Locating the Metal Ion in Calcium-Binding Proteins by Using Cerium(III) as a Probe

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The detection and assignment of NMR spectroscopic signals of carbon atoms from carbonyl and carboxylate groups in the loop hosting the Ce^{III} ion was performed for the cerium-substituted calcium-binding protein calbindin D_{9k}. This provided a tool to characterize in solution the first coordination sphere of the metal ion. Due to the well-documented possibility of replacing calcium with metal ions of the Ln^{III} series, this approach turns out to be extremely efficient for characterizing in solution the coordination of calcium ions in proteins, independently of the availability of X-ray crystal structures. The present approach completes the structural characterization of lanthanide-substituted calcium-binding proteins, for which the role of long-range constraints arising from hyperfine interaction and self-orientation has already been assessed.

KEYWORDS:

calcium-binding proteins · cerium · metalloproteins · NMR spectroscopy · structure elucidation

Introduction

A major problem in the solution structure calculation by NMR spectroscopy of metalloproteins in general and of calcium-binding proteins in particular is that of characterizing the metal-binding site and detecting the donor atoms.[1] In this work, the determination of the metal ligands in a cerium-substituted calcium-binding protein is accomplished by further extending the NMR spectroscopic approach to paramagnetic metalloproteins. The protein studied in this work is calbindin D_{9k}, which is a 75-amino-acid protein with two bound calcium ions.[2] It is possible to selectively replace[3, 4] Ca^{II} with Ce^{III} in site II (the archetypal EF C-terminal site) and use pseudocontact shifts (δ^{pc})[5, 6] and partial orientation,[7, 8] both induced by the paramagnetic ion, to refine the solution structure of the protein.[9–13] The pseudocontact-shift-based approach can locate the metal ion in the protein frame, but does not help in identifying the donor atoms. Signals of protons close to the metal ions are broadened, sometimes beyond detection, by the presence of the paramagnetic center.[14] A possibility to considerably extend the amount of NMR spectroscopic information around the paramagnetic site is offered by the availability of ^{15}N- and ^{13}C-labeled samples. The paramagnetic contribution to relaxation is scaled by a factor $(\gamma_H/\gamma_I)^2$ ($I = {}^{15}N$, ^{13}C), which favors direct ^{15}N and ^{13}C detection over ^{1}H detection for the residues in the immediate vicinity of the paramagnetic center.[15]

Here we report the identification of all the C=O groups coordinating the metal ion in the Ce^{III}-substituted protein through detection and assignment of their ^{13}C signals by using one-dimensional (1D) ^{13}C NMR and ^{13}C–^{13}C correlated spectroscopy (COSY) experiments. The identified signals were correlated to the already assigned ^{1}H signals by a combination of double- and triple-resonance experiments.

Results

Since we are interested in characterizing the first coordination sphere of the metal ion, we aim at a complete assignment of the protein nuclei and in particular at identifying signals of nuclei whose hyperfine parameters indicate strong interactions with the metal ion. To achieve this goal, the already available assignment of ^{1}H and ^{15}N signals[9] was extended to ^{13}C signals of backbone and side chains by means of triple-resonance experiments. HNCA, HNCO, HCACO, and (H)CCH-TOCSY data were therefore collected with 600- or 700-MHz spectrometers. Because of broadening effects induced by the paramagnetic center, no sequential backbone assignment is available in the region encompassing residues 55–62 (part of site-II binding loop). Tailored versions of ^{1}H–^{13}C HMQC,[16] ^{1}H–^{15}N HMQC,[17] and (H)CCH-TOCSY experiments[18] were used to identify signals in the proximity of Ce^{III}. Paramagnetic broadening is proportional to the square of the nuclear gyromagnetic ratio γ. The relative values of γ for ^{1}H, ^{13}C, and ^{15}N nuclei are 1:0.25:−0.1, respectively, and thus the relative contribution to overall relaxation arising from the hyperfine interaction is 1:0.0625:0.01, respectively. As a consequence, the loss of information for ^{13}C and ^{15}N assignments in the proximity of the

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paramagnetic center cannot be due to excessive broadening of the heteronuclear signal, but, in most cases, to the quenching of coherence transfer in inverse-detected experiments, due to ^1H T_2 relaxation. Therefore, to identify ^{13}C signals that escaped detection through inverse detection techniques, we used the direct detection of ^{13}C signals. This allowed us to identify those signals that were most affected by hyperfine relaxation, that is, carbon signals at shorter distances to the Ce^{III} ion.

Because sensitivity depends on $\gamma^{3/2}$ of the observed nucleus and on the γ value of the excited one, direct excitation and detection of ^{13}C nuclei is obviously unfavorable with respect to polarization transfer and indirect detection through proton nuclei for any standard application.[19] However, relaxation rates depend on γ^2 of the observed nucleus. Therefore, the loss in sensitivity related to the direct detection of ^{13}C should be more than compensated by a smaller contribution to relaxation due to hyperfine interaction. In the latter case, ^{13}C relaxation is operative during evolution times, while in experiments based on inverse detection, signal strength is lost during the coherence transfer from ^1H to ^{13}C and vice versa, mostly because of ^1H relaxation. To connect the observed fast-relaxing carbon signals to the already identified spin systems, a proton-decoupled $^{13}C-^{13}C$ COSY experiment was performed. Because of the relatively high values of the $^{13}C-^{13}C$ coupling constants and the limited effects of hyperfine relaxation on ^{13}C signals, COSY connectivities among ^{13}C spins were successfully detected. The quenching of coherence transfer induced by the paramagnetic center has been used as a marker to qualitatively assess relaxation properties of those ^{13}C carbon signals for which a precise estimate of their T_1 values was not possible in one-dimensional ^{13}C experiments.

Use of ^{13}C direct detection

Nuclear line broadening in paramagnetic systems arises from different electron–nucleus coupling mechanisms, that is, dipolar, contact, and Curie contributions.[20] Contact contributions are difficult to estimate a priori, and can be nonnegligible for ^{13}C nuclei of coordinated C=O groups. The relative weights of dipolar and Curie contributions can be obtained from reasonable estimates of τ_s (in the range of $1-6\times10^{-13}$ s) and τ_r (ca. 4×10^{-9} s), respectively.[20–22] Dipolar line broadening is essentially field-independent, whereas Curie broadening increases with the square of the field strength. The signal linewidth (in ppm), which should be minimal for maximal spectral resolution, is thus expected to decrease and then increase with increasing field. Among lanthanide ions, Ce^{III} is the one (except Eu^{III}, which has a diamagnetic ground state) where the minimum is centered at relatively high field strength and extends over a wide range of field strength values, at proton Larmor frequencies between 200 and 600 MHz (Figure 1). Therefore, ^{13}C direct detection experiments were collected using 400- and 600-MHz spectrometers (100 and 150 MHz ^{13}C Larmor frequency, respectively), with probes optimized for direct detection.

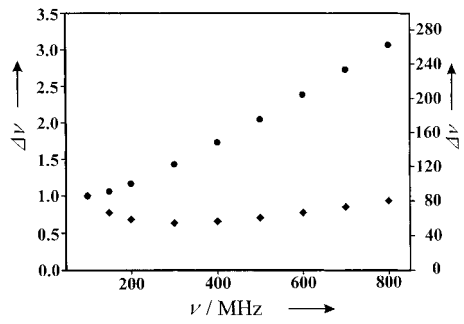

Figure 1. *Relative variation of ^{13}C-nuclear linewidth in ppm, due to paramagnetic effects of cerium(III) (◆, left-hand scale) and dysprosium(III) (●, right-hand scale) ions. The linewidths are normalized to the cerium(III) linewidth at 2.35 T (100 MHz in proton Larmor frequency). The shallower field dependence in the case of cerium(III) allows the use of relatively high magnetic field strengths.*

The 1D ^{13}C NMR spectrum of CaCeCb (Cb = calbindin D_{9k}) shows four resolved peaks (labeled A–D in Figure 2A) that appear to be hyperfine-shifted. To identify the occurrence of peaks not shifted out of the C=O envelope of diamagnetic resonances, but still affected by hyperfine interaction, a ^{13}C-detected weft experiment[23] was performed. The sequence ($180^\circ-\tau-90^\circ$) was used with the aim to tune the interpulse delay τ to minimize the intensity of diamagnetic resonances. The resulting ^{13}C NMR spectrum is shown in Figure 2B: Three peaks affected by hyperfine interaction can be detected in the range between $\delta=170$ and $\delta=185$ (signals E–G). Chemical shift

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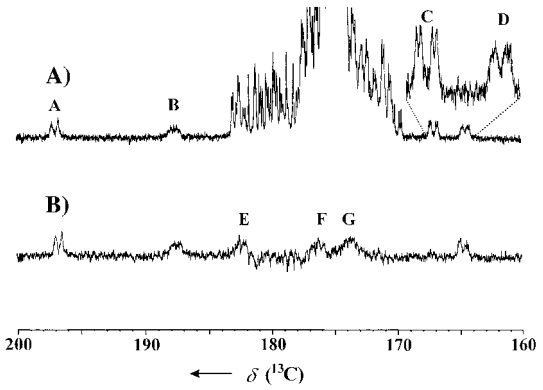
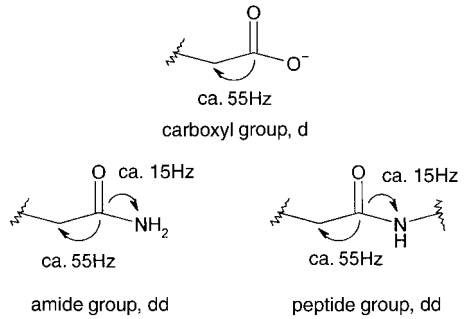


Figure 2. *A) Standard 150-MHz ^{13}C NMR experiment performed upon direct detection of ^{13}C. Only the region involving signals of quaternary carbon atoms is shown. The region involving signals C and D has also been expanded. This allows to better analyse the fine structure of signals C and D. B) ^{13}C-detected weft experiment; data were collected by using 1.5 and 0.5 s of recycle and interpulse delays, respectively. Signals are labeled as in Table 1.*

values of signals A – G are summarized in Table 1 (column 2). The T_1 values could be determined, by nonselective inversion recovery experiments, for the isolated signals A – D and they were found to be in the range of 100 – 250 ms (see Table 1, column 3). The T_1 values of signals E – G can be set smaller than 700 ms from a ^{13}C-detected weft experiment performed with a 500-ms interpulse delay (Figure 2 B). Signals E – G are observed with weak, positive intensity indicating a T_{null} time shorter than 500 ms. This allows to evaluate an upper limit of 700 ms for their T_1 values. Signals A – G show a significant temperature dependence.

According to Bleaney,[24] the temperature dependence of lanthanide-induced pseudocontact shifts should be of the type T^{-2}. Contributions arising from terms displaying T^{-3} temperature dependence should vary in the lanthanide series, but not exceed 10 – 20 %.[25] The intercepts at infinite temperature of the $1/T^2$ plot of the observed temperature dependence, experimentally obtained in the range 300 – 285 K, are listed in Table 1 (column 4). Despite the approximation of this approach and possible contact contributions, for which the temperature dependence is of the T^{-1} type,[26] the intercept of the plot indicates that peaks A – G belong to ^{13}C atoms of carbonyl and/or carboxyl moieties.

Some of the A – G signals show a fine structure that provides hints for their assignment. Signal A is a doublet (d) with a coupling constant of ca. 57 Hz, while C and D are two doublets of doublets (dd) with coupling constants of about 56 and 16 Hz, respectively. According to Scheme 1, signal A is assigned to a side-chain carboxyl C=O moiety, showing only the scalar coupling with the strongly scalar-coupled carbon atom of the chain. Signals C and D can correspond either to a side-chain amide or a peptide C=O group because of the additional observed coupling with a nitrogen spin.



Scheme 1. *Scalar couplings and multiplicity for ^{13}C=O moieties of amide, peptide backbone, and carboxyl groups.*

To obtain scalar correlations between C=O and aliphatic ^{13}C signals, a two-dimensional (2D) $^{13}C - ^{13}C$ COSY spectrum was recorded. An expansion of the region of interest is shown in Figure 3 A. All signals A – G show scalar correlations with carbon signals lying in the aliphatic region. Connectivities involving signals A – G are very weak, but they could be unambiguously detected through optimization of the number of data points to be used for the Fourier transformation in both dimensions.[27] The intensity of the cross peaks with respect to diamagnetic homonuclear correlations involving carbon resonances confirm that hyperfine-relaxation mechanisms are strongly operative. In the case of signals E and G, the cross peaks observed with their aliphatic carbon atoms are as weak as those observed from signals A – D, for which T_1 values are in the range of 100 – 250 ms. Therefore, although no quantitative estimate of T_1 for signals E and G was possible and only an upper limit of 700 ms was estimated from ^{13}C weft experiments (see Table 1), we can now,

Table 1. *^{13}C signals identified in the proximity of the metal center in calcium-binding site II.[a]*

Peak	δ (Ce)	T_1 [ms]	Intercept (δ) of T^{-2} plot	Assignment	δ (Ca)	δ (La)	δ^{hyp} (Ce-La)	δ^{pc} calc	δ^{con} calc	Coordination
A	196.6 (d)	232	178.5	Asp 54-COO^-	176.7	176.3	+20.3	1.2 to 3.9	19.1 to 16.4	bound
B	187.0	157	194.0	Glu 65-COO^-	185.0	188.0	−1.0	−9.3 to 1.0	–	bound
C	167.0 (dd)	251	–	Val 61-CO	173.3	171.9	−4.9	−3.2 to −2.9	−1.7 to −2	not bound
D	164.4 (dd)	126	177.4	Asn 56-$CONH_2$	175.6	177.2	−12.8	−87.0 to 11.5	–	bound
E	182.5	$<700^{[b]}$	171.2	Glu 60-CO	173.5	174.5	+8.0	−4.3 to −1.7	12.3 to 9.7	bound
F	176.3	$<700^{[b]}$	–	Gly 59-CO	170.5	171.0	+5.2	4.7 to 5.2	0.5 to 0	not bound
G	172.5	$<700^{[b]}$	189.6	Asp 58-COO^-	180.0	183.0	−10.5	−14.4 to 25.3	–	bound

[a] Characterization and assignments are discussed in the text. [b] Transverse relaxation of signals E and G appears comparable to that of signals A – D from a qualitative inspection of $^{13}C - ^{13}C$ COSY spectra. – hyp = hyperfine, pc = pseudocontact, con = contact.

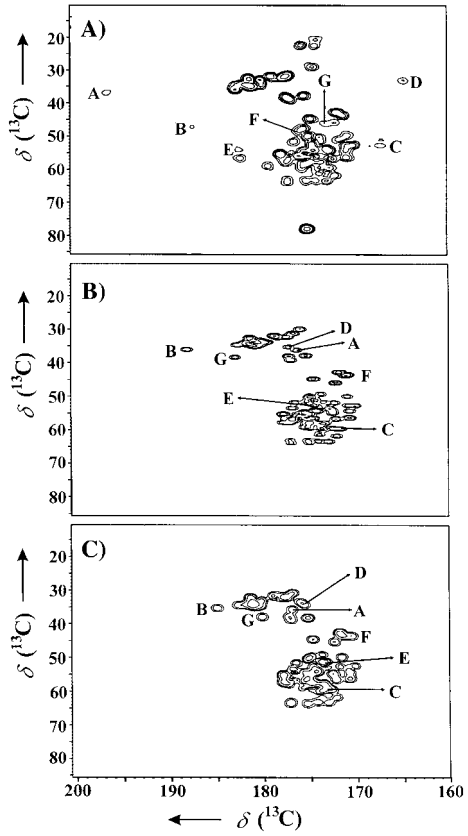


Figure 3. *$^{13}C-^{13}C$ COSY experiment; data were collected at 150 MHz (14.1 T) and 300 K for the derivatives CaCeCb (A), CaLaCb (B), and CaCaCb (C). Only the spectral region involving connectivities between quaternary carbon atom resonances (F2) and carbon atom resonances of the aliphatic region (F1) is shown. Cross peaks arising from previously identified quaternary carbon atom signals (A – G) for the three samples are indicated according to the labeling of quaternary carbon atom resonances. All experiments were performed over a spectral window of δ = 220 in both dimensions, using a 1024 × 256 data point matrix. 640 transients were acquired using a relaxation delay of 500 ms for CaCeCb and of 800 ms for both CaLaCb and CaCaCb, for a total experimental time of 26 and 30 h, respectively. Data were multiplied by a squared sine-bell weighting function in both dimensions before Fourier transformation.*

on the basis of their T_2 relaxation properties, unambiguously include signals E and G with the signals from those carbon atoms that are about as close to Ce^{III} as the ones that give rise to signals A – D.

Assignment strategy

A three-step approach was used to achieve the sequence-specific assignment of the signals of the atoms close to the paramagnetic Ce^{III} ion that had been identified through the direct ^{13}C experiments described in the previous section. The first step represents the unambiguous assignment of backbone ^{13}C and ^{15}N signals, starting from the available ^{1}H signal assignment. This was achieved by extending the backbone assignment already available for ^{1}H and ^{15}N signals[9] to $^{13}C^{\alpha}$ and ^{13}CO through HMQC (both $^{1}H-^{13}C$ and $^{1}H-^{15}N$), HNCA, and HNCO experiments. Additional ^{15}N signals of residues in the proximity of the metal center, previously unidentified, could be assigned. The second step was the assignment of ^{13}C signals of the side chains. This was largely achieved with (H)CCH-TOCSY, which was performed in the standard version[28] and also in a tailored, two-dimensional version optimized to detect fast-relaxing spin systems.[18] The final step consists of identifying coupling patterns between the last CH_2 moiety of the side chain and the coordinating side-chain C=O group. The previously described carbon-detected experiments were crucial for this step. As our aim is to identify metal-bound residues, we will first focus on the assignment of the seven ^{13}C signals affected by hyperfine contributions. Additional assignments, involving residues to which signals A – G belong, will also be discussed.

Signal A ($\delta = 196.6$) shows a well-defined cross peak in the $^{13}C-^{13}C$ COSY spectrum (Figure 3 A) with a carbon signal at $\delta = 36.6$. The latter is connected, in the $^{1}H-^{13}C$ HMQC spectrum, with a proton signal at $\delta = -1.2$ that is unambiguously assigned[9] to Asp 54-H^{β} (Figure 4). Signal A is therefore identified as the C=O moiety of the carboxyl group of Asp 54. This is consistent with the doublet fine structure of signal A (Table 1 and Scheme 1). Asp 54-C^{α} was assigned by $^{1}H-^{13}C$ HMQC, as the H^{α} proton is well shifted at $\delta = -7.5$ (Figure 4).

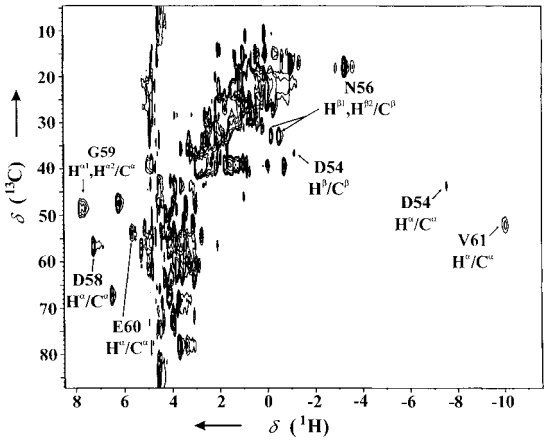


Figure 4. *$^{1}H-^{13}C$ HMQC experiment, performed at a proton Larmor frequency of 600 MHz and a temperature of 300 K. To identify connectivities involving fast-relaxing resonances, a delay of 1.67 ms (half of the delay for diamagnetic systems) was used for the double-quantum polarization transfer to compensate for transverse proton relaxation. A 2048 × 154 data point matrix was acquired, with 128 scans for each transient, by using a recycle delay of 250 ms. Signals that were found to be relevant to the assignment of metal-bound residues are indicated with arrows, together with their assignment.*

Signal B ($\delta = 187.0$) gives a $^{13}C-^{13}C$ COSY peak at $\delta = 47.1$ (Figure 3 A). The latter signal gives peaks in the $^{1}H-^{13}C$ HMQC and in the (H)CCH-TOCSY experiments (Figure 5) with a signal at $\delta = 10.68$, formerly assigned[9] to Glu 65-H^{γ}. This allows the identification of signal B as the side-chain C=O group of Glu 65.

Signal C ($\delta = 167.0$) shows a correlation in the $^{13}C-^{13}C$ COSY spectrum with a signal at $\delta = 51.7$ (Figure 3 A), which is assigned to Val 61-C^{α} on the basis of $^{1}H-^{13}C$ HMQC (Figure 4). The peculiar shift of Val 61-H^{α} (-10.28)[9] is such that the assignment is unambiguous. The whole C^{α}-C^{β}-C^{γ} coupling pattern is evident in the (H)CCH-TOCSY spectrum (Figure 5).

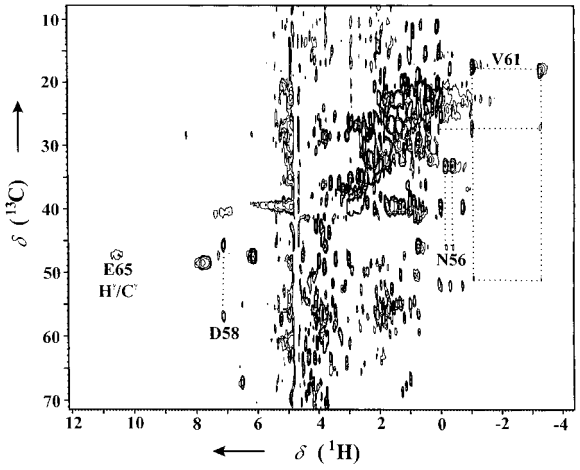


Figure 5. *700-MHz 2D (H)CCH-TOCSY experiment. The experiment was performed by using a spin-lock time and a relaxation delay of 2.9 and 280 ms, respectively. Polarization transfer delays for the ^{1}H-to-^{13}C INEPT, refocusing ^{13}C, and inverse-refocused INEPT were 700 μs (instead of 1.6 ms), 475 μs, and 500 μs (instead of 1.1 ms), respectively. A 1024 $\times$ 100 data point matrix was acquired, each transient consisting of 640 scans. Signals that were relevant to the assignment of metal-coordinated residues are labeled.*

Signal D ($\delta = 164.4$) is coupled in the ^{13}C – ^{13}C COSY spectrum with a carbon signal at $\delta = 33.2$ (Figure 3 A). The latter can be assigned to the C$^{\beta}$ atom of Asn 56 thanks to the peculiar shift of H$^{\beta}$ signals that can be easily recognized in a ^{1}H – ^{13}C HMQC experiment (Figure 4). The fine structure of signal D (a doublet of doublets) supports the assignment as the Asn 56 C=O side chain. The assignment of C$^{\alpha}$ was obtained on the basis of (H)CCH-TOCSY experiments, showing a weak connectivity between H$^{\beta}$ and C$^{\alpha}$ at $\delta = 46.5$ (Figure 5). The side-chain H^{δ}_{N} proton signal at $\delta = 2.78$ gives a ^{1}H – ^{15}N HMQC peak with a nitrogen signal, which is therefore assigned to N$^{\delta}$ (data not shown).

Signal E ($\delta = 182.5$) shows a connectivity with Val 61-NH in an HNCO experiment, and it is therefore assigned to the backbone C=O group of Glu 60. The C$^{\alpha}$ assignment of Glu 60 is obtained from HNCA. The ^{1}H – ^{3}C HMQC experiment (Figure 4) assigns the signal at $\delta = 5.6$ to the H$^{\alpha}$ proton.

Signal F ($\delta = 176.3$) shows, besides the ^{13}C – ^{13}C COSY connectivity with a carbon signal at $\delta = 48.8$ (Figure 3 A), a clear HCACO connectivity with a peak at $\delta = 7.77$ (data not shown). This correlation is confirmed from ^{1}H – ^{13}C HMQC (Figure 4). Therefore, signal F is assigned to Gly 59-C=O.

Signal G ($\delta = 172.5$) is connected in the ^{13}C – ^{13}C COSY spectrum with a peak at $\delta = 46.1$ (Figure 3 A). The latter is identified, from (H)CCH-TOCSY (Figure 5), as being part of the spin system of Asp 58. The H$^{\alpha}$ signal, found by sequential assignment at $\delta = 7.25$, is connected to two carbon signals at $\delta = 56.7$ and 46.1. The C$^{\alpha}$ signal was assigned to the resonance at $\delta = 56.7$ by analysis of ^{1}H – ^{13}C HMQC in the spectral region downfield with respect to the water signal (Figure 4). Therefore, the signal at $\delta = 46.1$ is assigned to C$^{\beta}$ and signal G to the Asp 58 carboxylate. A fast ^{1}H – ^{15}N HMQC experiment allowed also the assignment of the backbone nitrogen signal at $\delta = 122.0$.

The assignments of signals A – G are summarized in Table 1 (column 5). The overall assignment described above is summarized in Table 2.

Observed hyperfine shifts

To obtain the overall hyperfine contributions to the ^{13}C-investigated signals, the COO^{-} signals of Asp 54, Asp 58, and Glu 65, the $CONH_2$ signal of Asn 56, and the backbone C=O signals of Gly 59, Glu 60, and Val 61 were assigned also in the CaCaCb and in the CaLaCb derivative. Although the native form of the protein contains calcium, we decided to use the data obtained from the CaLaCb derivative as a diamagnetic reference for CaCeCb.[12] Indeed, the use of La^{III} as a blank eliminates the difference in charge between Ca^{II} and Ce^{III} and provides, in principle, a more reliable reference than the calcium form to calculate hyperfine contributions induced by the Ce^{III} ion.[1, 12, 29]

To assign signals of interest in both CaCaCb and CaLaCb, the same series of experiments performed to analyze the CaCeCb derivative was performed. Complete ^{1}H and ^{15}N NMR assigment was available for the CaCaCb protein,[30, 31] while an assignment of amide group ^{1}H and ^{15}N signals of CaLaCb was available from ^{1}H – ^{15}N HSQC experiments.[12] At variance with the case of the Ce^{III} derivative, in which fast proton relaxation limited the efficiency of proton-detected experiments, an HCACO experiment[32] turned out to be quite helpful.

Chemical shift values for Ca^{II} and La^{III} derivatives are reported in Table 1 (columns 6 and 7). The comparison of ^{13}C – ^{13}C COSY experiments performed on the CaCaCb and CaLaCb derivatives

Table 2. *Resonance assignment for nuclei in the proximity of the metal center in metal-binding site II for CaCeCb.[a]*

Residue	C=O$_{bb}$[b]	N	NH	C$^{\alpha}$	H$^{\alpha}$	C$^{\beta}$	H$^{\beta}$	C$^{\delta}$	H$^{\delta}$	H$^{\gamma}$	N$^{\gamma}$	C=O$_{sc}$[c]
Asp 54		115.1	6.33	43.7[d]	− 7.49	36.6[d]	− 1.20, 2.48[d]					**196.6**[d] (δ)
Asn 56			2.28	46.5[d]	2.54	33.2[d]	− 0.39, − 0.16			2.78, 7.53[d]	105.2[d]	**164.4**[d] (δ)
Asp 58	182.0[d]	122.0[d]	17.95	56.7[d]	7.25[d]	46.1[d]	3.81, 0.92					**172.5**[d] (δ)
Gly 59	176.3[d]	115.3[d]	20.84	48.8[d]	7.79, 7.77[d]							
Glu 60	**182.5**[d]		27.04	53.7[d]	5.63[d]		1.29, 3.03		2.12, 2.30			176.3[d] (γ)
Val 61	167.0[d]	121.6	7.35	51.7[d]	− 10.28	27.0[d]	− 0.32	18.1[d]	− 3.23, − 1.03			
Glu 65	176.2[d]	121.6	10.32	57.3[d]	5.29	27.9[d]	1.21, 3.06	47.1[d]	10.68			**187.0**[d] (γ)

[a] Only resonances of those residues that have been observed to have ^{13}C=O signals affected by hyperfine interactions are reported. Signals of the five metal-coordinated C=O groups are printed in boldface. [b] bb = backbone C=O, sc = side-chain C=O. [c] Carbon label (δ, γ) is indicated in brackets. [d] Assignments obtained in the present work.

with that performed on the CaCeCb derivative is shown in Figures 3 B and C. It is shown that, while the other features of the spectrum are only slightly changed when passing from Ca^{II} to La^{III}, at least two signals of metal-coordinated side chains (signals B and G) are strongly affected by the change of the metal ion.

Discussion

Identification of metal-coordinating residues

Seven signals of quaternary ^{13}C atoms were found to be affected by hyperfine interaction (either shift, relaxation, or both). Therefore, they are the most likely candidates to be involved in a direct bond with the Ce^{III} metal ion. As described in the previous section, these signals were assigned to three side-chain carboxyl groups (Asp 54, Asp 58, and Glu 65), one side-chain amide group (Asn 56), and three backbone C=O groups (Gly 59, Glu 60, Val 61).

For Asp 54-C^{γ} and Glu 65-C^{δ}, T_1 values were determined to be about 230 and 160 ms, respectively. Assuming 0.7 s^{-1} as a reasonable estimate for relaxation rates of quaternary ^{13}C resonances in the absence of hyperfine interaction,[33] hyperfine contributions from these resonances were 3.7 and 5.5 s^{-1}, respectively. Considering a correlation time in the range of 4 – 6 ns for CaCeCb and an electronic correlation time[20–22] for Ce^{III} in the range of $1 - 6 \times 10^{-13}$ s, the above values give metal-to-carbon distances in the range of 3.5 – 4.3 and 3.3 – 4.0 Å, respectively. These values have been calculated considering the contribution of the electron – nucleus dipole – dipole coupling[34] and the Curie spin relaxation,[35, 36] as the latter was found to be nonnegligible for ^{13}C relaxation. The above ranges are consistent with metal coordination of the C=O group. Although a quantitative estimate of Asp 58-C^{γ} T_1 is not available (see Table 1), the intensity in the $^{13}C - ^{13}C$ COSY experiment of its C^{β}-C^{γ} connectivity shows that Asp 58-C^{γ} has relaxation properties similar to those of Asp 54-C^{γ} and Glu 65-C^{δ}. Therefore, a range of 3.5 – 4.3 Å for the Asp 58-C^{γ} – Ce distance can safely be estimated. These short Ce–C distances are consistent with COO^- coordination (either in a monodentate or in a bidentate fashion) to the metal ion.[37, 38]

Similar considerations hold for Asn 56, for which a Ce–C^{γ} distance of 3.1 – 3.9 Å can be estimated. For this residue we observe that also the $^{15}N^{\delta}$ signal is barely detectable in the ^{15}N HMQC experiments, because significant hyperfine contributions contribute to T_2 ^1H relaxation of the terminal NH_2 group. Proton relaxation rate measurements for both H_N^{δ} account for Ce–H distances shorter than 5 Å and show, again, that the Asn 56 $CONH_2$ group is pointing toward the metal ion. Therefore, both T_1 and T_2 data from carboxylate groups of Asp 54, Asp 58, Glu 65, and the amide group of Asn 56 are fully consistent with the coordination of an oxygen donor atom to the Ce^{III} ion.

We are left with three C=O signals arising from backbone C=O groups (Gly 59, Glu 60, Val 61) that are affected by hyperfine interaction. For signals C and E (Glu 60 and Val 61), relaxation rates indicate metal – carbon distances in the range of 3.6 – 4.5 Å, while the analysis of $^{13}C - ^{13}C$ COSY data suggests that signal F (Gly 59) has slightly longer relaxation times. At variance with side chains, whose intrinsic mobility ensures the formation of a metal – oxygen bond whenever the metal – carbon distance is consistent with coordination, a short Ce–C distance for a backbone carbonyl group does not necessarily imply a chemical bond between the oxygen atom of the C=O group and the metal ion. Indeed, even when a Ce–C distance shorter than 4 Å is found, the peptide dipole may not be properly oriented to have the carbonyl oxygen atom at binding distance.

To solve this problem, information arising from hyperfine shifts can be exploited. The latter not only contain structural information arising from the pseudocontact contribution, but, through contact shifts, are immediate markers of whether or not the nucleus investigated bears unpaired electron density arising from electron delocalization from the metal ion to its ligands.

The available solution structure of CaCeCb is sufficiently resolved[39] to have a reliable backbone structure also in the proximity of the cerium-binding site. On this basis, an ensemble of 30 conformers can be used to calculate the expected pseudocontact contributions to C=O signals of residues 59 – 61. As the magnetic susceptibility tensor of the Ce^{III}-substituted protein is known,[9, 12] the program FANTASIAN[11] can be used to predict the expected pseudocontact shifts of all signals.

The experimental hyperfine shifts arising from the presence of the Ce^{III} ion, measured with respect to the shifts of the La^{III} derivative, are reported in Table 1 (column 8). Such shifts are the sum of a pseudocontact and a contact contribution, the former being predictable in principle from the structural model and by FANTASIAN calculations. However, as it appears from Table 1 (column 9), the predicted pseudocontact shifts for the carboxylate groups, except one, span too large a range to be useful. This is expected, due to the essential lack of constraints for these groups, which leads to a large root-mean-square deviation. On the other hand, within the ensemble of conformers of the solution structure, the ranges of pseudocontact values for the backbone C=O signals are far narrower, and therefore they can be used to estimate the contact contributions (Table 1, column 10). Contact values in the range of $\delta = 0$ to 0.5, 9.7 to 12.3, -1.7 to -2.0 were found for Gly 59, Glu 60, and Val 61, respectively. The above findings strongly suggest that only the backbone C=O group of Glu 60 is directly bound to the Ce^{III} ion. Indeed, it is quite unlikely (and unprecedented) that two backbone C=O groups from amino acids that are adjacent in primary sequence form two direct bonds with the same metal ion, and the results of FANTASIAN calculations are therefore consistent with expectations. Based on the above considerations, the residues coordinating the Ce^{III} ion are identified and summarized in Table 1 (column 11).

Comparison between X-ray and solution structures

The NMR solution structure does not provide direct information about the coordination number of the Ce^{III} ion. The possibility to unambiguously identify binding residues from NMR data allows us, by solution structure calculations, to link the metal ion to its ligands and to impose oxygen-to-metal distances according to the evidence of a chemical bond. The available NMR solution

structures of calcium-loaded calbindin D_{9k},[40] as well as other NMR structures of calcium-binding proteins,[41–45] do not include the Ca^{II} ion in the structure calculations, and no experimental structural constraints involving the metal ion are included. Another strategy that has been used is the incorporation of distance restraints from Ca^{II} to oxygen carboxylate ligands, based on a coordination model or obtained from other indirect spectroscopic techniques.[46] The proposed approach allows us now to have experimental, NMR-derived evidence of chemical bonds between Ce^{III} and oxygen ligands. Although previous studies already proposed methods to assess, by means of NMR spectroscopy, whether or not a metal ion is coordinated to backbone carbonyl oxygen atoms within an EF-hand motif,[47] this is the first time that all coordinating groups could be identified by NMR spectroscopy. This allows the use of much more reliable and restrictive constraints between the metal ion and its ligands.

The five residues that have been found to coordinate the Ce^{III} ion are those residues that have been found to bind the Ca^{II} ion in the X-ray crystal structure of calbindin D_{9k}.[37, 38] This is, in a way, an expected finding, because no dramatic changes are expected in the chromophore. Given the high affinity of Ca^{II} and Ln^{III} for oxygen-containing ligands,[48] a possible structural change, when passing from Ca^{II} to Ce^{III}, could be a variation in the number of water molecules bound to the metal center. Such a change in the coordination number cannot be addressed by a high-field NMR spectroscopic study, and should be investigated by relaxometry techniques.[49] However, our aim here was the direct identification of metal-bound residues without any structural assumption. This is an important finding when dealing with calcium-binding proteins, in the light of the increasing popularity of the use of lanthanides as a tool to induce self-orientation.[10, 12, 50–52] It is crucial to have tools to characterize coordination number and coordination properties of lanthanide ions to be able to reliably exploit any structural information arising from their presence. Within this frame, the loss of detailed structural information in the proximity of the metal center could be a reasonable price to pay for exploiting long-range constraints arising from the paramagnet. On the other hand, it is important to assess the location of the metal ion and the number and position of metal-binding groups to properly place the metal center within the protein structure.

A comment on the observed shifts

One of the questions that are always associated with the use of pseudocontact shifts is the reliability of the diamagnetic reference used to evaluate the hyperfine contributions.[29, 53] The difference in charge between Ca^{II} and La^{III} gives rise to nonnegligible changes in chemical shift values for at least some of the signals of residues that are bound to the metal center, as shown in Figures 3 B and C. With the only exception of signal A, for which a small change of $\delta = 0.4$ is observed, the other four C=O signals of the chromophore experience changes from $\delta =$ 1.5 to 3.0. Noticeably, these effects are also nonnegligible for those carbonyl groups that are not directly coordinated to the metal ion such as Val 61, which experiences a change of $\delta = 1.4$ without being directly coordinated to the metal ion. It seems, therefore, that the effects on the observed shifts are due to a combination of electrostatic factors (which are observed also in the absence of direct Me–O–C bonds) and deshielding effects arising from charge polarization induced by changes in the Me–O bond strength. Changes in π polarization of the peptide bond upon formation of the bond between metal ion and backbone carbonyl oxygen atom in EF-hand proteins have been pointed out from ^{15}N chemical shift differences between the apo and the holo forms of several calcium-binding proteins.[47]

Therefore, the replacement of Ca^{II} with La^{III} is of little utility in identifying metal ligands. On the other hand, substitution with a paramagnetic metal is much more informative, thanks to the presence of contributions to both shift and relaxation. Moreover, the occurrence of nonnegligible contact shifts, which can be assessed whenever reliable estimates of the pseudocontact shifts are possible, further contributes to the identification of those signals belonging to metal-bound residues.

The comparison of chemical shift values of the Ce^{III} derivative extrapolated at infinite temperature with those obtained for the Ca^{II} and La^{III} derivatives basically supports the idea that La^{III} is a better diamagnetic reference for Ce^{III} than is Ca^{II}, although in the case of Glu 60, the extrapolated value of Ce^{III} is closer to that of Ca^{II} than to that of La^{III} (see Table 1). The agreement is only qualitative, due to the fact that, even under the assumption that pseudocontact shifts obey a T^{-2} temperature dependence, contact contributions, which depend on T^{-1}, are expected to occur for signals of metal-coordinated residues. Due to the limited temperature range available, the estimate of the effective T^{-n} order of the temperature dependence cannot be performed on experimental grounds.

Finally, the separation of the observed hyperfine shifts into contact and pseudocontact contributions, which has been attempted (see Table 1, columns 9, 10) on the basis of FANTASIAN[11, 54] calculations over the available solution structure,[39] deserves a comment. The behavior of backbone C=O groups has already been commented upon. The separation turned out to be helpful to unambiguously identify Glu 60 as the only metal-coordinated backbone carbonyl group. Its contact contribution to hyperfine shifting was found to be around $\delta = +10$. A similar result is obtained in the case of Asp 54, whose contact shift is estimated to be around $\delta = +17$. It is possible that a positive value of the contact shift of about $\delta = 10 - 20$ should also be expected for the other coordinated C=O groups. If this were true, estimated contact shifts of this order of magnitude could be subtracted from the hyperfine shifts, and the resulting pseudocontact shift values could be used to further refine the position of the coordinated side chains around the metal ion. This could be attempted in the future, as more data on contact shifts of Ce^{III}-coordinated carboxylates will become available.

Conclusions

Combination of direct ^{13}C detection and paramagnetic versions of classic double- and triple-resonance experiments permits the full signal assignment for the C=O groups around the paramagnetic ion in CaCeCb, together with the identification of the

coordinated ones. An extension of side-chain CH_2 assignment is also performed. This enables the characterization of the chromophore of calcium-binding proteins by the simple tool of lanthanide substitution. Of course, the investigation can be extended to the other calcium-binding site by using a CeCeCb sample.

Such information can be used to obtain structure refinement through molecular dynamics simulations by exploiting further structural constraints in the proximity of the paramagnetic center. This method can be applied to solve structures far more complicated than that of CaCeCb and can contribute to mapping the spin distribution over the first coordination sphere of the paramagnetic ions.

Experimental Section

Sample preparation: Protein expression[55] and purification[56] of both the Ca^{II} and the apo form of the bovine Pro 43 →Met 43 (P43M) mutant[57, 58] of calbindin D_{9k} was performed as previously reported. The expression system was a generous gift from Prof. S. Forsén. Uniformly [^{13}C,^{15}N]-labeled overexpressed P43M was obtained from M9 minimal medium containing $(^{15}NH_4)_2SO_4$ as the sole nitrogen source and [$^{13}C_6$]D-glucose as the sole carbon source. The cerium substitution procedure has been described elsewhere.[9] The pH was adjusted to 6.0 with 0.1 M NaOH or 0.1 M HCl. The samples were kept at 4 °C between measurements.

NMR spectroscopy: All NMR experiments were performed at 300 K, except when otherwise specified, on 1.5 – 2.0 mM samples on Bruker Avance 400, 600, and 700 spectrometers. One-dimensional ^{13}C NMR experiments were performed on Bruker Avance 600 or Bruker Avance 400 spectrometers. To identify fast-relaxing resonances, typical ^{13}C NMR spectra were collected with about 190 000 scans, using an overall recycle delay of 110 ms. Weft experiments[23] were collected using a recycle delay of 1.5 s and an interpulse delay of 500 ms. To measure T_1 of ^{13}C resonances, inversion recovery data were collected, each with 8096 scans, using a recycle delay of 2.3 s and interpulse delays of 7, 15, 30, 60, 120, 250, 500, and 1000 ms, respectively. To obtain the assignment the following set of experiments was performed on Bruker Avance 600 and 700 spectrometers: HNCA,[32] HNCO,[32] HCACO,[32] 2D (H)CCH-TOCSY,[28] $^{13}C-^{13}C$ COSY, $^{1}H-^{13}C$ HSQC, $^{1}H-^{15}N$ HSQC.[59] For most of these pulse sequences, experiments were performed by using established parameters for the investigation of biomolecules. (H)CCH-TOCSY, $^{13}C-^{13}C$ COSY, and HSQC experiments were also tailored to the identification of fast-relaxing signals. In the case of $^{13}C-^{13}C$ COSY, the acquired experiments were processed by using different combination of the effective number of data points. These parameters have been chosen in such a way as to optimize, in the final 2D spectra, the signal-to-noise ratio of the signals of interest.[27] The usual delays required to ensure the evolution of $^{13}C-^{1}H$ and $^{15}N-^{1}H$ couplings (typically $^{1}/_{2}J = 3.33$ ms for CH couplings and 5.5 ms for NH couplings) have been shortened to 1.4 and 1.0 ms, respectively. The $^{13}C-^{13}C$ transfer during the isotropic mixing in (H)CCH-TOCSY has been shortened to 2 ms. The same series of experiments used to assign signals not affected by the hyperfine interaction in the CaCeCb derivative was used to assign signals of interest in the native form containing two Ca^{II} ions (CaCaCb) and in the derivative in which Ca^{II} has been replaced by La^{III} at site II (CaLaCb). All NMR spectroscopic data were processed with the Bruker XWINNMR software package.

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