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ARTICLE

100% complete assignment of non-labile ^{1}H, ^{13}C, and ^{15}N signals for calcium-loaded calbindin D_{9k} P43G

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Abstract Here we present the 100% complete assignment chemical shift of non-labile ^{1}H, ^{15}N and ^{13}C nuclei of Calbindin D_{9k} P43G. The assignment includes all non-exchangeable side chain nuclei, including ones that are rarely reported, such as LysNζ as well as the termini. NMR experiments required to achieve truly complete assignments are discussed. To the best of our knowledge our assignments for Calbindin D_{9k} extend beyond previous studies reaching near-completeness (Vis et al. in Biochem 33:14858–14870, 1994; Yamazaki et al. in J Am Chem Soc 116:6464–6465, 1994; Yamazaki et al. in Biochem 32:5656–5669, 1993b).

Keywords Calbindin D_{9k} · 100% Complete assignment · Assignment strategy · NMR spectroscopy

Biological context

Calbindin D_{9k} is a small monomeric protein (Mr 8.5 kDa, 76 amino acids) which belongs to the EF-hand family, and consists of two helix-loop-helix motifs that bind one calcium ion each (Kretsinger and Nockolds 1973). Calbindin D_{9k} undergoes small structural changes upon calcium-binding, involving the rearrangement of non-polar side chains (Ikura 1996). The protein is predominantly found in the mammalian epithelial cells of the small intestine and placenta, and it has been implicated to facilitate the transport of calcium across the intestinal epithelial cells (Christakos et al. 1989).

High resolution three dimensional structures of Calbindin D_{9k} in various calcium-loaded states have been characterized extensively by X-ray crystallography and solution state NMR spectroscopy (Kordel et al. 1997; Kordel et al. 1993; Szebenyi and Moffat 1986). Although the complete resonance assignment of ^{13}C, ^{15}N and ^{1}H nuclei for this protein was not used for structure determination, it will facilitate a comprehensive study of its dynamics, structure, dihedral angle distributions and electrostatic interactions, as well as supplying data for comparisons with solid-state NMR and chemical shift calculations by quantum chemical methods.

Methods and experiments

MM294 *E. coli* cells transformed by the PCBWR plasmid containing the *calbindin* Pro43Gly gene (Bos taurus) were used for protein expression. A single colony was picked from agar plate and grown overnight in 100 mL LB medium with ampicillin at 30°C. 20 mL of the overnight culture was added to 500 mL minimal medium containing U-^{13}C-glucose and U-^{15}N ammonium chloride at 30°C. Protein production was started by ten-fold dilution of the cells into medium containing U-^{13}C glucose, U-^{15}N ammonium chloride and 0.1 mg/mL IPTG at 37°C. Purification of Calbindin D_{9k} P43G was performed as in a previous study (Thulin 2002).

All experiments (see Table 1) were carried out on Varian Unity INOVA 500 and 600 MHz spectrometers

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Table 1 List of experiments

No	Experiments	Connectivities	Experimental time (h)
1	$^{1}H–^{15}N$ HSQC-SE-wfb[a,b,c]	$N^{H}–H^{N}$	0.2
2	Sensivity-enhanced HA(CA)CO[d,e]	Hα–C′	0.6
3	3D–HN(C′)N[f]	$N^{H}_{(i)}–H^{N}_{(i)}–N^{H}_{(i+1)}$	20.5
		$N^{H}_{(i)}–H^{N}_{(i)}–N^{H}_{(i)}$	
4	3D–HNN[f]	$N^{H}_{(i)}–H^{N}_{(i)}–N^{H}_{(i)}$	55.8
		$N^{H}_{(i)}–H^{N}_{(i)}–N^{H}_{(i-1)}$	
		$N^{H}_{(i)}–H^{N}_{(i)}–N^{H}_{(i+1)}$	
5	H(N)CO[g]	$H^{N}_{(i)}–C'_{(i-1)}$	0.3
6	HACA(N)[h]	$H\alpha_{(i)}–C\alpha_{(i)}$	1.75
7	H2(C)N[i]	N^{H}–Hα	0.3
		Nζ–Hε(Lysine)	
		N–Hα (Proline)	
8	H2(CA)N[i]	Nterminus–Hα	0.6
9	3D $^{1}H–^{15}N$–TOCSY−HSQC,[j,k,l,m]	$N^{H}_{(i)}H^{N}_{(i)}$–all aliphatic side chain protons $_{(i)}$	8.25
10	3D HCCH–COSY[n,o]	$C_{(i)}–H_{(i)}–H_{(i)}$ (through one bond coupling of aliphatic resonances)	17
11	3D C–TOCSY–N(C)H2[i]	Hε–Nε and all side chain carbons of lysine	20
12	3D H(CCO)NH–TOCSY	$N^{H}_{(i)}–H^{N}_{(i)}$–all aliphatic side chain protons $_{(i-1)}$	21.25
13	3D (H)C(CO)NH–TOCSY[q]	$N^{H}_{(i)}–H^{N}_{(i)}$–all aliphatic side chain carbons $_{(i-1)}$	14
14	$^{1}H–^{13}C$ constant time HSQC[r]	$C_{(i)}–H_{(i)}$ of aliphatic resonances	0.2
15	(HBGCBG)CO(CBGCABCON)H[t]	$C\gamma_{(i)}–H^{N}_{(i+1)}$for asparagine and aspartic acid	4
		$C\delta_{(i)}–H^{N}_{(i+1)}$for glutamine and glutamatic acid	
16	H2(C)CO[u]	C′–Hα	2
		Cγ–Hβ (for asparagine and aspartate)	
		Cδ–Hγ (for glutamate and glutamine)	
17	$^{3}J^{s}_{NC\gamma}$	$C\gamma_{(i)}–H^{N}_{(i)}$	8.6
		$C\beta_{(i)}–H^{N}_{(i)}$	
		$C\alpha_{(i)}–H^{N}_{(i)}$	
		$C\alpha_{(i-1)}–H^{N}_{(i)}$(if i−1 is glycine)	
		$C\gamma_{(i)}–H\varepsilon 2_{(i)}$ for glutamine	
		$C\beta_{(i)}–H\delta 2_{(i)}$ for asparagine	
18	$^{3}J^{s}_{C'C\gamma}$	$C'_{(i-1)}–H^{N}_{(i)}$	2.16
		$C\gamma_{(i-1)}–H^{N}_{(i)}$	
		$C\beta_{(i)}–H^{N}_{(i)}$	
		$C\beta_{(i-1)}–H^{N}_{(i)}$	
		$C\alpha_{(i-1)}–H^{N}_{(i)}$ (for proline)	
19	$3D^{1}H–^{13}C$ HSQC NOESY[v]	$C_{(i)}–H_{(i)}$-all protons within 5Å	38
20	3D HCCH-COSY aromatic[o,p]	C–H–H	16.8
21	CG(CB)HB[w]	Cγ–Hβ for aromatic side chain	9.3
22	CB(CGCD)HD[x]	Cβ–Hδ for aromatic side chain	10.8
23	CB(CGCDCE)HE[x]	Cβ–Hε for aromatic side chain	10.8
24	$^{1}H–^{13}C$ HSQC aromatic[r]	Cδ–Hδ	0.8
		Cε–Hε	
		Cζ–Hζ	
		for aromatic side chain	

Table 1 continued

No	Experiments	Connectivities	Experimental time (h)
25	^{1}H–^{13}C HSQC CP aro[y]	Cδ–Hδ	7
		Cδ–Hε	
		Cε–Hε	
		Cε–Hζ	
		Cζ–Hζ	
		for aromatic side chain	
26	^{1}H–^{13}C HMQC aromatic[z]	Cδ–Hδ	0.3
		Cε–Hε	
		Cζ–Hζ	
		for aromatic side chain	

[a] Cavanagh et al. 1991; [b] Palmer et al. 1991; [c] Palmer et al. 1992; [d] Kay et al. 1990b; [e] Powers et al. 1991; [f] Panchal et al. 2001; [g] Muhandiram and Kay 1994; [h] Ottiger and Bax 1997; [i] Andre et al. 2007; [j] Fesik and Zuiderweg 1990; [k] Marion et al. 1989a; [l] Marion et al. 1989b; [m] Zhang et al. 1994; [n] Ikura et al. 1991; [o] Kay et al. 1990a; [p] Ikura et al. 1991; [q] Logan et al. 1993; [r] Vuister and Bax 1992; [s] Konrat et al. 1997; [t] Tollinger et al. 2002; [u] Oda et al. 1994; [v] Majumdar and Zuiderweg 1993; [w] Prompers et al. 1998; [x] Yamazaki et al. 1993a; [Y] Zuiderweg et al. 1996; [z] Bax et al. 1990

equipped with pulsed field gradient probes. The spectra were recorded at 301 K. The NMR sample contained ~2.5 mM [^{13}C,^{15}N]-enriched Calbindin D_{9k} P43G, pH 6.0, 7% D_2O. The spectra were processed using NMRPipe (Delaglio et al. 1995) and analyzed using Sparky (Goddard and Kneller 2003).

Assignments and data deposition

Relation to previous assignment (BMRB entry 327): Only ^{1}H signals of Calbindin D_{9k} P43G had been assigned. Here the 100% complete assignment of non-labile ^{1}H, ^{13}C and ^{15}N signals for calcium-loaded D_{9k} P43G was achieved using an extensive suit of standard and non-standard 2D and 3D NMR experiments.

Backbone and aliphatic side chains

Backbone assignments were obtained using 3D experiments such as HN(C′)N and HNN to obtain the amide proton and nitrogen chemical shifts (Fig. 1 displays the dispersion of N^{H}–H^{N} chemical shift of Calbindin D_{9k} from ^{1}H–^{15}N HSQC). Several 2D projections of triple resonance experiments; H(N)CO, HA(CA)CO, and HACA(N) were recorded to assign the carbonyl, alpha proton and alpha carbon resonances. Most aliphatic side chain signals were assigned using 3D experiments; (H)C(CO)NH-TOCSY and H(CCO)NH-TOCSY, which correlate the backbone nitrogen and amide proton shifts to aliphatic carbon and proton frequencies of residue i − 1. For residues which are preceded by proline, the side chain nuclei were assigned using a 3D ^{15}N–^{1}H TOCSY-HSQC experiment which correlates

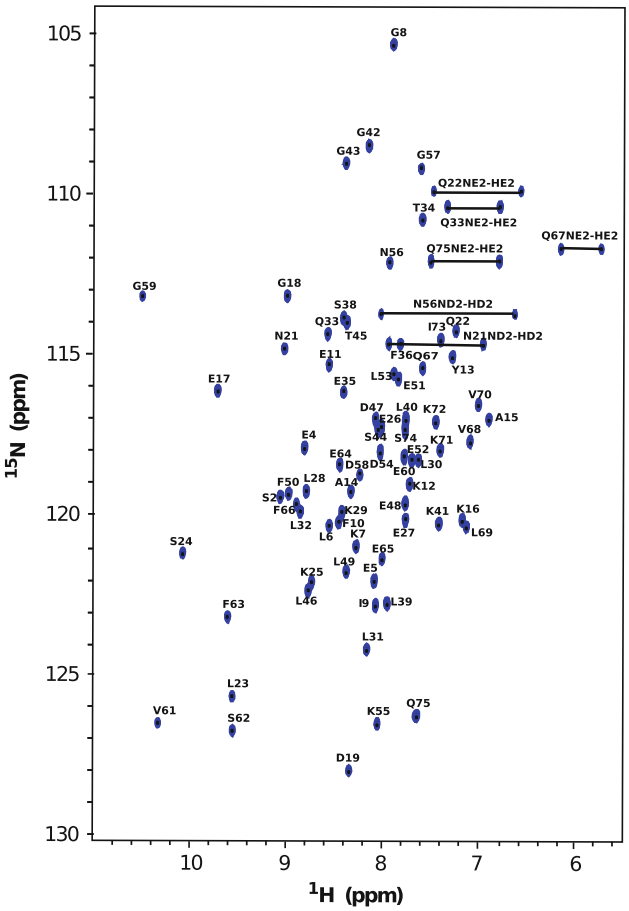

Fig. 1 2D ^{1}H–^{15}N HSQC spectrum of uniformly ^{15}N/^{13}C-labelled Calbindin D_{9K}. All peaks are annotated with the one letter amino acid symbol and their position in the sequence. All amide proton and nitrogen nuclei in the backbone of Calbindin D_{9k} were observed. The $^{15}N\varepsilon$ and $^{1}H\varepsilon$ chemical shits of glutamine and the chemical shifts of $^{15}N\delta$ and $^{1}H\delta$ of asparagines are also indicated

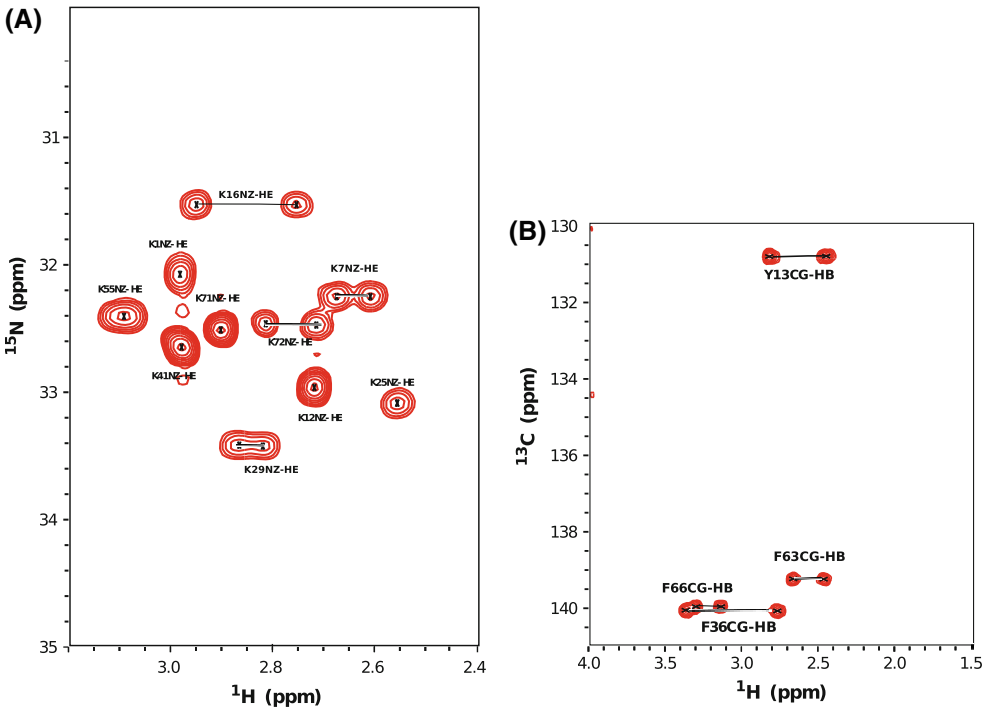

Fig. 2 Some specific assignments of Calbindin D_{9k} side chains. **a** 2D H2(C)N spectrum showing Lys Nζ–Hε correlations. **b** 2D CG(CB)HB spectrum to assign aromatic side chain resonances

N^H, H^N, and proton aliphatic side chain in the same residue. The HCCH-COSY experiment, which gives information about chemical shifts of protons bound to carbon, enabled us to verify the assignment for long side chains like lysine, leucine, glutamate, isoleucine, valine and proline. The specific assignment of lysine Nζ were obtained using the 2D H2(C)N experiment. The signals in this spectrum are well dispersed between 31 and 34 ppm (see Fig. 2a). The ^{15}N shift of the *N* terminal methionine residue was obtained using 2D H2(C)N pulse sequence where the final shaped carbon inversion pulse was replaced by a full power rectangular 180° pulse (Andre et al. 2007). The carbonyl side chains of glutamate, glutamine, aspragine and aspartate were detected using (HBGCBG)CO(CBGCABCACON)H experiments which correlates the side chain carbonyl of residue i to the amide proton of residue i+1 (see Fig. 3). This experiment is very powerful to get the unambiguous chemical shift assignment of carbonyl/carboxyl side chains due to the excellent dispersion of amide proton chemical shifts in folded proteins. However, the sensitivity of the experiments was not sufficient to detect all signals (Q22 was absent) and a H2(C)CO experiment was used to detect the Q22 Cδ–Hγ correlation.

Aromatic side chains

Calbindin D_{9k} P43G contains 5 phenylalanines and 1 tyrosine residue. Some specific strategies were required to assign the aromatic ring ^{1}H and ^{13}C resonances. Aromatic Cγ resonances were assigned using a combination of CG(CB)HB,

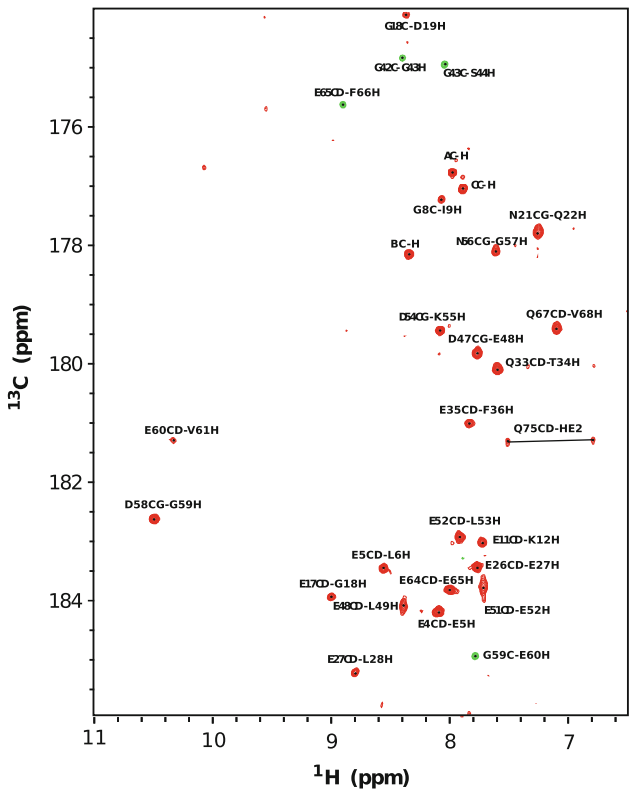

Fig. 3 2D ^{1}H–^{13}C (HBGCBG)CO(CBGCABCACON)H spectrum of uniformly labelled ^{15}N/^{13}C Calbindin D_{9k}. Correlation can be observed for carboxyl/carbonyl side chain ^{13}C′ of glutamate, glutamine, asparagine and aspartate of residue i with the amide proton of residue i+1. Peaks labeled AC–H, BC–H and CC–H refer to carbonyl and amide proton peaks from the soluble cyclic enterobacterial common antigen, ECA_{CYC} (Erbel et al. 2003)

$^{3}J_{NC\gamma}$ and $^{3}J_{C'C\gamma}$ experiments. The sequence-specific side chain ^{1}H assignment of the aromatic side chains was obtained via CB(CGCD)HD and CB(CGCDCE)HE experiments. The information of Hδ and Hε in the aromatic rings from these experiments were used to assign ^{1}H–^{13}C HSQC CP aro, aromatic ^{1}H–^{13}C CT HSQC and ^{1}H–^{13}C HMQC aromatic experiment. F10 Cδ–Hδ was only observed in the non constant time ^{1}H–^{13}C HSQC or ^{1}H–^{13}C HMQC experiment due to the strong coupling within the aromatic ring. A 3D ^{1}H–^{13}C HSQC-NOESY experiment was used to verify the assignment of the aromatic side chains.

To summarize, the ^{1}H, ^{13}C and ^{15}N resonance assignments of Calbindin D_{9k} P43G have been deposited in the BioMagResBank (accession number 16340). Although complete side chain resonance assignments were obtained for Calbindin D_{9k} P43G, it should be mentioned that it does not contain any cysteine, arginine, histidine and tryptophan residues. Those residues, in particular, require specific strategies for their side chain assignment. For arginine guanidine groups, sequence specific assignment of ^{15}N and ^{1}H chemical shifts have been presented by Yamazaki et al. 1995, and for histidine and tryptophan ring, sequence specific assignment of ^{1}H, ^{13}C, ^{15}N have been established by Löhr et al. (2005).

Our study shows that complete assignments of all NMR-active nuclei in small protein can be obtained and describes a suitable strategy for this purpose. In particular, lysine Nζ chemical shifts appear to be difficult to get correct, as witnessed by the BMRB database. Currently (grid update of August 16th, 2010) 110 chemical shift assignments for lysine Nζ are available, but this list contains as many as 25 erroneous assignments. In three cases, a ^{1}H chemical shift was entered for Nz and in 22 instances chemical shifts between 67 and 133 ppm have been listed, either as a result of exchanging the assignment with that of backbone nuclei, or by the incorrect account of spectral aliasing. Even today, the lysine Nζ statistics are heavily polluted and yield 47.8 ± 32.9 ppm for the full set. A restricted set of 14 entries now gives 34.1 ± 3.0 ppm, as opposed to 73.8 ± 50.3 ppm for 7 entries in 2004.

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