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# Letter to the Editor: Sequential $^{1}H$, $^{15}N$ and $^{13}C$ NMR assignment of human calbindin D28k

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## Biological context

Calbindin D28k is a highly conserved $Ca^{2+}$-binding protein with approximately 98% sequence identity between human and most other mammals. The protein is abundant in brain and sensory neurons, showing differential distribution among functionally distinct subpopulations of neurons. It has been demonstrated that calbindin D28k protects against apoptosis by suppressing the concentration of free calcium, by inhibiting caspase-3 (Bellido et al., 2000), and by inhibiting cytokine-induced free radical formation (Rabinovitch et al., 2001). In addition, *in vivo* studies have revealed that calbindin D28k present in the Purkinje cells of the cerebellum is a critical determinant for motor coordination in mice (Barski et al., 2003).

Calbindin D28k consists of 261 residues organized in six EF-hands (helix-loop-helix calcium binding motifs) that are packed into a single globular domain (Linse et al., 1997), the atomic-resolution structure of which is still unknown. The protein was originally believed to function as a $Ca^{2+}$-buffer, but recent studies have shown that the protein exhibits properties characteristic of a signaling protein (Berggård et al., 2002a), and interactions with cellular targets have been identified, including caspase-3 (Bellido et al., 2000), Ran-binding protein M (Lutz et al., 2003), and *myo*-inositol monophosphatase (Berggård et al., 2002b). The latter target is activated by calbindin D28k, suggesting an intriguing role for calbindin D28k in regulating the phosphatidylinositol-signaling pathway.

Here we report the sequential assignment of an engineered form of calbindin D28k with improved properties for high-resolution NMR studies. Initial NMR studies of the wild-type protein revealed severe problems with linebroadening and spectral heterogeneity associated with non-specific oligomerization/aggregation and heterogeneous post-translational modifications. The mutant protein effectively eradicates these obstacles (see further below), enabling detailed structure-function studies at the level of atomic resolution. The sequence-specific resonance assignments form the necessary basis for structure determination and interaction studies by NMR to identify binding sites for putative targets.

## Methods and experiments

Human calbindin D28k contains five cystein residues, all of which were mutated to serine to avoid heterogeneous oligomerization caused by intra- and intermolecular disulfide bridges. In addition, Q182 was mutated to E and N203 to D, so as to avoid deamidation and the consequent heterogeneity of the covalent structure (Vanbelle et al., in preparation). The resulting protein displays a well-dispersed [$^{15}N$, $^{1}H$]-HSQC spectrum with limited exchange broadening, as expected for a well-folded protein (Figure 1). The mutated form is active and binds $Ca^{2+}$ in a manner highly similar to the native protein (Cedervall et al., in preparation).

Recombinant protein was expressed from a modified Pet3a plasmid in the *Escherichia coli* strain BL21 (DE3) pLysS Star in minimal medium with $^{15}N$-ammonium chloride and $^{13}C$ glucose. The protein was purified by heat treatment followed by two subsequent DEAE anion exchange chromatography steps,

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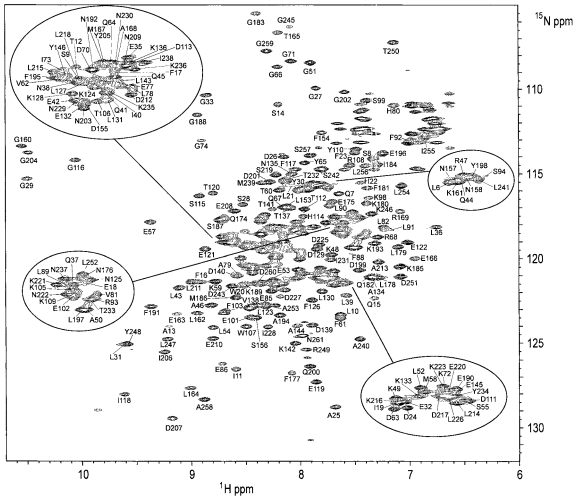


*Figure 1.* The [$^{15}$N, $^{1}$H]-HSQC spectrum of calcium-saturated human calbindin D28k (C94S, C100S, C187S, C219S, C257S, Q182E, N203D). Calcium binding is indicated by the characteristic down-field shifted amide proton resonances of G29, G116, G160 and G204 in EF-hands 1, 3, 4 and 5, respectively. The identification of four functional calcium binding sites is in agreement with previous results (Berggård et al., 2002a).

in $Ca^{2+}$ and EDTA, respectively, and desalting on a G25 gel filtration column. The NMR sample contained 1 mM of uniformly $^{15}$N-labeled or $^{13}$C/$^{15}$N-labeled calbindin D28k in 20 mM Mes buffer, 11 mM $CaCl_2$, 1 mM EDTA, 0.02% $NaN_3$ and 90% $H_2O$/10% $D_2O$ at pH 6.5 (uncorrected for the isotope effect).

NMR measurements were performed at 50 °C on Varian Inova 800 MHz and 600 MHz spectrometers. Proton chemical shifts were referenced to water at 4.534 ppm. $^{13}$C and $^{15}$N chemical shifts were referenced indirectly using absolute frequency ratios (Cavanagh et al., 1995). Sequence specific assignments of the polypeptide backbone resonances were obtained primarily from the following spectra: 2D [$^{15}$N, $^{1}$H]-HSQC, 3D HNCA, 3D HNCACB, 3D CBCA(CO)NH, 3D HNCO, 3D (HCA)CO(CA)NH, 3D C(CO)NH-TOCSY and 3D H(CCO)NH-TOCSY (Cavanagh et al., 1995). Remaining ambiguities and gaps in the sequential assignments were resolved using 3D $^{15}$N-resolved [$^{1}$H, $^{1}$H]-NOESY (Cavanagh et al., 1995). The NMR spectra were processed using NMRPipe (Delaglio et al., 1995). Spectral analysis and sequential assignment were performed in a semi-automated manner using Ansig for Windows (Helgstrand et al., 2000).

## Extent of assignment and data deposition

The resonance assignments comprise $^{1}H^{N}$, $^{15}N$, $^{13}C'$, $^{13}C^{\alpha}$ and $^{13}C^{\beta}$. The level of completeness is: 93% of all amide groups, 91% of C′, 94% of $C^{\alpha}$ and 92% of $C^{\beta}$. Missing assignments, due to severe overlap or exchange broadening, are limited to four short stretches of residues: M1–H5, I75–V76, T147–K152 and L170–L171, and the single residues K34, T84 and Q95.

The $^{1}$H, $^{15}$N and $^{13}$C chemical shifts have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under the BMRB accession number 5853.

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