, respectively, produced a second set of signals with the well-dispersed chemical shifts that are characteristic of a folded peptide. The complexes were in slow exchange with the metal-free LBPs, as expected for a stable metal complex. NOESY spectra recorded for the Lu^{3+}–LBP1-(T4C) complex at 5 °C were in agreement with the crystal structure of Tb^{3+}–LBP1.[11]

Covalent attachment of LBP2 to the N-terminal domain of the *Escherichia coli* arginine repressor (ArgN)

LBP2 was attached to the uniformly ^{15}N-labelled ArgN by a disulfide bond to Cys68, the single cysteine residue in ArgN, according to Scheme 1. First, the thiol group of Cys68 was activated by treatment with Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in tris(hydroxymethyl)aminomethane (Tris) buffer at pH 7.8 (Scheme 1 A). Second, LBP2 was added to the ArgN–TNB complex to exchange the disulfide bond (Scheme 1 B). Both reaction steps produced yellow colour from the TNB anion released. Finally, the ligated protein was purified by ion-exchange chromatography. This approach produced the derivatised protein in more than 90% yield. This is a generally applicable method for the attachment of thiol-containing compounds to cysteine side chains.



Scheme 1. Scheme for disulfide-bond formation between a cysteine thiol group of a protein and a thiol-group-containing low-molecular-weight compound. A) Activation of the thiol group of the protein with DTNB. B) Formation of a disulfide bond between the protein and peptide thiol groups.

NMR characterisation of the diamagnetic ArgN–LBP2 complex

The ^{15}N-HSQC spectra of ArgN and ArgN–LBP2 were very similar, with chemical shift changes limited to the residues in close proximity to Cys68. The structural integrity of ArgN in the ArgN–LBP2 construct was confirmed by a 3D NOESY–^{15}N-HSQC spectrum recorded with a 0.4 mM solution; this spectrum showed the long-range NOEs expected for ArgN.[12] This result indicated that the attachment of LBP to the protein had not significantly altered the protein structure. The ^{15}N-HSQC spectrum of ArgN–LBP2 was compared with that of ArgN–LBP2 in complex with Y^{3+} ions. Y^{3+} ions have a similar ionic radius to Dy^{3+} ions and can thus be considered as diamagnetic homologues of Dy^{3+} ions. No significant chemical shift changes were detected in the ^{15}N-HSQC spectra, which indicates that the structural reorganisation of LBP around the metal ion did not affect the structure of the protein backbone and that the protein did not contain other metal-binding sites that could compete with that of LBP2.

The presence of free Ln^{3+} ions in solutions of ArgN–LBP2 was found to catalyse the hydrolysis of peptide bonds to a significant extent even after two hours at 10 °C. This degradation process could effectively be inhibited by the addition of 4 mM glycine. Glycine has been reported to bind Ln^{3+} ions with a dissociation constant of about 300 μM.[13] We subsequently carried out all NMR measurements of ArgN–LBP2 with lanthanides in solutions with 4 mM glycine. Even in the presence of a threefold excess of Ln^{3+} ions (fivefold for the complex with Y^{3+} ions), there was no evidence of protein degradation in these samples after two weeks at 10 °C.

Lanthanide-binding affinity of the ArgN–LBP2 complex

Titration of a 50 μM solution (pH 6.5, 10 °C) of ^{15}N-ArgN–LBP2 with $ErCl_3$ to a ratio of 1:1 yielded two sets of signals in the ^{15}N-HSQC spectrum, which corresponded to the ArgN–LBP2–Er^{3+} complex and the apoprotein. The relative cross-peak intensities were about 5:1, thereby indicating a dissociation constant of about 1 μM. Addition of an excess of Er^{3+} ions further decreased the size of the cross-peaks from the apoprotein. The decreased binding affinity of Er^{3+} ions for ArgN–LBP2 compared to the 50 nM binding affinity of Tb^{3+} ions for LBP1[10] may be explained by the lower pH value (6.5 versus 7.5), the slightly different amino acid sequences of the peptides, the competition with the glycine present in solution and the steric restraints imposed by interactions between ArgN and LBP2. Notably, derivatisation of ArgN with LBP1(T4C) did not result in a construct with significant Ln^{3+}-binding affinity (data not shown).

^{15}N-HSQC spectra with paramagnetic lanthanides

We subsequently prepared complexes of ^{15}N-ArgN–LBP2 with Tb^{3+}, Dy^{3+}, Ho^{3+}, Er^{3+}, Tm^{3+} and Yb^{3+} ions, respectively. All Ln^{3+} ions were used in two- to threefold excess. A diamagnetic reference was prepared with fivefold excess of Y^{3+} ions. PCS

values were determined as the difference between the ^{1}H chemical shifts observed in the ^{15}N-HSQC spectra of the paramagnetic and diamagnetic complexes, respectively. Figure 1

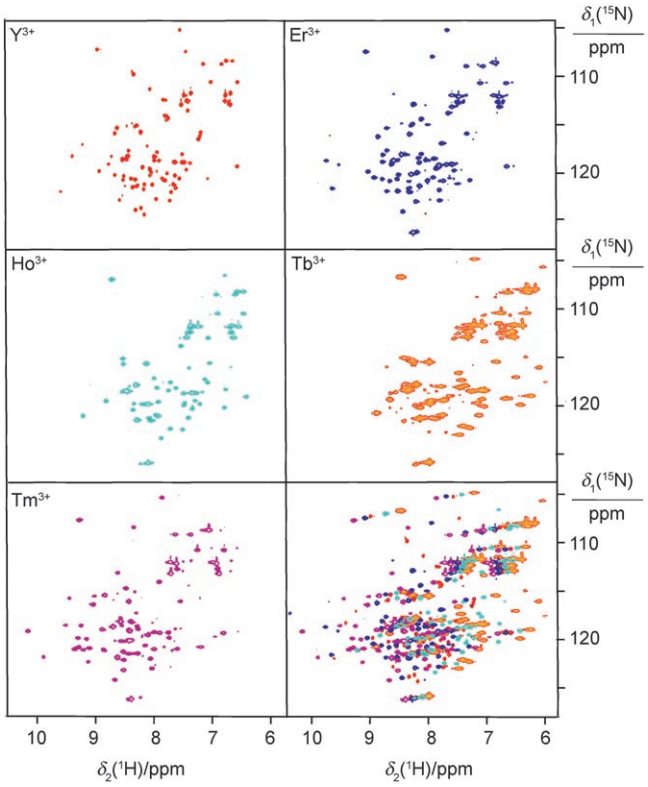


Figure 1. ^{15}N-HSQC spectra of complexes between the uniformly ^{15}N-labelled N-terminal domain of the *E. coli* arginine repressor derivatised with LBP2 at Cys68 and the metal ions indicated on the plots. The spectra were recorded at 283 K in 20 mM β-morpholinoethanesulfonic acid (MES) buffer (pH 6.5) with 4 mM glycine. The spectra were colour coded for improved visual display of the superposition of all spectra in the lower right-hand panel.

shows ^{15}N-HSQC spectra of the complexes with Y^{3+}, Tb^{3+}, Ho^{3+}, Er^{3+} and Tm^{3+} ions. All paramagnetic Ln^{3+} ions induced pronounced paramagnetic shifts. No peak doubling was observed. The PCS induced by the Tb^{3+}, Dy^{3+} and Ho^{3+} ions were of opposite sign compared to those of the Er^{3+}, Tm^{3+} and Yb^{3+} ions, as expected.[14] The spectrum with Dy^{3+} ions was similar to that with Tb^{3+} ions but the signals were significantly broader, as predicted for the larger magnetic susceptibility of Dy^{3+} ions (data not shown). The Yb^{3+} complex showed no significant chemical shift changes in the ^{15}N-HSQC spectrum (data not shown). The magnetic moment of Yb^{3+} ions is relatively small and LBP2 was not enriched with ^{15}N. In the crystal structure of the LBP1–Tb^{3+} complex,[11] the Ln^{3+} ion is located >10 Å from the N terminus. Apparently, the Ln^{3+} ions are equally well separated from the other ArgN residues in the ArgN–LBP2 complex.

Magnitude of observed pseudocontact shifts

On average, Tb^{3+} and Dy^{3+} ions generated the largest PCS but also the largest paramagnetic line broadening, which resulted in the loss of over a third of the ^{15}N-HSQC cross-peaks. The largest PCS values were observed for the amide proton resonance of Leu69 in the complexes with Er^{3+} and Tm^{3+} ions ($\Delta\delta = 1.2$ and 2.9 ppm, respectively). The complexes with these ions showed smaller PCS values on average, but the reduced paramagnetic relaxation enhancements associated with Er^{3+} and Tm^{3+} ions allowed the observation of almost all of the ^{15}N-HSQC cross-peaks. Most importantly, larger PCS were observed than for any previously reported Ln^{3+}-binding tag,[5,7,8,15] a result indicating that LBP2 attached to a cysteine residue maintains the orientation of the $\Delta\chi$ tensor of the Ln^{3+} ion with respect to the protein much better than Ln^{3+}-binding tags with flexible linkers. In the case of the ^{15}N-ArgN–LBP2–Tm^{3+} complex, PCS values greater than 0.2 ppm were measured even for amide protons farther than 35 Å from the Tm^{3+} ion.

Assignment of the paramagnetic ^{15}N-HSQC spectra and $\Delta\chi$-tensor determination

The ^{15}N-HSQC cross-peaks of the paramagnetic samples were assigned based on the assignments of the diamagnetic spectrum. Since the ^{1}H and ^{15}N spins of each amide group are close in space, they experience similar PCS, which results in a displacement of the paramagnetic peak from the diamagnetic peak by similar ppm values in the ^{1}H and ^{15}N dimensions of the spectrum (Figure 1). By using this criterion, between 10–15 resolved paramagnetic peaks could readily be assigned and used for an initial estimate of the $\Delta\chi$ tensor. For additional assignments, we used the 3D structure of ArgN,[12] by comparing back-calculated PCS with the experimental paramagnetic ^{15}N-HSQC spectrum. Use of the program Echidna[16] showed that the paramagnetic data could be explained by a wide range of metal-ion positions and $\Delta\chi$ tensors. In order to determine the metal-ion positions, we reduced the number of variables by calculating the axial and rhombic components of the $\Delta\chi$ tensor from the alignment tensor arising from the paramagnetically induced alignment of the protein with respect to the magnetic field. This was done by using Equation (1),[1] where A_{ax}, A_{rh}, $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ denote the axial or rhombic components of the alignment tensor and magnetic susceptibility tensor, respectively, B_0 is the magnetic field strength, μ_0 is the induction constant, k is the Boltzmann constant and T is the temperature.

$$\Delta\chi_{ax,rh} = \frac{15\,\mu_0 kT}{B_0^2} A_{ax,rh} \qquad (1)$$

RDC values of backbone one-bond ^{1}H–^{15}N couplings ($^{1}D_{HN}$) were determined by subtracting the couplings measured for the amide nitrogen doublets in ^{15}N-ArgN–LBP2 complexes with diamagnetic Y^{3+} ions from the couplings measured with Er^{3+} and Tm^{3+} ions, respectively. The program Module[17] was used to determine the alignment tensors with reference to the structurally well-defined part of the 3D structure of ArgN.[12] The tensor parameters were insensitive with respect to the omission of up to one third of the RDC data. The $\Delta\chi$ tensor pa-

rameters calculated with Equation (1) were used to optimise the metal-ion position and to increase the number of assignments made in the paramagnetic spectra in an iterative manner by using a Mathematica (Wolfram Research) routine.[18] The positions of the Er^{3+} and Tm^{3+} ions found in this way were the same to within 1.5 Å. Subsequent calculations used the position found for the Tm^{3+} ions because it was based on larger PCS values. This metal-ion position was chemically plausible, that is, models of ArgN–LBP1 could be built in agreement with this lanthanide position.[19] By using this metal position, the ^{15}N-HSQC spectra recorded with Tb^{3+}, Dy^{3+}, Ho^{3+}, Er^{3+} and Tm^{3+} ions were assigned in several iterations with optimisation of the $\Delta\chi$-tensor magnitude and orientation.

Table 1 presents an overview over the $\Delta\chi$-tensor parameters obtained for the different Ln^{3+} ions. The magnitudes of the axial components were similar to those reported for calbindin–Ln^{3+} complexes,[20] where the Ln^{3+} ion occupies a natural metal-binding site buried inside the protein structure; this result reflects the rigid attachment of the LBP moiety to ArgN. For comparison, an almost fourfold smaller $\Delta\chi_{ax}$ value was reported for the 1-epi-4/Dy^{3+} tag attached to the cysteine residue of the S100C mutant of trigger factor;[7] this illustrates the averaging effect arising from a flexible tether. The conclusion of a rigid LBP attachment to ArgN was further supported by the magnitude of the RDCs observed. For example, the ArgN–LBP2–Tm^{3+} complex yielded fourfold larger $^1D_{HN}$ RDCs (ranging from about −12 to 21 Hz on an 800 MHz NMR spectrometer) than those reported previously for an N-terminal fusion of LBP1 to ubiquitin.[5] The relatively small $\Delta\chi_{rh}$ values observed with ArgN–LBP2 may be a consequence of the specific metal-coordination sphere provided by the LBP.

Figure 2 depicts the orientations of the $\Delta\chi$ tensors of the different Ln^{3+} ions with respect to the 3D structure of ArgN. Similarly to previous results with other protein–lanthanide complexes,[20] the *z* axes of the tensors varied only a little between different lanthanides. The orientations of the *x* and *y* axes were more variable but also less certain. For example, the rhombic component of the $\Delta\chi$ tensor found for Dy^{3+} ions was relatively small (Table 1) and corresponded to a near axially symmetric $\Delta\chi$ tensor and, hence, less well-defined *x* and *y* axes. The significance of the differences between the different $\Delta\chi$ tensors can be assessed by pairwise correlations between the PCS induced by Ho^{3+}, Er^{3+} and Tm^{3+} ions (Figure 3). While the PCS from Er^{3+} and Tm^{3+} ions are clearly correlated and the PCS from Ho^{3+} and Tm^{3+} ions are clearly anticorrelated, the deviations from straight lines were significant. This indicates that the different $\Delta\chi$ tensors were not simply linearly proportional with respect to one another. Even small differences in the orientation of the tensors of two different metal ions are sufficient to model the 3D structures of protein–ligand complexes from PCS alone, in an unambiguous way.[3]

Table 1. Axial and rhombic components of the $\Delta\chi$ tensors of different lanthanide ions in complexes with ArgN–LBP2 and calbindin, as determined from pseudocontact shifts.[a]

Protein	Tm^{3+}	Er^{3+}	Ho^{3+}	Tb^{3+}	Dy^{3+}
ArgN–LBP2	−21.3, −8.2	−9.9, −3.1	13.7, 4.7	45.9, 4.7	34.7, 4.4
calbindin[20]	−21.9, −20.1	−11.6, −8.6	18.5, 5.8	42.1, 11.2	34.7, 20.3

[a] $\Delta\chi_{ax}$, $\Delta\chi_{rh}$ values in 10^{-32} m^{-3}.

Conclusion

In conclusion, we have established a method for site-specific attachment of lanthanide ions to proteins, produced a protein–LBP complex where the LBP is rigidly attached without significantly affecting the structure of the protein, and measured for the first time a set of $\Delta\chi$-tensor parameters for an LBP–lanthanide complex. The magnitude of the RDC values measured suggests that LBP–Ln^{3+} complexes present a useful tool for protein alignment in a magnetic field in the absence of alignment media. Since the tag is not isotope enriched, it is invisible in isotope-edited NMR spectra, thereby simplifying the NMR analysis of isotope-labelled proteins. The LBP2

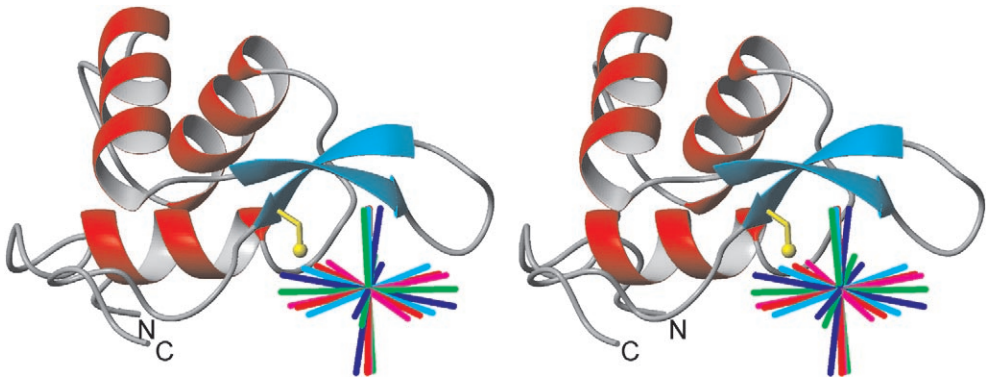


Figure 2. Stereoimage of ArgN with the χ-tensor axes of different lanthanide ions bound to LBP2. Red: Er^{3+}, magenta: Tm^{3+}, cyan: Ho^{3+}, green: Tb^{3+}, blue: Dy^{3+}. All tensor axes are displayed with the same length. The *z* axes of the tensors are oriented approximately vertical. The metal-ion position is 12.4 Å from the sulfur atom of Cys68 (highlighted in yellow). The figure was prepared with the program Molmol.[23]

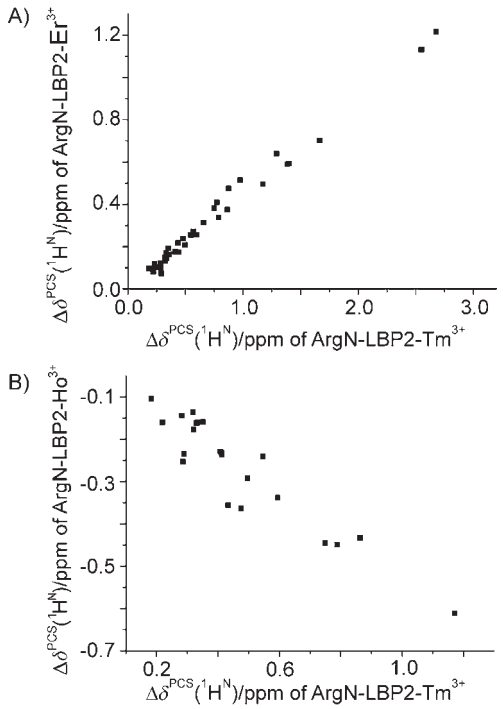


Figure 3. Correlation plot of the pseudocontact shifts of the backbone amide protons of ArgN–LBP2 loaded with different paramagnetic lanthanide ions. A) PCS from Er^{3+} ions versus PCS from Tm^{3+} ions. B) PCS from Ho^{3+} ions versus PCS from Tm^{3+}ions. The range of PCS from Tm^{3+} ions is smaller in (B) than in (A) because the most strongly shifted amide resonances were broadened beyond detection in the Ho^{3+} sample. The uncertainty in chemical shift measurements was about 0.02 ppm.

tag thus presents a most promising general tool for the rigid attachment of Ln^{3+} ions to proteins with single cysteine residues. Future studies will focus on the PCS induced by Ln^{3+}-tagged ArgN in complexes with operator DNA, with the aim of determining their 3D structures.

Experimental Section

Materials: Peptides were synthesised chemically by using the 9-fluorenylmethyloxycarbonyl (FMOC) method on a Rainen Symphony/Multiplex peptide synthesiser and were supplied by the Biomolecular Resource Facility at the Australian National University. Uniformly ^{15}N-labelled ArgN was expressed and purified as previously described,[12] with modifications. An autoinduction system[21] was used for cell growth in a minimum medium containing $^{15}NH_4Cl$ as the only nitrogen source. Cells were grown at room temperature for three days, harvested and lysed with a French press. The protein was purified by anion-exchange chromatography on a diethylaminoethyl-fractogel column, followed by chromatography on a SP-Sepharose column with a shallow NaCl gradient. Typical yields were 30–40 mg of purified protein per litre of culture medium. DTNB in analytical purity was purchased from Aldrich. Lanthanide trichlorides (Aldrich) were weighed and dissolved in 20 mM MES buffer at pH 6.5.

Protein–LBP ligation: ^{15}N-ArgN was first treated with an excess of 1,4-dithiothreitol (DTT) to reverse any formation of intermolecular disulfide bonds and the DTT was removed by ultrafiltration with an Amicon centrifugal concentrator (molecular weight cut off of 5000). A 30-fold excess of DTNB in 50 mM Tris buffer (pH 7.8) was added to the protein and the solution was stirred at room temperature for 2 h. Subsequent ultrafiltration removed excess DTNB and TNB and left behind a light-yellow ^{15}N-ArgN–TNB complex. The Ln^{3+}-binding peptide was added in fourfold excess in the same reaction buffer and the mixture was treated at room temperature for 4 h. The solution was then concentrated and centrifuged to remove traces of precipitate. The ArgN–LBP complex was purified by FPLC with a Mono-Q column in 20 mM Tris buffer; it was eluted with a gradient of 0–1 M NaCl.

NMR spectroscopy: NMR experiments were recorded at 10 °C in 20 mM MES buffer (pH 6.5) with 4 mM glycine at a ^{1}H NMR frequency of 800 MHz on a Bruker AV800 NMR spectrometer equipped with a TCI cryoprobe. A 3D NOESY–^{15}N-HSQC spectrum (80 ms mixing time; t_{1max} = 13.3 ms, t_{2max} = 12 ms, t_{3max} = 106 ms; 20 h total recording time) was recorded with a 0.5 mM solution of diamagnetic ^{15}N-ArgN–LBP2 in H_2O/D_2O (90:10). All ^{15}N-HSQC spectra with paramagnetic Ln^{3+} ions were recorded at a protein concentration of 50 μM. Residual dipolar couplings ($^1D_{HN}$) were measured as observed differences in ^{15}N-doublet splittings between paramagnetic and diamagnetic samples, by using the IPAP pulse sequence.[22] PCS were measured from ^{15}N-HSQC spectra as differences in ^{1}H chemical shifts between samples prepared with paramagnetic Ln^{3+} ions and diamagnetic Y^{3+} ions.

Determination of $\Delta\chi$-tensor parameters: The $\Delta\chi$-tensor parameters were determined as described in the text. Only residues located in regions of well-defined secondary structure were included in the fits. In particular, no data from the flexible nine N-terminal and seven C-terminal residues were used.

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Keywords: lanthanides · NMR spectroscopy · peptides · proteins · structure elucidation

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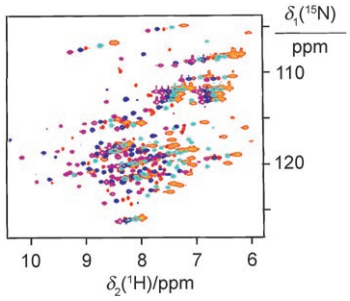
Playing tag with proteins. Paramagnetic lanthanide ions rigidly attached to proteins provide a rich source of long-range NMR restraints for protein-structure determination and modelling protein–ligand complexes. This paper presents a generic method for lanthanide labelling and the acquisition of paramagnetic data for proteins without natural metal-binding sites (see ^{15}N-HSQC spectrum).



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Site-Specific Labelling of Proteins with a Rigid Lanthanide-Binding Tag

