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 LysN$_{\alpha}$ 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; Szébenyi 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.
Table 1  List of experiments

| No | Experiments | Connectivities | Experimental time (h) |
|----|-------------|----------------|-----------------------|
| 1  | $^1$H–$^{15}$N HSQC-SE-wfb<sup>a,b,c</sup> | $N^H$–$H^N$ | 0.2 |
| 2  | Sensitivity-enhanced HA(ICA)CO<sup>d,e</sup> | Hz–C<sup>e</sup> | 0.6 |
| 3  | 3D–HN(C<sup>c</sup>)N<sup>f</sup> | $N^H_0$–$H^N_0$, $N^H_{i}$–$H^N_{i}$ (through one bond coupling of aliphatic resonances) | 20.5 |
| 4  | 3D–HNN<sup>f</sup> | $N^H_{i}$–$H^N_{i}$–$N^H_{i+1}$, $N^H_{i}$–$H^N_{i}$–$N^H_{i-1}$, $N^H_{i}$–$H^N_{i}$–$N^H_{i+1}$ | 55.8 |
| 5  | H(N)CO<sup>g</sup> | $H^N_0$–$C^i$<sub>(i–1)</sub> | 0.3 |
| 6  | HACA(N)<sup>h</sup> | Hz<sub>0</sub>–$Cz_0$ | 1.75 |
| 7  | H2(C)<sup>i</sup> | $N^H$–Hz (Proline) | 0.3 |
| 8  | H2(CA)<sup>i</sup>N<sup>j</sup> | Nterminus–Hz | 0.6 |
| 9  | 3D $^1$H–$^{15}$N–TOCSY–HSQC<sup>j,k,l,m</sup> | $N^H_0$–$H^N_0$–all aliphatic side chain protons<sup>i</sup> | 8.25 |
| 10 | 3D HCCH–COSY<sup>n,o</sup> | $C_0$–$H_0$–$H_{i}$ | 17 |
| 11 | 3D C–TOCSY–N(C)H<sub>2</sub><sup>i</sup> | Hz–$N^e$ and all side chain carbons of lysine | 20 |
| 12 | 3D H(CC)NH–TOCSY<sup>q</sup> | $H_{i}$–$H^N_0$–all aliphatic side chain protons<sup>i</sup>–$H_{i-1}$ | 21.25 |
| 13 | 3D (H)(CC)NH–TOCSY<sup>q</sup> | $H_{i}$–$H^N_0$–all aliphatic side chain carbons<sup>i</sup>–$H_{i-1}$ | 14 |
| 14 | $^1$H–$^{13}$C constant time HSQC<sup>q</sup> | $C_0$–$H_0$ of aliphatic resonances | 0.2 |
| 15 | (HBGCBG)(CO)(CBGCABCON)H<sup>f</sup> | $C_{i}$–$H^N_{i+1}$ for asparagine and aspartic acid | 4 |
| 16 | H2(C)<sup>i</sup>CO<sup>g</sup> | $C^c$–Hz | 2 |
| 17 | $^3$J<sub>NC<sub>7</sub></sup> | $C_{i}$–$H^N_{i}$<sup>o</sup> | 8.6 |
| 18 | $^3$J<sub>C<sub>7</sub></sup> | $C_{i}$<sub>0</sub>–$H^N_{i}$<sup>o</sup> | 2.16 |
| 19 | 3D$^1$H–$^{13}$C HSQC NOESY<sup>v</sup> | $C_0$–$H_0$–all protons within 5Å | 38 |
| 20 | 3D HCCH-COSY aromatic<sup>p</sup> | C–H–C | 16.8 |
| 21 | CG(CB)HB<sup>o</sup> | $C_{y}$–H$^p$ for aromatic side chain<sup>o</sup> | 9.3 |
| 22 | CB(CGCD)HD<sup>o</sup> | $C_{p}$–H$^z$ for aromatic side chain<sup>o</sup> | 10.8 |
| 23 | CB(CGCDCE)HE<sup>o</sup> | $C_{p}$–H$^z$ for aromatic side chain<sup>o</sup> | 10.8 |
| 24 | $^1$H–$^{13}$C HSQC aromatic<sup>o</sup> | C$^c$–H$^c$ | 0.8 |

<sup>a</sup> for aromatic side chain |

<sup>b</sup> for aromatic side chain |

<sup>c</sup> for aromatic side chain |

<sup>d</sup> for aromatic side chain |

<sup>e</sup> for aromatic side chain |

<sup>f</sup> for aromatic side chain |

<sup>g</sup> for aromatic side chain |

<sup>h</sup> for aromatic side chain |

<sup>i</sup> for aromatic side chain |

<sup>j</sup> for aromatic side chain |

<sup>k</sup> for aromatic side chain |

<sup>l</sup> for aromatic side chain |

<sup>m</sup> for aromatic side chain |

<sup>n</sup> for aromatic side chain |

<sup)o</sup> for aromatic side chain |

<sup>p</sup> for aromatic side chain |

<sup>q</sup> for aromatic side chain |

<sup>r</sup> for aromatic side chain |

<sup>s</sup> for aromatic side chain |

<sup>t</sup> for aromatic side chain |

<sup>u</sup> for aromatic side chain |

<sup>v</sup> for aromatic side chain |

<sup>w</sup> for aromatic side chain |

<sup>x</sup> for aromatic side chain |

<sup>y</sup> for aromatic side chain |

<sup>z</sup> for aromatic side chain |

<sup>{</sup> for aromatic side chain |

<sup>}</sup> for aromatic side chain
equipped with pulsed field gradient probes. The spectra were recorded at 301 K. The NMR sample contained 2.5 mM [\(^{13}\text{C},^{15}\text{N}\)]-enriched Calbindin D\(_{9k}\) P43G, pH 6.0, 7% D\(_2\)O. 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 \(^1\text{H}\) signals of Calbindin D\(_{9k}\) P43G had been assigned. Here the 100% complete assignment of non-labile \(^1\text{H},^{13}\text{C}\) and \(^{15}\text{N}\) 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\(^{15}\)–H\(^\text{N}\) chemical shift of Calbindin D\(_{9k}\) from \(^1\text{H}–^{15}\text{N}\) 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 \(^{15}\text{N}–^{1}\text{H}\) TOCSY-HSQC experiment which correlates...
NH, HN, 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 N\textsubscript{F} 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 15N shift of the N\textsubscript{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, asparagine and aspartate were detected using (HBGCBG)CO(CBG-CABCACON)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 C\textalpha–H\textgamma correlation.

Aromatic side chains

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

Fig. 2 Some specific assignments of Calbindin D\textsubscript{9k} side chains. a 2D H2(C)N spectrum showing Lys N\textsubscript{F}–H\textepsilon correlations. b 2D CG(CB)HB spectrum to assign aromatic side chain resonances.

Fig. 3 2D $^{1}$H–$^{13}$C (HBGCBG)CO(CBGCA)ACON)H spectrum of uniformly labelled $^{15}$N/$^{13}$C Calbindin D\textsubscript{9k}. Correlation can be observed for carboxyl/carbonyl side chain $^{13}$C\textsuperscript{0} 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\textsubscript{CYC} (Erbel et al. 2003)
$^3$J_{NC}$ and $^3$J_{C'C}$ 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 H0 and Hc 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 C0–H0 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 Löhrl 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 $N_c$ 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 $N_c$ are available, but this list contains as many as 25 erroneous assignments. In three cases, a $^1$H chemical shift was entered for Nc 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 $N_c$ 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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References

Andre I, Linse S, Mulder FAA (2007) Residue-specific pKa determination of lysine and arginine side chains by indirect $^{15}$N and $^{13}$C NMR spectroscopy: application to apo calmodulin. J Am Chem Soc 129:15805–15813

Bax A, Ikura M, Kay LE, Torchia DA, Tschudin R (1990) Comparison of different modes of two-dimensional reverse-correlation nmr for the study of proteins. J Magn Reson 86:304–318

Cavanagh J, Palmer AG, Wright PE, Rance M (1991) Sensitivity improvement in proton-detected two-dimensional heteronuclear relay spectroscopy. J Magn Reson 91:429–436

Christakos S, Gabrieldes C, Rothen WB (1989) Vitamin D-dependent calcium binding proteins: chemistry, distribution, functional considerations, and molecular biology. Endocr Rev 10:3–26

Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A (1995) NMPrpipe—a multidimensional spectral processing system based on unix pipes. J Biomol NMR 6:277–293

Erbel PJA, Barr K, Gao N, Gerwig GJ, Rick PD, Gardner KH (2003) Identification and biosynthesis of cyclic enterobacterial common antigen in escherichia coli. J Bacteriol 185:1995–2004

Fesik SW, Zuidervog ERP (1990) Heteronuclear three-dimensional nmr-spectroscopy of isotopically labeled biological macromolecules. Q Rev Biophys 23:97–131

Goddard TD, Kneller DG (2003) SPARKY 3. University of California, San Francisco

Ikura M (1996) Calcium binding and conformational response in EF-hand proteins. Trends in Biochem Sci 21:14–17

Ikura M, Kay LE, Bax A (1991) Improved three-dimensional $^1$H–$^{13}$C–$^1$H correlation spectroscopy of a 13C-labeled protein using constant-time evolution. J Biomol NMR 1:299–304

Kay LE, Ikura M, Bax A (1990a) Proton proton correlation via carbon carbon couplings—a three-dimensional NMR approach for the assignment of aliphatic resonances in proteins labeled with carbon-13. J Am Chem Soc 112:888–889

Kay LE, Ikura M, Tschudin R, Bax A (1990b) Three-dimensional triple-resonance NMR-spectroscopy of isotopically enriched proteins. J Magn Reson 89:496–514

Kontrat R, Muhandiram DR, Farrow NA, Kay LE (1997) Pulse schemes for the measurement of $^3$J_{C'C} and $^3$J_{NC} in $^{15}$N, $^{13}$C uniformly labeled proteins. J Biomol NMR 9:409–422

Kordel J, Skelton NJ, Akke M, Chazin WJ (1993) High-resolution solution structure of calcium-loaded calbindin-D$_{9k}$. J Mol Biol 231:711–734

Kordel J, Pearlman DA, Chazin WJ (1997) Protein solution structure calculations in solution: solvated molecular dynamics refinement of calbindin D9 k. J Biomol NMR 10:231–243

Kretschmer RH, Nockolds CE (1973) Carp muscle calcium-binding protein: II. Structure determination and general description. J Biol Chem 248:3313–3326

Logan TM, Olejnizckaz ET, Xu RX, Fesik SW (1993) A general method for assigning NMR-spectra of denatured proteins using 3D HC(CO)NH-TOCSY triple resonance experiments. J Biomol NMR 3:225–231

Löhrl F, Rogov VV, Shi M, Bernhard F, Dötsch V (2005) Triple-resonance methods for complete resonance assignment of aromatic protons and directly bound heteronuclei in histidine and tryptophan residues. J Biomol NMR 32:309–328

Majumdar A, Zuidervog ERP (1993) Improved 13C-Resolved HSQC-NOESY spectra in H$_2$O, using pulsed-field gradients. J Magn Reson Series B 102:242–244

Marion D, Driscoll PC, Kay LE, Wingfield PT, Bax A, Gronenborn AM, Clore GM (1989a) Overcoming the overlap problem in the assignment of proton nmr spectra of larger proteins by use of three-dimensional heteronuclear proton and nitrogen hartmann-hahn multiple quantum coherence and nuclear overhauser multiple quantum coherence spectroscopy—application to interleukin 1.beta. Biochemistry 28:6150–6156
Marion D, Kay LE, Sparks SW, Torchia DA, Bax A (1989b) Three dimensional heteronuclear NMR of nitrogen-15 labeled proteins. J Am Chem Soc 111:1515–1517

Muhandiram DR, Kay LE (1994) Gradient-enhanced triple-resonance 3-dimensional NMR experiments with improved sensitivity. J Magn Reson Series B 103:203–216

Oda Y, Yamazaki T, Nagayama K, Kanura Y, Nakamura H (1994) Individual ionization-constants of all the carbonyl groups in ribonuclease HI from Escherichia-Coli determined by NMR. Biochemistry 33:5275–5284

Ottiger M, Bax A (1997) An empirical correlation between amide deuterium isotope effects on $^{13}$C chemical shifts and protein backbone conformation. J Am Chem Soc 119:8070–8075

Palmer AG, Cavanagh J, Wright PE, Rance M (1991) Sensitivity improvement in proton-detected two-dimensional heteronuclear correlation NMR-spectroscopy. J Magn Reson 93:151–170

Palmer AG, Fairbrother WJ, Cavanagh J, Wright PE, Rance M (1992) Improved resolution in three-dimensional constant-time triple resonance NMR spectroscopy of proteins. J Biomol NMR 2:103–108

Panchal SC, Bhavesh NS, Hosur RV (2001) Improved 3D triple resonance experiments, HNN and HN(C)N, for HN and 15 N sequential correlations in $^{13}$C, $^{15}$N labeled proteins: application to unfolded proteins. J Biomol NMR 20:135–147

Powers R, Gronenborn AM, Clore GM, Bax A (1991) 3-Dimensional triple-resonance NMR of $^{13}$C/$^{15}$N enriched proteins using constant-time evolution. J Magn Reson 94:209–213

Prompers JJ, Groenewegen A, Hilbers CW, Pepermans HAM (1998) Two-dimensional NMR experiments for the assignment of aromatic side chains in 15N-labeled proteins. J Magn Reson 130:68–75

Szebenyi DM, Moffat K (1986) The refined structure of vitamin D-dependent calcium-binding protein from bovine intestine. Molecular details, ion binding, and implications for the structure of other calcium-binding proteins. J Biol Chem 261:8761–8777

Thulin E (2002) Purification of recombinant calbindin D$_{37}$k. Humana Press inc, Totowa

Tollinger M, Forman-Kay JD, Kay LE (2002) Measurement of side-chain carboxyl pKa values of glutamate and aspartate residues in an unfolded protein by multinuclear NMR spectroscopy. J Am Chem Soc 124:5714–5717

Vis H, Boelens R, Mariani M, Stroop R, Vorgias CE, Wilson KS, Kaptein R (1994) $^{1}$H, $^{13}$C, and $^{15}$N resonance assignments and secondary structure analysis of the HU protein from Bacillus stearothermophilus using two- and three-dimensional double- and triple-resonance heteronuclear magnetic resonance spectroscopy. Biochemistry 33:14858–14870

Vuister GW, Bax A (1992) Resolution enhancement and spectral editing of uniformly $^{13}$C enriched proteins by homonuclear broad-band $^{13}$C decoupling. J Magn Reson 98:428–435

Yamazaki T, Forman-Kay JD, Kay LE (1993a) 2-Dimensional NMR experiments for correlating carbon-13Beta and proton-delta/epsilon chemical-shifts of aromatic residues in 13C-labeled proteins via scalar couplings. J Am Chem Soc 115:1054–11055

Yamazaki T, Yoshida M, Nagayama K (1993b) Complete assignments of magnetic resonances of ribonuclease-H from escherichia coli by double-resonance and triple-resonance 2D and 3D NMR spectroscopies. Biochemistry 32:5656–5669

Yamazaki T, Lee W, Revington M, Mattiello DL, Dahlquist FW, Arrowsmith CH, Kay LE (1994) An HNCA pulse scheme for the backbone assignment of $^{15}$N, $^{13}$C, $^{2}$H-labeled proteins—application to a 37-kDa trp repressor DNA complex. J Am Chem Soc 116:6464–6465

Yamazaki T, Pascal SM, Singer AU, Forman-Kay JD, Kay LE (1995) NMR pulse scheme for the sequence-specific assignment of arginine guanidino $^{15}$N and $^{1}$H chemical shifts in proteins. J Am Chem Soc 117:3556–3564

Zhang OW, Kay LE, Olivier JP, Forman-Kay JD (1994) Backbone $^{1}$H and $^{15}$N resonance assignments of the N-terminal Sh3 Domain of drk in folded and unfolded states using enhanced-sensitivity pulsed-field gradient NMR techniques. J Biomol NMR 4:845–858

Zuiderweg ERP, Zeng L, Brutscher B, Morshauer RC (1996) Band-selective hetero- and homonuclear cross-polarization using trains of shaped pulses. J Biomol NMR 8:147–160