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Validation of a Lanthanide Tag for the Analysis of Protein Dynamics by Paramagnetic NMR Spectroscopy

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Abstract: Paramagnetic lanthanide tags potentially can enhance the effects of microsecond to millisecond dynamics in proteins on NMR signals and provide structural information on lowly populated states encoded in the pseudocontact shifts. We have investigated the microsecond to millisecond mobility of a two-point attached lanthanide tag, CLaNP-5, using paramagnetic ^{1}H CPMG relaxation dispersion methods. CLaNP-5 loaded with Lu^{3+}, Yb^{3+}, or Tm^{3+} was attached to three sites on the surface of two proteins, pseudoazurin and cytochrome *c*. The paramagnetic center causes large relaxation dispersion effects for two attachment sites, suggesting that local dynamics of the protein at the attachment site causes mobility of the paramagnetic center. At one site the relaxation dispersions are small and limited to the immediate environment of the tag. It is concluded that paramagnetic relaxation dispersion could represent a sensitive method to probe protein dynamics. However, the selection of a rigid attachment site is of critical importance.

The function of proteins is tightly linked to their dynamical properties. Conformational dynamics on the μs−ms time scale is of particular interest, because numerous biochemical processes, such as enzyme catalysis and signal transduction, occur on this time scale.[1] Such molecular motions can be studied by NMR relaxation dispersion experiments, which provide quantitative information about the thermodynamics and kinetics of dynamic processes.[2] It is more difficult to derive information about the structures of lowly populated protein states. Chemical shift differences between interconverting protein conformations can be obtained from relaxation dispersion experiments, but it remains difficult to interpret them in structural terms. Dipolar couplings and anisotropic shifts of the lowly populated states can be derived from relaxation dispersion measurements[3] and provide more powerful structural constraints of transient protein conformations. Also pseudocontact shifts (PCSs), which result from the magnetic susceptibility of paramagnetic metal ions, encode structural information.[4] The PCS is solely determined by the position of the nucleus within the frame of the magnetic susceptibility tensor of the metal. Hence, the PCS gradient, produced by a paramagnetic metal, across the protein can be utilized as a reference frame for studying protein dynamics on the μs−ms time scale. When a protein segment moves relative to the paramagnetic metal, the nuclei will experience a fluctuating PCS, which causes lines to broaden if the motion occurs on the μs−ms time scale (Figure 1).[5,6] The fluctuation in PCS can be analyzed in more detail using relaxation dispersion techniques,[6] offering a potentially powerful approach to determine structures of transiently populated protein conformers. It is important to realize that in this approach observation of line broadening implies motion with respect to the magnetic susceptibility tensor frame. Such motion may be a consequence of the mobility of either the nucleus or the paramagnet.

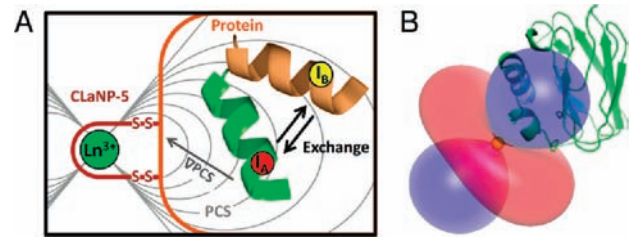

Figure 1. (A) Schematic illustration of a nucleus moving in a PCS gradient produced by a lanthanide ion. (B) The magnetic susceptibility tensor of Tm^{3+} loaded CLaNP-5 attached to PAZ(E100C/S104C) is shown as red and blue isosurfaces corresponding to a PCS of −1 and +1 ppm, respectively. The structure of PAZ (PDB entry code 1PAZ)[11] is shown in green ribbons. The program Numbat[12] was used to calculate the isosurface.

For most proteins the paramagnetic metal needs to be introduced artificially, for example, by using lanthanide binding tags.[7] The two-armed lanthanide tag CLaNP-5 was reported to give minimal averaging of PCSs and residual dipolar couplings[8,9] and is thus a good candidate for paramagnetic relaxation dispersion experiments. Here, we assessed the mobility of this tag on two proteins known to be mostly rigid,[10] pseudoazurin (PAZ) from *Alcaligenes faecalis* S-6 and *iso*-1-cytochrome *c* from yeast (Cyt *c*). CLaNP-5, loaded with Tm^{3+}, Yb^{3+}, or Lu^{3+}, was attached to ^{15}N labeled, double cysteine mutants, PAZ(E51C/E54C) and PAZ (E100C/S104C), via Cys residues located in a loop region and a small α-helix, respectively. Cyt *c*(N56C/L58C), in which the Cys residues are located in a short β-strand, was similarly prepared. The PCSs, observed in ^{15}N, ^{1}H HSQC experiments, were used to determine the position of the lanthanide ion and the size and orientation of the magnetic susceptibility tensor.[12] Then, the PCS gradient was predicted (Supporting Information for details), as illustrated for PAZ in Figure 2 (blue lines in A−C and color-coded structures in D−F). For Cyt *c*, see Figure S5.

To observe exchange contributions from fluctuations in PCSs, the relaxation rates of protons were measured. Protons are the most sensitive nuclei, because the PCS induced relaxation effect is proportional to the square of the gyromagnetic ratio (eq S5, assuming fast exchange). A sensitive, though qualitative, ^{1}H CPMG relaxation dispersion experiment (Supporting Information) was used, based on the CPMG-INEPT experiment.[13] This experiment has a high signal-to-noise ratio and allows for the detection of large dispersions (10−200 s^{-1}). A quantitative analysis of the shape of the dispersion curves may not be reliable, but to evaluate the mobility of the tag mere estimation of the amplitude of the paramagnetic dispersion effect, ΔR_{ex}*, (Supporting Information) is sufficient.

We measured no significant dispersion effects for Yb-CLaNP-5 tagged PAZ(E100C/S104C) (Figure 2, panels B and H), while moderate dispersions could be detected for the Tm variant (largest ΔR_{ex}* values ∼30 s^{-1} in the vicinity of the tag, panels C and I).

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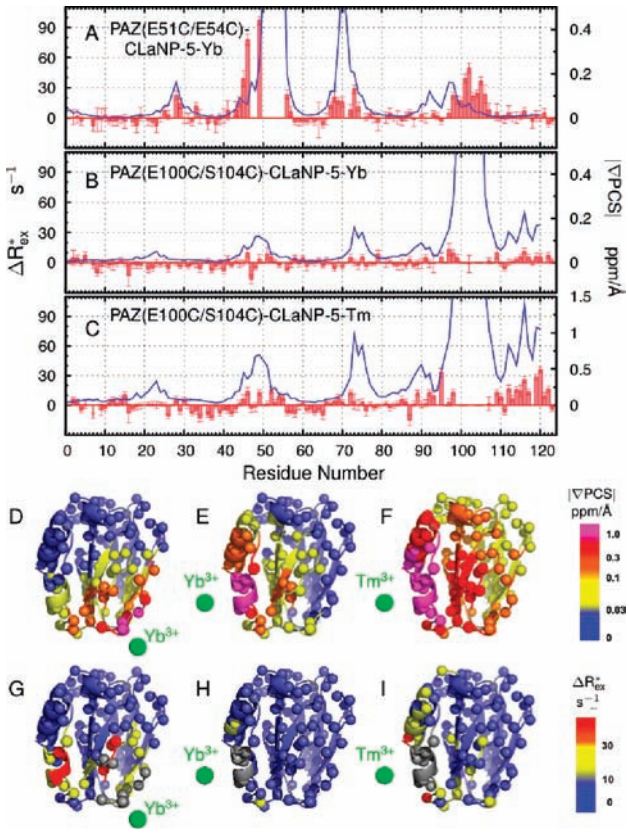

Figure 2. (A−C) Bars show the paramagnetically induced ΔR_{ex}*, lines show the PCS gradient calculated from the magnetic susceptibility tensor and the X-ray structure of *A. f.* PAZ. (D−F) The structure of PAZ[11] colored by the strength of the PCS gradient and (G−I) the structure of PAZ colored by ΔR_{ex}* obtained for (D, G) PAZ(E51C/E54C)-Yb, (E, H) PAZ(E100C/E104C)-Yb, and (F, I) PAZ(E100C/E104C)-Tm. The green spheres indicate the position of the lanthanides. Residues for which no data were obtained are colored gray.

These observations are consistent with the 5.8-fold greater paramagnetism of Tm with respect to Yb, which magnifies exchange effects up to 34-fold. In contrast, extensive dispersions were observed when the Yb-CLaNP-5 tag was attached to PAZ(E51C/E54C) (Figure 2, panels A and G) and for both Yb- and Tm-CLaNP-5 tagged Cyt *c*(N56C/L58C) (Figure S5). Many signals within ∼20 Å of the lanthanide ion experience large line broadening and ΔR_{ex}* values up to ∼120 s^{-1}. With Tm-CLaNP-5 tagged Cyt *c*(N56C/L58C), almost all observable residues show dispersion (Figure S5 B,F). Fluctuations in the PCS experienced by the nuclei must be the cause of the additional line broadening, since the dispersions are not observed for the diamagnetic proteins (Figures S3, S4). These findings lead us to conclude that large, paramagnetically induced dispersions can be obtained, indicating that this approach is very sensitive to μs−ms mobility, as predicted by the strong PCS gradients within the proteins (Figure 2). However, the widespread dispersion observed for PAZ(E51C/E54C) and Cyt *c*(N56C/L58C) are likely due to the mobility of the lanthanide tag rather than domain motions within the protein. This is surprising, given the excellent agreement between observed and calculated shifts (Figure S6) and the magnitude of the magnetic susceptibility tensors of CLaNP-5,[9] which indicate minimal averaging of the PCSs. Several potential sources of the observed exchange effects can be considered. NMR data demonstrate that the free probe slowly interconverts between two enantiomers that exhibit the same magnetic susceptibility tensor (see Supporting Materials and Methods and Figure S8), suggesting that it is unlikely that exchange between different coordination geometries of the lanthanide causes the observed fast exchange effects. However, interconversion between rotamers of the linkers or disulfide bonds could cause tag movement. Alternatively, the effects could be caused by local protein dynamics at the site of CLaNP-5 attachment. The relaxation dispersion effects of CLaNP-5 linked to PAZ(E100C/E104C) are modest considering the very strong PCS gradient in the affected region (∼1 ppm/Å for Tm^{3+}; Figure 2), where movements of only 2 Å can cause R_{ex} values of 100s s^{-1} (see Figure S7 for simulations). Interestingly, residues 100 and 104 are part of a rigid α-helix, whereas 51 and 54 are within a loop. ^{15}N relaxation studies of PAZ have shown that this loop region is affected by conformational exchange.[10] Our data suggest that the location of the tag on the protein is critical to obtain a rigid attachment. For the purpose of studying protein dynamics, ideally, both the tag and attachment site should be completely rigid so that the susceptibility tensor frame provides a fixed reference for observing fluctuations of the protein structure. In reality, any part of a protein and the tag has a degree of flexibility. A systematic analysis of the dynamics of the tag attached to different types of secondary structures is clearly required. Structure determination of the lowly populated state will likely require the attachment of the tag at several different positions on the protein, which may allow for the deconvolution of the mobility of the paramagnetic center from that of the protein. Such studies are now in progress on perdeuterated proteins known to undergo large conformational fluctuations.

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Supporting Information Available: This contains Materials and Methods, Theory, Tables (S1−S3), and Figures (S1−S8). This material is available free of charge via the Internet at http://pubs.acs.org.

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