Resolving Resonance Overlap in the NMR Spectra of Proteins from Differential Lanthanide-Induced Shifts

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Two major problems limit the study of large proteins by NMR spectroscopy. These are the broad line widths due to the slow tumbling of big proteins in solution and the signal overlap produced by the large number of resonances. Recent improvements in NMR methods and the use of deuteration have increased the molecular weight limit of proteins that can be studied by NMR by significantly reducing the line widths and improving the signal-to-noise.[1−5] However, signal overlap is still a major problem for determining the three-dimensional structures of larger proteins (>30 kDa) by NMR.

In the past, paramagnetic lanthanides and lanthanide complexes have been used for resolving resonance overlap for nuclear spins within the coordination sphere of a lanthanide ion due to the lanthanide-induced shifts (LIS).[6−9] Most of these shift reagents were designed for small molecules in organic solvents.[7] Some applications have been reported for utilizing Ln(III) salts in aqueous solution as shift/relaxation agents[10−13] and surface probes[14−16] for biological macromolecules. However, these studies have been mostly confined to smaller molecules, such as nucleotides,[17] or to metal binding peptides/proteins where the lanthanide interacts with a natural[18,19] or chemically introduced[20] metal binding site. Moreover, the use of lanthanides in aqueous solution has been limited due to the formation of insoluble lanthanide hydroxides. Here we introduce a general approach for resolving signal overlap in the NMR spectra of proteins by the differential shifts induced upon the addition of a stable paramagnetic lanthanide complex. The approach should greatly facilitate the structure determination of proteins with molecular weights higher than 30 kDa.

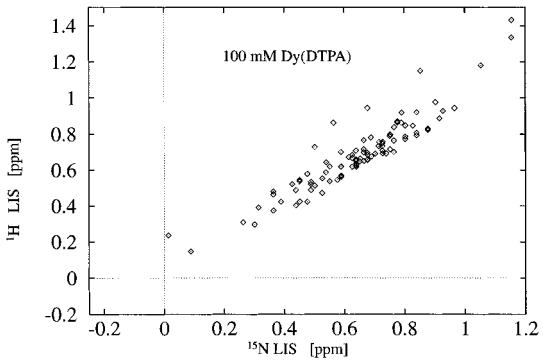

Figure 1. Lanthanide-induced shifts (LIS) for the ^{1}H and ^{15}N amide resonances of FKBP upon the addition of 100 mM Dy(DTPA). The data were obtained from water flip back sensitivity enhanced ^{1}H,^{15}N-HSQC experiments[25] recorded at 303 K on 1 mM solutions of ^{15}N-labeled FKBP in 50 mM sodium phosphate and 100 mM sodium chloride buffer (H_2O:D_2O, 9:1) at pH 6.5.

Since the application of aqueous solutions of $LnCl_3$ is limited due to the precipitation of Ln(III) hydroxides at pH >6.5, we investigated lanthanide complexes involving ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetate (EDTA) and diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetate (DTPA). These negatively charged, multidentate chelators are known to form soluble, highly stable, chemically inert 1:1 complexes.[7,17] For all lanthanides tested, the Ln(EDTA) complexes were found to be partially dissociated in the NMR buffer, leading to precipitation of lanthanide hydroxides, and were therefore unsuitable. In contrast, complexes with DTPA[21,22] proved to be satisfactory with the NMR samples being stable for months.

To evaluate the optimum choice of lanthanide that would induce the largest chemical shift changes without increasing the relaxation rates, dysprosium (Dy), europium (Eu), and praseodynium (Pr) were examined. Dy is expected to induce the largest LIS but may cause unfavorable relaxation, whereas Eu and Pr are expected to induce smaller shifts but should have less relaxation effects based on the shorter electronic relaxation times and smaller magnetic moments.[23] For each of these lanthanides, the LIS was examined upon the addition of 0−100 mM Ln(DTPA) (Ln = Eu, Pr, Dy) to a 1 mM solution of ^{15}N-labeled FKBP. Large *differential* shifts were observed for the individual amides of the protein upon the addition of the Dy(DTPA) complex (Figure 1). The average LIS at 100 mM Ln(DTPA) were found to be −0.033/−0.025, 0.109/0.119, and 0.643/0.683 ppm (^{15}N/^{1}H) for Eu, Pr, and Dy, respectively (see Supporting Information for data on Eu and Pr). The relative LIS correlate well with the pseudocontact shift constants C_D of the corresponding lanthanide ions.[23]

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(23) The main LIS contribution for ligands in the second coordination sphere of a lanthanide is the dipolar (pseudocontact) shift that results from a through space interaction between the electron and nuclear magnetic dipoles. The dipolar shift is characterized by a coupling constant C_D and is proportional to $1/r^3$. Therefore, the LIS has a larger effective radius than the lanthanide-induced relaxation (LIR) that scales as $1/r^6$. The LIR depends on the longitudinal electronic relaxation time T_{1e} and the effective magnetic moment μ_{eff} of the lanthanide ion. From the known shift and relaxation constants for the various lanthanides, the LIS and LIR effects can be estimated. For Dy, Eu, and Pr, $C_D = -100$, 4, and -11; $T_{1e} = 2.99$, 0.09, and 0.57×10^{-13} s, and $\mu_{eff} = 10.6$, 3.5, and 3.6 μ_B.[7]

(24) Interestingly, the LIS for the C^α cross peak of Q^{65} is large and in the opposite direction compared to the average shift (Figure 2a,b). This can be explained by the dependence of the dipolar LIS on the spherical angle θ between a vector between the lanthanide and the nuclear spin and the principal magnetic susceptibility axis of the lanthanide center, which is proportional to $(3 \cos^2 \theta - 1)$ and thus may assume negative values.

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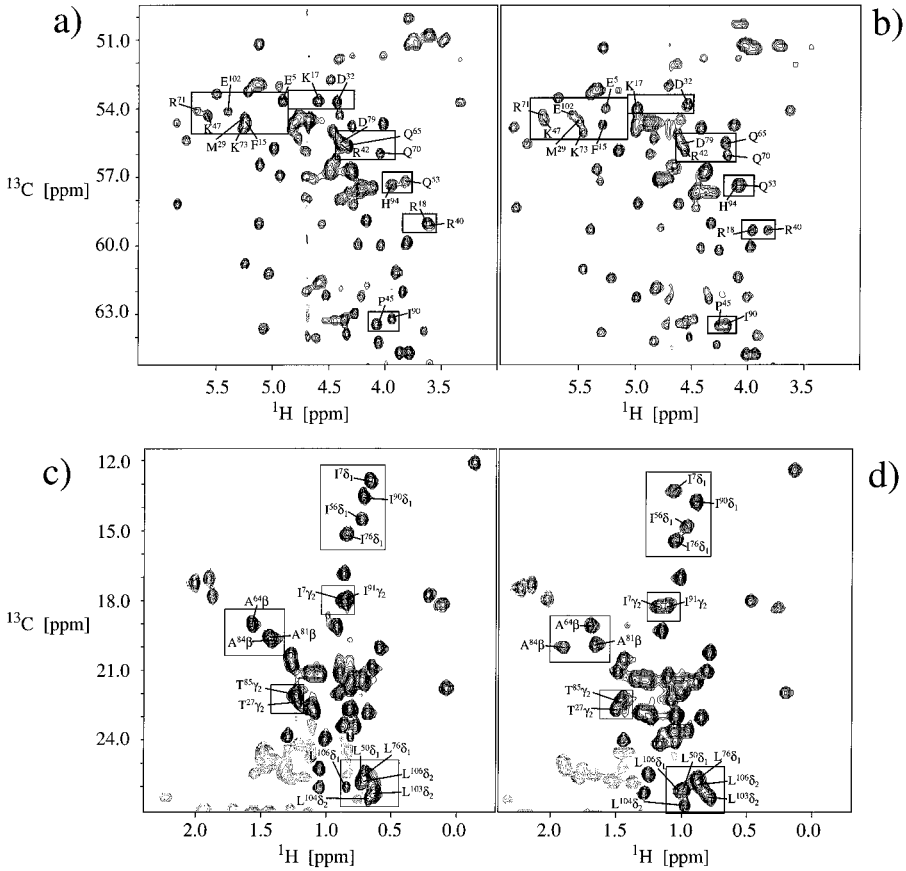

Figure 2. ^{1}H,^{13}C-HSQC spectra from sensitivity enhanced constant-time experiments with gradient coherence selection[26] recorded on uniformly ^{13}C,^{15}N-labeled FKBP with and without 30 mM Dy(DTPA) in the same buffer as described in Figure 1. Cross peaks where differential LIS resolves spectral overlap are indicated in boxes. The H$^{\alpha}$,C$^{\alpha}$ region of the spectrum (a) without and (b) with Dy(DTPA) and the methyl region (c) without and (d) with Dy(DTPA) are shown. The chemical shift assignments for FKBP were previously reported.[27,28] The assignments at 30 mM Dy(DTPA) were obtained by following the chemical shifts in the HSQC spectra during the titration with Dy(DTPA) and confirmed by three-dimensional HCCH-TOCSY and H(CCO)NH-TOCSY experiments at the final concentration of the lanthanide complex.

Dy(DTPA) induces the largest chemical shift changes but did not appear to cause unfavorable relaxation effects as evidenced by the lack of any change in the ^{1}H/^{13}C line widths of the protein upon the addition of Dy(DTPA) (Figure 2). In addition, the ^{15}N T_1 values were largely unaffected by the addition of Dy(DTPA) to the buffer, while the average ^{15}N T_2 relaxation times decreased by 4 and 7% in the presence of 30 and 50 mM Dy(DTPA), respectively (Supporting Information). This rather small effect may be a result of exchange line broadening due to the weak binding of Dy(DTPA) rather than a relaxation rate enhancement induced by the lanthanide.

The utility of Dy(DTPA) for resolving signal overlap is demonstrated in Figure 2, which depicts constant-time ^{1}H,^{13}C-HSQC spectra recorded on a 1 mM solution of uniformly ^{13}C/^{15}N-labeled FKBP with and without 30 mM Dy(DTPA). As expected from the negative charge of the Dy(DTPA) complex, large differential LIS effects are found for spins that are in the vicinity of positively charged arginine and lysine side chains in the three-dimensional structure of FKBP, suggesting a predominant interaction with these residues. For example, the C$^{\alpha}$ signals of K^{17}, R^{18}, K^{47}, Q^{53}, Q^{65}, and K^{73} experience large shifts compared to the neighboring cross peaks (Figure 2a,b).[24] As a result, the chemical shift degeneracy between F^{15}/K^{73}, Q^{65}/R^{42}, and R^{18}/R^{40} is removed (Figure 2a,b). On the other hand, the C$^{\alpha}$ cross peaks of R^{71}/K^{47}, H^{94}/Q^{53}, and P^{45}/I^{90}, which are better resolved in the spectrum without Dy(DTPA), are partially overlapped upon addition of the shift reagent (Figure 2a,b). However, by using the combined information from both spectra, the resonance overlap is resolved. In the methyl region of the spectrum (Figure 2c,d), the signal of I$^{7}\delta_1$ experiences a significantly larger LIS than the neighboring cross peaks of I$^{56}\delta_1$, I$^{76}\delta_1$, and I$^{90}\delta_1$. Furthermore, from the differential lanthanide-induced shifts, the overlap between I$^{7}\gamma_2$/I$^{91}\gamma_2$, A$^{81}\beta$/A$^{84}\beta$, T$^{85}\gamma_2$/T$^{27}\gamma_2$, L$^{103}\delta_2$/L$^{104}\delta_2$, and L$^{50}\delta_1$/L$^{106}\delta_2$ is eliminated upon the addition of the lanthanide complex (Figure 2c,d). This type of spectral overlap between side chain signals of the same amino acid type is the major problem in assigning side-chain chemical shifts and extracting NOEs for larger proteins and cannot be resolved even by four-dimensional NMR experiments.

In summary, we have shown that a Dy(DTPA) complex can induce large differential chemical shifts which can be useful for resolving spectral overlap in the NMR spectra of proteins. This approach should greatly facilitate the assignment of the side-chain signals and NOE data and thus extend the molecular weight range of proteins whose structures can be determined by NMR.

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Supporting Information Available: LIS for the ^{1}H and ^{15}N amide resonances of FKBP upon the addition of 100 mM Eu(DTPA) and Pr(DTPA), and T_1 and T_2 ^{15}N relaxation data of ^{15}N-labeled FKBP measured at Dy(DTPA) concentrations of 0, 30, and 50 mM (5 pages). See any current masthead page for ordering and Internet access instructions.

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