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Direct Carbon Detection in Paramagnetic Metalloproteins To Further Exploit Pseudocontact Shift Restraints

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In paramagnetic metal complexes, Curie relaxation broadens the NMR lines, even beyond detection, depending on the inverse sixth power of the distance, r, between the resonating nucleus and the metal ion, on the molecular tumbling rate τ_R, on the square of the external magnetic field, B_0, on the square of the magnetogyric ratio of the resonating nucleus, γ_I, and on the fourth power of the effective electron magnetic moment μ_{eff} (eq 1).[1,2]

$$R_{2M} = \frac{1}{5}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_I^2 B_0^2 \mu_{eff}^4}{(3kT)^2 r^6}\left[4\tau_R + \frac{3\tau_R}{1+\omega_I^2 \tau_R^2}\right] \qquad (1)$$

For metalloproteins at high magnetic field, the line broadening is thus particularly severe, especially for protons (large γ_I) and for metal ions with large μ_{eff} (e.g., lanthanides such as Tb^{3+} or Dy^{3+}, where Curie relaxation is the dominant mechanism[2]). In such cases, line broadening is severe already at distances of about 15 Å from the metal ion in a globular protein of 75 aa at 600 MHz.[3] On the other hand, paramagnetic metal ions yield precious structural restraints that are not available for their diamagnetic analogues.[4,5] An interesting way to reduce the adverse effect of line broadening is that of directly detecting nuclei with a smaller γ_I than proton. Recently, direct heteronuclear detection has been pursued in our and other labs,[6−14] after the pioneering work of J. Markley.[15] For example, ^{13}C experiences a line broadening about 16 times smaller than ^1H, and therefore the observability of signals is expected to increase sensibly. The lower sensitivity of ^{13}C (and ^{15}N) nuclei can be compensated at least in part by high field instruments, cryoprobes, special probes, and taylored experiments.[16] As a result, purely heteronuclear NMR represents a new challenge for both large proteins and paramagnetic proteins.

Paramagnetic species can be obtained from diamagnetic calcium binding proteins by replacing the Ca^{2+} ions with a paramagnetic Ln^{3+} ion. The difference in chemical shifts between the paramagnetic form and the Ca^{2+} or La^{3+} derivatives provides a direct measurement of pseudocontact shifts (PCS). PCS can be used as restraints as such and can be used to obtain the magnetic anisotropy tensor which, in turn, is proportional to the alignment tensor in a high enough external magnetic field.[17] The alignment tensor is responsible for the detection of residual dipolar couplings (RDC).[5,18,19] Recently it has been shown that proteins constituted by two independent domains such as calmodulin can be conveniently investigated if a lanthanide is placed on one domain to induce PCS and RDC on the other domain, provided that the alignment tensor is obtained from the PCS of the former domain.[20]

Within this frame, we want to make available to the scientific community the direct detection of ^{13}C nuclei in paramagnetic biomolecules, and we show how different and tailored experiments allow us to significantly extend the signal detection close to the metal ion. This is a strategic advancement for those exploiting paramagnetism-based constraints for structure calculations.

The Tb^{3+} substituted form of the human oncomodulin (OM hereafter) for which the solution structure has been solved in our laboratory (Babini et al., in preparation) is used. OM is a 12 kDa protein (109 aa) with two Ca^{2+} binding sites. The C-terminus Ca^{2+} was replaced by Tb^{3+} to an extent of 80% (to avoid the formation of the Tb_2OM species) by simple titration of Ca_2OM with $TbCl_3$.

In Figure 1, the direct $^{13}C-^{13}C$ multiple quantum correlation experiment COCAMQ[9,11] and $^1H-^{15}N$ HSQC spectra at 283 K are reported for Ca_2OM and CaTbOM. It appears that many backbone signals (58 out of 107 (two of the 109 aa are prolines)) are lost in the $^1H-^{15}N$ HSQC spectrum because of ^1H Curie relaxation, while only 37 out of 109 signals are lost in the COCAMQ spectrum.

Taylored $^{13}C-^{13}C$ NOESY[11,13] spectra were then recorded, with relatively short mixing times (360 ms), a large number of scans (512), and a minimal number of t_1 (256) increments (Figure 2).

The $^{13}C-^{13}C$ NOESY is interesting because during the mixing time, the longest time in the sequence, cross-peaks are attenuated by longitudinal relaxation, which is much slower than transverse relaxation, especially if Curie relaxation is dominant.

With respect to the COCAMQ, 26 additional cross-peaks are observed in the NOESY spectrum (some of these are enclosed in boxes), which leaves a total of only 11 out of 109 unobserved C′−Cα cross-peaks. NOESY is apparently the best sequence to detect the broadest signals, even if, in the most crowded regions, the COCAMQ provides both a better signal/noise ratio and resolution.

The assignment of ^{13}C nuclei is based on an iterative procedure[3] which starts from some easily assigned ^1H and ^{13}C signals experiencing PCS (10 signals in our case), then calculating the anisotropy of the magnetic susceptibility tensor, χ, and predicting the observed shifts for the other ^{13}C nuclei on the basis of the 3D structure. Under the present experimental conditions,[21] $^1H-^{15}N$ HSQC cross-peaks are detectable only beyond ca. 16 Å from the metal, $^{13}C-^{13}C$ COCAMQ cross-peaks beyond ca. 11 Å, and $^{13}C-^{13}C$ NOESY beyond about 8 Å.

The 1D ^{13}C spectrum reveals very strongly shifted and very broad signals spread from 270 to −140 ppm. The assignment of these peaks is difficult in the absence of connectivities, also because their shifts are strongly dependent on small variations in geometry around the metal ion. However, some of them can be assigned from their predicted PCS values,[22] moving the detectability threshold down to 5.5 Å (Figure 3).[23]

The final magnetic susceptibility tensor anisotropy parameters agree, within their indetermination and after scaling for the different temperatures,[24] with those already obtained (Babini et al., in preparation): $\Delta\chi_{ax} = (50 \pm 3)\ 10^{-32}$ m^3 and $\Delta\chi_{rh} = (11 \pm 1)\ 10^{-32}$

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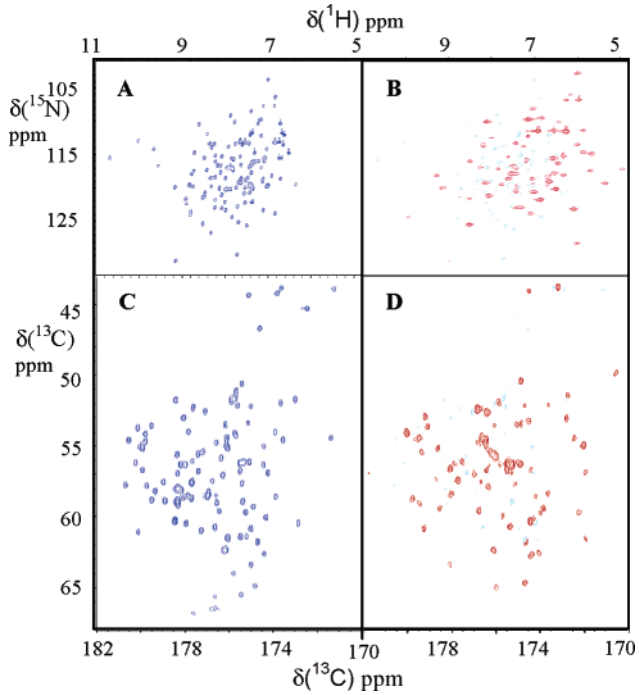

Figure 1. (A) $^{1}H-^{15}N$ HSQC spectrum of Ca_2OM (blue). (B) $^{1}H-^{15}N$ HSQC spectrum of CaTbOM.[21] (C) $^{13}C-^{13}C$ COCAMQ spectrum of Ca_2-OM (blue). (D) $^{13}C-^{13}C$ COCAMQ spectrum of CaTbOM.[21] In B and D, residual diamagnetic resonances are light blue.

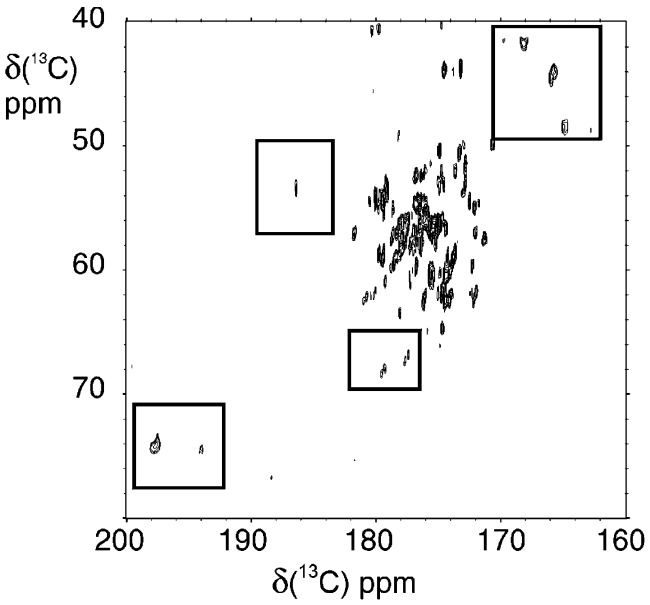

Figure 2. C′−Cα region of $^{13}C-^{13}C$ NOESY spectrum of CaTbOM[21] (0.5 s recycle delay). Signals in boxes are observed on the same spectrum but processed with different parameters.

m^3; the *z*-axis of the tensor points from the metal in the direction of C′ 94, and the *x*-axis points in the direction of C′ 92.

In conclusion, ^{13}C direct detection allows one to "fill the hole" of paramagnetic information around the metal center. The complementary use of $^{13}C-^{13}C$ COCAMQ, NOESY, and 1D experiments reveals signals as close as 5.5 Å from Tb^{3+}. Therefore, this strategy provides assignments and restraints for nuclei in a region where protons are undetectable. These restraints are precious for structure determination or refinement. The results are particularly significant for (i) the investigation of large proteins, where τ_R makes Curie relaxation a quite effective source of broadening and (ii) the

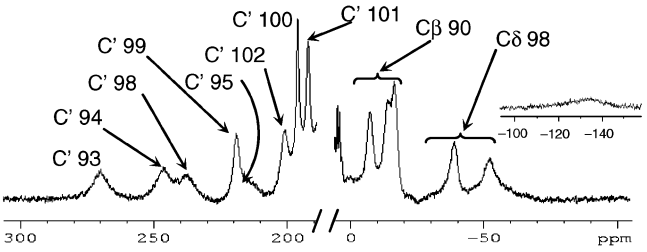

Figure 3. Some assigned peaks in the 1D ^{13}C spectrum of CaTbOM:[21] ambiguities are indicated with brackets.

assignment of nuclei in small domains containing the paramagnetic center and belonging to a polydomain protein.

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Supporting Information Available: Assigned resonances of ^{1}H and ^{15}N in $^{1}H-^{15}N$ HSQC and of backbone ^{13}C of Ca_2OM and CaTbOM. This material is available free of charge via the Internet at http://pubs.acs.org.

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(21) Spectra were acquired at 283 K, 700 MHz of proton Larmor frequency, and 175 MHz of carbon Larmor frequency; protein concentration was 5 mM in a 0.1 M of NaCl in H_2O solution (with 10% D_2O), pH = 6.
(22) The 1D ^{13}C resonances are assigned though comparison with peaks calculated over a family of 20 structures with 0.51 RMSD from the mean.
(23) In the case of lanthanides, the deviations observed between calculated and observed PCS of heteronuclei are small. The main variations are due to an incorrect choice of the diamagnetic reference (ref 7).
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