Three-Dimensional Protein Fold Determination from Backbone Amide Pseudocontact Shifts Generated by Lanthanide Tags at Multiple Sites

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SUMMARY

Site-specific attachment of paramagnetic lanthanide ions to a protein generates pseudocontact shifts (PCS) in the nuclear magnetic resonance (NMR) spectra of the protein that are easily measured as changes in chemical shifts. By labeling the protein with lanthanide tags at four different sites, PCSs are observed for most amide protons and accurate information is obtained about their coordinates in three-dimensional space. The approach is demonstrated with the chaperone ERp29, for which large differences have been reported between X-ray and NMR structures of the C-terminal domain, ERp29-C. The results unambiguously show that the structure of rat ERp29-C in solution is similar to the crystal structure of human ERp29-C. PCSs of backbone amides were the only structural restraints required. Because these can be measured for more dilute protein solutions than other NMR restraints, the approach greatly widens the range of proteins amenable to structural studies in solution.

INTRODUCTION

Conventional three-dimensional (3D) structure determinations of proteins with nuclear magnetic resonance (NMR) spectroscopy rely on a very large number of nuclear Overhauser effects (NOE) to provide a dense network of short-range distance restraints (Wüthrich, 1986). Additional NMR parameters, such as scalar and residual dipolar couplings (RDC), cross-correlation effects, and paramagnetic relaxation enhancements (PRE), provide powerful restraints for further structure refinements but none of these parameters is sufficient on its own to determine 3D structures of proteins, except for RDCs, where this has been achieved for small proteins with a large number of experimental RDCs (Hus et al., 2001; Tian et al., 2001; Wang et al., 2007; Rasia et al., 2011; Kontaxis, 2012). The measurement of small heteronuclear RDCs, however, becomes increasingly difficult for proteins of limited solubility or proteins yielding broad NMR line widths due to mobility effects or increased molecular weight. Here, we present an alternative approach to 3D structure determinations of proteins that relies on pseudocontact shifts of backbone amide protons induced by paramagnetic lanthanide ions attached to multiple different sites of the target protein. PCS data can be measured with much greater sensitivity than NOEs or RDCs, opening the door to detailed structural studies of a much greater range of proteins in solution. Furthermore, we show that PCSs of backbone amides are sufficient for 3D fold determination. Combined with the remarkable capability of the program Rosetta to predict correct amino acid side chain conformations even if experimental data are only available for backbone atoms (Raman et al., 2010), complete protein structures can be obtained also without cumbersome analysis of poorly resolved NMR resonances of amino acid side chains.

PCSs stand out for their long-range effects (up to 40 Å for lanthanides; Allegrozzii, 2000). PCSs are generated by many paramagnetic metal ions (Bertini and Luchinat, 1986) and have long been used successfully for structure refinement of metalloproteins (Bertini et al., 2001, 2008; Arnesano et al., 2005; Otting, 2010) and interaction studies (Pintacuda et al., 2007; Keizers and Ubbink, 2011). Arguably, lanthanide ions are most suited as paramagnetic centers for generating PCSs because many of them are highly paramagnetic and once a lanthanide binding site has been established, it will bind any lanthanide similarly well, with yttrium as a suitable diamagnetic reference (Wu¨ thrich, 1986). With the advent of efficient protocols for site-specific attachment of lanthanide ions (for reviews, see Rodriguez-Castañeda et al., 2006; Su and Otting, 2010, 2011; Koehler and Meiler, 2011), including methods that do not depend on single cysteine residues (Wöhnert et al., 2003; Su et al., 2008; Barthelmes et al., 2011; Loh et al., 2013), large PCSs can be routinely generated in proteins amenable to genetic engineering. The PCS $\Delta \delta_{\text{PCS}}$ of a nuclear spin is measured in ppm as the difference in chemical shift observed between a sample with a paramagnetic lanthanide and a reference sample with a chemically similar diamagnetic metal (e.g., $\delta^{\text{Y}}$). It depends on the polar coordinates $r$, $\theta$, and $\phi$ of the nuclear spin with respect to the principal axes of the $\Delta \chi$ tensor that describes the pertinent paramagnetic properties of the lanthanide ion:

$$\Delta \delta_{\text{PCS}} = \frac{1}{12 \pi r^3} \left[ \Delta \chi_{xx} (3 \cos^2 \theta - 1) + \frac{3}{2} \Delta \chi_{nn} \sin^2 \theta \cos 2 \phi \right].$$

(1)
and where the $\Delta \chi$ tensor is defined as the $\chi$ tensor minus its isotropic component. Therefore, if the $\Delta \chi$ tensor and its location and orientation with respect to the protein are known, the PCS of a nuclear spin provides useful information about its location in the coordinate frame of the $\Delta \chi$ tensor.

It is instructive to visualize the PCSs described by Equation 1 as isosurfaces of constant PCS, where each isosurface depicts all coordinates at which nuclear spins would display a certain PCS value. While the PCS of a nuclear spin ties its location to a certain isosurface, a second PCS measured for a sample with a lanthanide attached at a different site confines the nuclear spin further to lie on the line defined by the intersection of the respective PCS isosurfaces. A third PCS value from a sample with a lanthanide attached at yet another site would pin down the position of the nuclear spin even more.

Using PCSs in this manner for 3D structure determination poses the dilemma that the $\Delta \chi$ tensor parameters of a metal ion are usually not known a priori. Without 3D coordinates of the protein, however, it is not possible to fit a $\Delta \chi$ tensor that reproduces the experimentally observed PCSs. To circumvent this conundrum, we used the Rosetta algorithm (Qian et al., 2007) to model the protein structure to fit initial $\Delta \chi$ tensors. For PCSs from a single site, this strategy has been implemented at the fragment assembly step in the program PCS-Rosetta, which discards ill-fitting structures before any further time-consuming refinement to improve the final yields of correct structures (Schmitz et al., 2012).

To avoid the ambiguities associated with PCSs from a paramagnetic tag at a single site and to achieve complete coverage of the polypeptide chain with PCSs, we expanded the concept of PCS-Rosetta to multiple tagging sites. The results show that PCSs generated by lanthanide tags attached at several different sites offer a robust route to 3D fold determinations of proteins that can be more accurate than structure determinations by NOEs, even if only PCSs of backbone amide protons are considered.

The approach was applied to the C-terminal domain of the rat protein ERp29, ERp29-C (106 residues, 12 kDa). ERp29 is a chaperone expressed in the endoplasmatic reticulum, where it facilitates the processing and transport of proteins. Its chaperone activity underpins numerous biological functions, including roles in virus infections, cystic fibrosis, and tumor suppression and treatment (Rainey-Barger et al., 2009; Das et al., 2009; Farmaki et al., 2011; Suaud et al., 2011; Zhang and Richardson, 2011; Bambang et al., 2013; Wu et al., 2012). The 3D structure of the C-terminal domain of rat ERp29 was first determined by NMR spectroscopy using the conventional NOE approach. This structure is henceforth referred to as the NOE structure of ERp29-C (Liepinsh et al., 2001). Subsequently, the 3D structure of the full-length human protein was determined by X-ray crystallography (Barak et al., 2001), revealing a significantly different structure of ERp29-C, with a C$^\circ$ root mean square deviation (rmsd) of 4.5 Å to the NOE structure (residues 158–244). Specifically, helices 8 and 9 of the crystal structure form fewer contacts with the rest of the protein, giving it a less globular appearance (Figure 1). The present work thus not only establishes a method for 3D structure determinations of proteins in solution, but also reassesses the structure of the rat ERp29-C domain.

RESULTS

Sample Preparation

To generate PCSs from multiple metal positions and to obtain PCSs for most of the backbone amides, ERp29-C was tagged at four different sites with lanthanide ions. Wild-type rat ERp29-C contains a single cysteine residue, Cys157, which was ligated with the C1 tag (Graham et al., 2011) to generate PCSs. To create additional lanthanide binding sites, Cys157 was mutated to serine and the three additional double mutations S200C/K204D, A218C/A222D, and Q241C/N245D were superimposed for minimal rmsd.
were introduced for attachment of the IDA-SH tag via a disulfide bond. (For simplicity, these triple mutants are referred to in the following as C200, C218, and C241.) All double mutation sites were in α helices (Figure 1A). The aspartate residue in position $i + 4$ served to create a specific lanthanide binding site in which the metal is immobilized by coordination to the IDA-SH tag and by additional coordination to the aspartate residue (Swarbrick et al., 2011; Yagi et al., 2013). Samples of the mutants with the IDA-SH tag loaded with Y$^{3+}$, Tm$^{3+}$, or Tb$^{3+}$ were prepared by titration with the respective lanthanide chlorides. Corresponding samples of the wild-type protein with a C1 tag were prepared by ligation with C1 tags preloaded with the respective metal ions because high temperatures are required to install lanthanides in the C1 tag.

NMR Measurements

All four constructs displayed significant PCSs in $^{15}$N-HSQC spectra (Figures 2A–2D). The cross-peaks in the paramagnetic states were assigned using the fact that the PCSs of the $^1$H and $^{15}$N spins of any single amide group are very similar because their coordinates are close compared with their distance from the paramagnetic center (cross-peaks from amides close to the metal are broadened beyond detection due to PREs; Allegrozzi et al., 2000; John and Otting, 2007). 3D HNCO spectra recorded of $^{13}$C/$^{15}$N-labeled samples of the wild-type protein and the C200 mutant, both labeled with paramagnetic lanthanides, confirmed the reliability of the resonance assignments obtained from the two-dimensional (2D) $^{15}$N-HSQC spectra.

A total of 212 PCSs were measured for the backbone amide protons from the 2D $^{15}$N-HSQC spectra, using Tm$^{3+}$ and Tb$^{3+}$ (Table S1 available online). Figure 2E shows that at least one PCS was obtained for almost 90% of residues 161–256, i.e. excluding the flexible N- and C-terminal segments (Liepinsh et al., 2001), and two or more PCSs were observed for over half of the protein.

GPS-Rosetta Calculations

The PCSs served as input for 3D structure calculations using a new version of the PCS-Rosetta algorithm (Schmitz et al., 2012). Like CS-Rosetta (Shen et al., 2008), PCS-Rosetta uses the backbone chemical shifts of the protein to assist the initial fragment selection from the Rosetta library of proteins. The PCSs are used only in the subsequent fragment assembly step to fit $\Delta \gamma$ tensors and thus probe the agreement of the emerging model with the PCS data. The long-range nature of the PCSs effectively discriminates between successful and unsuccessful models in this early stage of calculations. Our version of the PCS-Rosetta program, called GPS-Rosetta, uses the same structures.
approach but is no longer limited to a single paramagnetic center, accepting PCSs generated by multiple lanthanides at different sites and optimizing their respective Dc tensors during the structure calculation.

About 100,000 full-atom models were generated with GPS-Rosetta. The structure identified by the red point in Figure 3A was selected for its low Rosetta energy and high quality of back-calculated versus experimental PCSs. This structure is shown in red in Figure 3B, also marks the four positions of the lanthanide ions by blue spheres labeled with the residue number of the cysteine they are attached to and highlights the sites of the respective cysteine residues on the ribbon in blue. The Cα rmsd between the GPS-Rosetta structure and the crystal structure of human ERp29-C is 2.4 Å (where the rmsd calculation included residues 158–244, while omitting Lys229, which is inserted in the human protein (Figure 1A), and excluding the C-terminal segment that forms an extended helix in the GPS-Rosetta structure although residues 254–260 are known to be highly flexible; Liepinsh et al., 2001). The superimposition in (C) also shows the five structures with the lowest PCS rmsd values, represented in shades of blue. Their Cα rmsd to the crystal structure (residues 158–228 and 230–244) is 2.0–2.9 Å. A comparison of the side chain conformations of conserved amino acids between the GPS-Rosetta and crystal structures is given in Figure S2. Structures combining a low combined PCS + Rosetta energy with low PCS rmsd values are represented in shades of green (D). They have a Cα rmsd range of 2.2–2.6 Å to the crystal structure. The NOE structure shown in yellow (PDB ID 1G7D) has a Cα rmsd of 6 Å (residues 158–244) to the GPS-Rosetta structure shown in red (E).

In contrast, the GPS-Rosetta structure is markedly different from the original NOE structure (Figure 3E). The disagreement with the NOE structure is highlighted by the correlation between the back-calculated and experimental PCSs, which is much better for the GPS-Rosetta structure than for the NOE structure (Figure 4). Cross-validation with PCSs not used in the structure calculation (measured with C1-Yb and C1-Dy tags attached to C157) confirm this conclusion (Figure S3 and Tables S2–S4). The results thus clearly show that the fold of rat ERp29-C in solution is similar to the crystal structure of the human protein, superseding the original NOE structure.

To check whether the Rosetta program would have found the same structure without the help of PCSs, we repeated the same calculations with CS-Rosetta (Shen et al., 2008; see Supplemental Experimental Procedures). Much fewer models were in agreement with the experimental PCSs (Figure S1) and the two models with the lowest Rosetta energy displayed very different folds, with Cα rmsd values to the GPS-Rosetta structure greater...
than 13 Å (Figure S2). The PCSs were thus critical for the identification of the correct fold.

**DISCUSSION**

The present study highlights the value of PCSs delivered by multiple site-specific lanthanide tags and the pitfalls that can be associated with the misinterpretation of NOEs, especially in the case of helical proteins where contacts between the helices are made by side chain rather than backbone atoms, requiring careful interpretation of crowded spectral regions. PCSs from multiple rather than single lanthanide sites offer particularly valuable structural restraints. First, paramagnetic relaxation enhancements (PRE) in the vicinity of a lanthanide broaden the NMR signals of many residues beyond detection. PCSs from different tagging sites thus significantly improve the coverage of the amino acid sequence with experimental PCSs (Figure 2E).

Second, a single PCS value measured of a nuclear spin provides the information that the spin must be located on a PCS isosurface defined by the $\Delta \chi$ tensor. This presents a relatively weak structural restraint because the isosurface extends all around the metal ion. In contrast, if the nuclear spin must fulfill the PCSs generated by lanthanides at two different sites, it must be positioned on the line defined by the intersect of the isosurfaces of the corresponding two different $\Delta \chi$ tensors. PCSs from lanthanides at three different sites restrict the possible locations of the spin to the two points, where the line described by the intersect defined by the first two isosurfaces intersects the isosurface from the third $\Delta \chi$ tensor. The PCS from a fourth lanthanide site unambiguously locates the spin at a single point. In this way, PCSs from multiple tags allow positioning of a nuclear spin in 3D space in a manner analogous to the global positioning systems that calculate coordinates on earth by distance measurements from satellites. Similar to the situation with RDCs, which convey detailed structural information if measured with multiple alignment media (Meiler et al., 2001; Ruan et al., 2008), the availability of PCSs from four different sites presents a fundamental advance over data from a single paramagnetic center, which are compatible with multiple structure solutions as shown in previous attempts of using PCSs for protein structure determination (Bertini et al., 2002b). In the case of ERp29-C, one amide (Gln212) displayed PCSs from four sites, and PCSs from two or more sites were observed for about one-third of the amide resonances (Figure 2E).

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The present study shows that, if PCS data are available only from a single lanthanide tagging site, large structural differences may not be picked up even if different lanthanides are used. This
is apparent in the correlation plots obtained for the C218 mutant, which were of similar quality for the crystal, NOE, and GPS-Rosetta structures (Figure 4B). In part, this can be attributed to the similarity in $\Delta\gamma$, tensor orientations for different lanthanides, which is a consequence of the conserved ligand field presented by the compound that chelates the metal.

Nonetheless, using two different paramagnetic metal ions at each site is useful. In the present work, we tagged each of the four sites with either Tb$^{3+}$, Tm$^{3+}$, or Y$^{3+}$ to generate two sets of PCSs from each site. Tb$^{3+}$ and Tm$^{3+}$ tend to shift the cross-peaks in opposite directions, supporting the resonance assignments of the paramagnetic states. In addition, Tb$^{3+}$ is more strongly paramagnetic than Tm$^{3+}$, yielding PCSs at different distances from the metal ion.

The IDA-SH tag is particularly powerful for the present application because it defines the position of the lanthanide if it is located in an $\alpha$-helix with an aspartate in position $i+4$ (Swarbrick et al., 2011; Yagi et al., 2013). Therefore, while a $\Delta\gamma$, tensor determination generally requires the fit of eight parameters ($\Delta\gamma_{xx}$, $\Delta\gamma_{yy}$, $\Delta\gamma_{zz}$, $\Delta\gamma_{xy}$, $\Delta\gamma_{yx}$, $\Delta\gamma_{xz}$, $\Delta\gamma_{zx}$, $\Delta\gamma_{yz}$, and $\Delta\gamma_{zy}$) to relate the orientation of the tensor to the protein structure), prior knowledge of the metal coordinates reduces the number of fitting parameters to five. Notably, however, the GPS-Rosetta algorithm can also fit the metal coordinates, and this was used to locate the C1 tag for which no prior information about its location was available.

The GPS-Rosetta structure of rat ERP29-C supersedes the earlier NOE structure of the same protein. The magnitude of the structural differences suggests wrong NOE assignments. The NOE structure relied on NOE assignments made in 2D NOESY and 3D NOESY-15N-HSQC spectra recorded on a 600 MHz NMR spectrometer (Liepinsh et al., 2001). In particular, the 2D spectrum allowed the observation of over a dozen NOEs with the side chain hydroxyl resonance of a buried tyrosine residue. The current GPS-Rosetta structure indicates that this hydroxyl resonance, which could only be assigned by NOEs, should have been assigned to Tyr 161 but was in fact misassigned to Tyr 202. Structure calculations using this missassignment would necessarily have resulted in further misassignments of NOEs which, in the case of poorly resolved resonances of aromatic and methyl side chains, could easily have gone undetected due to the absence of a 3D NOE$^{15}$C-HSQC spectrum of a $^{15}$C-labeled sample.

The results obtained in the present work carry great promise for 3D structure determinations of perdeuterated proteins for which no side chain resonances can be observed. Selectively $^{15}$N-labeled samples can be used to achieve the required spectral resolution in $^{15}$N-$^1$H correlation spectra for PCS measurements of backbone amides in larger proteins. An extension of the present work could also exploit the fact that any lanthanide that generates PCSs also causes partial alignment of the protein with the magnetic field axis, giving rise to RDCs (Bertini et al., 2002a). It is well known that alignment tensors with different orientations, as obtained from lanthanide tags at different sites, greatly enhance the structural information content of RDCs (Bax et al., 2001; Tolman and Ruan, 2006; Bouvignies et al., 2007; Lange et al., 2008). Multiple orientations will augment the experimental data set particularly if only one-bond RDCs between the $^{15}$N and $^1$H spins of backbone amides can be measured.

In conclusion, paramagnetic lanthanides attached at multiple different sites offer a potent strategy to pinpoint the global fold of a protein by the use of PCSs, even in the absence of additional structural restraints. Most importantly, only PCSs of backbone amide protons are required, opening the door to solution structure determinations of proteins of limited solubility, for which other NMR parameters are difficult to measure.

**EXPERIMENTAL PROCEDURES**

### Sample Preparation

Wild-type and mutant samples (S200C/K204D/C157S, A218C/A222D/C157S, and Q241C/N245D/C157S) of the C-terminal domain of rat ERP29-C (Liepinsh et al., 2001) were expressed with a His$_6$-tag at the C terminus, using the plasmid pETMCSi (Neylon et al., 2000) under control of the T7 promoter. We refer to the three different triple mutants as C200, C218, and C241, respectively. Uniformly $^{15}$N- and $^{13}$C/$^{15}$N-labeled samples were expressed in _Escherichia coli_ Rosetta (I(DE3)/pRARE cells. For protein production, cells were grown overnight in 10 ml of LB medium. This preculture was used to inoculate 1 l of minimal medium containing $^{15}$NH$_4$SO$_4$ as the sole nitrogen source, or $^{15}$-labeled glucose and $^{15}$NH$_4$Cl as the sole carbon and nitrogen sources. Cells were harvested 6 hr after induction. All proteins were purified using a HisTrap HP column (GE Healthcare, 5 ml). The mutants C200, C218, and C241 were ligated with the IDA-SH tag following a previously published protocol (Swarbrick et al., 2011), except that the reaction buffer contained 20 mM Tris-HCl, pH 7.6. As a fourth sample, the wild-type protein, which contains a single cysteine residue at position 157, was ligated with the C1 tag (Graham et al., 2011) as described previously (Yagi et al., 2011).

### NMR Spectroscopy

All NMR spectra were recorded of about 0.3–0.6 mM solutions of the wild-type and mutant samples of rat ERP29-C in 20 mM MES (pH 4.9) at 31°C using a Bruker Avance 800 MHz NMR spectrometer with a TCI cryoprobe. PCSs were measured as the $^1$H chemical shifts observed in $^{15}$N-NOESY spectra of ERP29-C tagged with paramagnetic lanthanides (Tm$^{3+}$, Tb$^{3+}$) minus the corresponding chemical shifts observed for the diamagnetic reference (Y$^{3+}$). For the wild-type protein and the C200 mutant, the paramagnetic shifts were verified for all samples by 3D HNCO spectra of the $^{13}$C/$^{15}$N-labeled protein. To minimize residual anisotropic chemical shifts that can be associated with the chemical shifts of heteronuclei with large chemical shift anisotropies (John et al., 2009), we measured PCSs only for $^1$H spins.

### GPS-Rosetta Calculations

GPS-Rosetta is an extended version of PCS-Rosetta (Schmitz et al., 2012), which uses experimental sets of PCSs from multiple metal binding sites for the simultaneous optimization of all $\Delta\gamma$, tensors during the low-resolution, backbone-only folding simulation of Rosetta. The PCS data from each of the metal binding centers were scored using

$$S = \sum_{k=1}^{m} \sum_{l=1}^{n} (PCS_{exp}^{k,l} - PCS_{model}^{k,l}),$$  

where $m$ is the number of PCS data sets (one data set per metal ion) per binding site $k$ and $n_{PCS}$ is the number of PCSs in the data set. A total score $S_{total}$ was then calculated as the weighted sum of the individual scores $S_k$ and added to the low-resolution energy function of Rosetta:

$$S_{total} = \sum_{k=1}^{n} S_k w_k,$$

where $n$ is the total number of metal binding centers and $w$ denotes the weighting factor relative to the Rosetta ab initio scoring function.

Rosetta 9- and 3-residue fragment libraries were generated using the experimental backbone chemical shifts of the NOE structure of rat ERP29-C (BMRB accession code 4920; Liepinsh et al., 2001) to aid in selecting fragments of...
higher quality, as in the standard CS-Rosetta protocol (Shen et al., 2008). In addition, to help resolve the differences between the NOE structure (Protein Data Bank [PDB] ID 1G7D; Liepinsh et al., 2001) and the crystal structure (PDB ID 2QCT; Barak et al., 2009) of rat and human ERp29-C, respectively, the backbone dihedral angles of both structures were explicitly added to the otherwise nonhomologous fragment library.

The PCS scoring was simultaneously optimized during the backbone folding simulation for the lanthanide tags attached at Cys157, C200, C218, and C241, and the weighting factor w for each of the n centers was calculated independently by

\[ w = \frac{\alpha_{\text{high}} - \alpha_{\text{low}}}{\sigma_{\text{high}} - \sigma_{\text{low}}} \]

where \( \alpha_{\text{high}} \) and \( \alpha_{\text{low}} \) are the averages of the highest and lowest 10% of the values of the Rosetta ab initio score, and \( \sigma_{\text{high}} \) and \( \sigma_{\text{low}} \) are the averages of the highest and lowest 10% of the PCS score value obtained by scoring 1000 decoys with a unity weighting factor.

In α-helical secondary structure, lanthanide ions bound to an IDA-SH tag in position i also coordinate to the side chain carboxyl group of an aspartate in position i+4. Therefore, the metal ion was positioned 5.9 Å from the C\(^{\alpha}\) atom of residue i+4 on a line that joins the backbone amide nitrogen of residue i+6 with the C\(^{\alpha}\) atom of residue i+4. In contrast, the position of the lanthanide in the C1 tag, which was ligated to Cys157, was dynamically optimized during the folding simulation, using the strategy implemented in PCS-Rosetta (Schmitz et al., 2012).

**ACCESSION NUMBERS**

The coordinates of the GPS-Rosetta structure of rat ERp29-C were deposited in the Protein Data Bank with the accession code 2M66.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four tables, Supplemental Experimental Procedures, and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.str.2013.04.001.

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